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How environmental enrichment affects behavioral and glucocorticoid responses in captive blue-and-yellow macaws (*Ara ararauna*)

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ABSTRACT

Captive animals are susceptible to chronic stress due to restricted space, lack of hiding places, presence of visitors, or the lack of resources that promote physical and mental stimuli. In birds, chronic stress can promote stereotypes, self-mutilation, feather picking, chewing on cage bars and walls, fearfulness and excessive aggression. Environmental enrichment (EE) becomes an important management tool to decrease chronic stress in captive animals. In our study, captive blue-and-yellow macaws (n = 22) kept in zoos in Brazil were exposed to EE in three experimental phases (pre-enrichment – no objects added to the cage; enrichment – physical, occupational and food items added; and post-enrichment – no objects added to the cage). Their behaviors were monitored by focal sampling and excreta were collected three times a week. Frequency of the behavior occurrence and correlation between behavioral data and corticosterone metabolites (CM) levels in droppings were assessed. "Vocalization", "Movement" and "Resting" were the behaviors that occurred more frequently during the three phases. Abnormal behaviors showed no significant difference between phases, but "Biting itself" and "Pacing" decreased significantly from the pre-enrichment to the enrichment phase ($p = 0.042$ and $p = 0.012$, respectively), while "Standing on grid ceiling" increased significantly from pre-enrichment to enrichment phase ($p = 0.002$). Locomotion ($p = 0.018$) and movement ($p = 0.003$) were increased, whereas vocalization ($p = 0.007$), preening ($p < 0.001$) and feather bristling ($p < 0.001$) were reduced during the EE phase compared to the pre-enrichment phase. Macaws interacted more frequently with "Sunflower rolls" (24.4%), "Stuffed pinecones" (21.1%), "Dried corncobs" (17.0%) and "Egg boxes" (13.3%). Individual differences in preference for EE items were also observed. CM levels did not differ between the three phases ($p = 0.798$). "Locomotion" and CM levels were negatively correlated in the pre-enrichment phase ($r = -0.58$; $p = 0.033$), suggesting that more active macaws cope better with stress. "Abnormal behaviors" were not correlated to CM levels. Our results provide evidence for the positive effects of EE on behavior and suggest that it could be used for improving the quality of life in parrots and other captive birds.

1. Introduction

Captive animals are susceptible to chronic stress due to restricted space, lack of hiding places, the presence of visitors, or the lack of resources that promote physical and mental stimuli (Morgan and Tromborg, 2007). In many animal species, chronic stress (mostly mediated by elevated glucocorticoid levels) causes suppression of the immune system, increases blood glucose levels, inhibits growth and reproduction, causes behavioral changes (Romero, 2004), and especially in birds, negatively affects spatial cognition and song learning (Farrell et al., 2015). In birds, abnormal behaviors such as stereotypies, self-mutilation, feather picking, chewing on cage bars and walls, fearfulness and excessive aggression are examples of those behavioral changes (Meehan and Mench, 2006). They are usually signs of low quality of life (QOL). According to Yeates (2016), QOL is related to an animal's subjective experiences (affect and motivation), taking into consideration health, environment and welfare. Duration and frequency of particular conditions can impact animal's life and depend on the individual response.

QOL can be assessed by behavior (Yeates, 2016) and/or endocrinology (e.g. by measuring stress hormones or their metabolites; Möstl and Palme, 2002). In birds, the adrenocortical stress response can be evaluated noninvasively via corticosterone metabolites (CM) in droppings (Goymann, 2005; Möstl et al., 2005; Rettenbacher et al.,

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Table 1
Information about blue-and-yellow macaws (*Ara ararauna*) and housing conditions in Cascavel Municipal Zoo and Conservation Center of Assis Gurgacz Faculty.

Study site	Enclosure	Size (area x height)	Macaw ID	Sex	Additional Information about enclosure
Conservation Center of Assis Gurgacz Faculty	Enclosure BB	16.80 m^2 × 3 m	FAG 141	F	–
			J1	F	
			J2	F	
	Enclosure BC	16.80 m^2 × 3 m	FAG 150	F	Also containing 1 scarlet macaw
	Enclosure D4	7.12 m^2 × 3 m	N1	F	–
			N2	F	
	Enclosure D5	7.12 m^2 × 3 m	N3	F	Also containing 2 blue-fronted amazons, 1 white-eyed parakeet and 1 chopi blackbird
	Enclosure D12	7.12 m^2 × 3 m	N4	F	Also containing 1 blue-fronted amazon and 1 chestnut-eared araçari
Cascavel Municipal Zoo	Enclosure 7	31.81 m^2 × 2.9 m	ZOO CVEL 146	M	–
			ZOO CVEL 147	M	
			ZOO CVEL 148	F	
			ZOO CVEL 159	F	
			ZOO CVEL 160	M	
			ZOO CVEL 164	F	
			ZOO CVEL 165	M	
	Enclosure 20B	28.79 m^2 × 3 m	ZOO CVEL 012	F	–
			ZOO CVEL 141	M	
			ZOO CVEL 143	M	
	Enclosure 22A	38.52 × 3 m	ZOO CVEL 140	F	Also containing 3 scarlet macaws
			ZOO CVEL 161	F	
			ZOO CVEL 162	M	
			ZOO CVEL 163	M	

2004). Measuring glucocorticoid metabolites in droppings has many advantages: (i) sample collection is simple; (ii) there is no animal manipulation, without influence that would alter the results, and does not interfere with natural behavior; (iii) allows the collection of a large number of samples; (iv) enables longitudinal studies; and (v) their concentrations reflect the dynamics of active blood hormones after some hours (Goymann, 2005; Möstl and Palme, 2002; Palme, 2005; Sheriff et al., 2011). However, a sound physiological validation of the utilized method for measuring CM is a prerequisite before its application in a given species (Palme, 2012; Sheriff et al., 2011; Touma and Palme, 2005).

Behavior has been the subject of several studies, which allow researchers to obtain relevant information to help conservation, captive breeding programs and assessment of the quality of life in captivity, and to develop a proper species management (Snowdon, 1999). In this way, environmental enrichment (EE) becomes an important management tool to decrease chronic stress in captive animals. EE consists of procedures that improve physical, nutritional, occupational, sensory and social conditions (Bloomsmith et al., 1991; Boere, 2001), providing opportunities to hide, socialize, forage and exercise (Rupley and Simone-Freilicher, 2015). Birds, especially parrots, have high cognitive abilities and need stimulation to maintain proper neural function. Studies have shown that the cognitive ability of Gray parrots (*Psittacus erithacus*) is comparable to marine mammals and young children, and the communicative competence is equivalent to many primates due to the similarity in brain structure and information processing (Pepperberg, 2006). In a recent study, Olkowicz et al. (2016) found that the brains of many bird species show a higher number and density of neurons than brains of similarly sized mammals. Because of these features, captive birds are susceptible to boredom and stress in conditions with insufficient stimuli for cognition exercise.

Studies on captive birds have shown that EE stimulates neurogenesis in adults (LaDage et al., 2010; Melleu et al., 2015), reduces stereotypic behaviors (Dias et al., 2010), fearfulness (Brantsæter et al., 2016), idleness (Assis et al., 2016; Dias et al., 2010) and preening and feather picking (Lumeij and Hommers, 2008; Meehan et al., 2003; Telles et al., 2015; van Hoek and King, 1997). EE stimulates foraging, enclosure exploration, and increases activity (Andrade and Azevedo, 2011; van Hoek and King, 1997). EE also promotes conditions that meet psychological and physical needs, which contribute positively to an increase in reproductive potential (Moreira et al., 2007).

The blue-and-yellow macaw (*Ara ararauna*) is one of the most prevalent species found in captivity in Brazil (Bianchi, 1998). So far, studies on stress and EE in captive blue-and-yellow macaws have only assessed behavior (Santos et al., 2011; Yepes, 2015). Thus, this study aimed to evaluate behavioral and glucocorticoid responses in captive blue-and-yellow macaws as a tool to improve QOL in captivity. The objectives and corresponding hypotheses of this study were as follows:

(i) To evaluate behavioral and hormonal responses to EE in captive blue-and-yellow macaws. If macaws suffer chronic stress, they will show higher frequencies of abnormal behaviors and higher levels of corticosterone metabolites in droppings. In addition, we hypothesized that both would decrease when EE is offered;
(ii) To validate a noninvasive method for measuring corticosterone metabolites in blue-and-yellow macaws' droppings;
(iii) To associate behavioral data and levels of corticosterone metabolites in droppings. We hypothesized that macaws with higher levels of corticosterone metabolites would show a higher frequency of abnormal behaviors, and vice versa.

2. Material and methods

2.1. Animals and husbandry

The study took place in Cascavel Municipal Zoo and in Conservation Center of Assis Gurgacz Faculty (FAG) in Cascavel, Paraná, Brazil. The Brazilian Environment Agency (IBAMA) Normative Instruction n. 07/2015 categorizes both institutions as Zoo Gardens, allowing public visitation and having scientific, conservation, educational and sociocultural purposes (IBAMA, 2015). The Cascavel Municipal Zoo was open to the public from Tuesday to Sunday from 8:00 am to 6:00 pm and the FAG Conservation Center was open to the public from Mondays to Fridays from 7:00 am to 6:00 pm and on Saturdays from 7:00 am to 12:00 am The number of visitors was higher in Cascavel Municipal Zoo than FAG Conservation Center during the whole study period.

Macaws (n = 22) were housed in a total of eight enclosures at both study sites (Table 1). To facilitate individual recognition, in the enclosures 7 and 22A, macaws received different colored rings (males banded on the right leg and females on the left leg). In other enclosures, individual characteristics and presence/absence of ring were used. During the whole experiment, birds received regular feeding and water *ad libitum*, the same management and the same health care. Feeding, maintenance and cleaning of enclosures were carried out every day in the morning between 7:00 a.m. and 10:00 a.m. in both study sites.

2.2. Clinical examination

A week before starting the experiments, macaws were weighed and a coproparasitological analysis was performed using Faust method (Faust et al., 1970), evaluating the presence of helminth eggs and larvae and protozoan cysts. This health assessment was necessary to exclude diseases, which may affect behavior and corticosterone excretion and alter the results from the experiments.

2.3. Treatment and behavioral data collection

Behavioral and hormonal responses to EE were evaluated in 22 captive blue-and-yellow macaws (Table 1) during three phases (pre-enrichment, enrichment and post-enrichment), each one with a duration of two months from November 2014 to April 2015. Macaws were observed using focal sampling (Martin and Bateson, 2007) for 30 min per bird per day. Types and frequencies of behaviors were recorded every 30 s in six sessions of 5 min. Each bird was observed for a total of 8 h in the pre-enrichment phase (all birds together 176 h), 9 h in the enrichment phase (198 h), and 9 h in the post-enrichment phase (198 h). Data collection was performed three times a week in each study site from 7:00 am to 12:00 am and from 1:00 pm to 5:30 pm during open days. In the pre-enrichment phase, behaviors displayed by birds were observed without EE interference.

In the enrichment phase, physical, occupational and food items were applied in all enclosures three times a week in different hours to avoid habituation of birds to EE. EE were composed of sisal ropes as physical enrichment, pinecones as occupational enrichment, and stuffed pinecones (filled with fruits), surprise boxes (fruits, seeds, dried leaves and bamboo leaves inside cardboard boxes), sunflower rolls (sunflower seeds glued to toilet paper roll), fruit popsicles, dried corncobs and egg boxes (filled with fruits, seeds and bamboo leaves) as food enrichment (Fig. 1). Sisal ropes were applied throughout this phase, while a rotation of food and occupational enrichments was applied in the enclosures. Only one type of these EE was offered per day and the same item was not offered on two consecutive days to avoid habituation. Only fruits and seeds regularly offered to birds were used as food enrichment in order to avoid health problems. In the post-enrichment phase, birds were again observed without EE interference.

2.4. Excreta collection

Excreta were collected during the same periods as behavioral observations. Samples were collected after spontaneous defecation at the end of behavioral observations in the morning, identifying the macaw that provided the sample, totaling three collections per week in each enclosure. Because the sample collection was difficult in enclosures 22A (risk of causing stress due to the aggressive behavior of scarlet macaws), D4, D5 and D12 (low frequency of defecation during the observation period due to feeding logistics, i.e. food was served after the observation because the animal keeper spent time cleaning the enclosures and serving food), samples were collected only in four enclosures (7, 20B, BB and BC). Each sample was individualized in a plastic zip-lock bag previously identified and frozen at $-20\,^{\circ}C$ until steroid analysis.

2.5. Physiological validation

To validate the method physiologically, an ACTH challenge was performed by intramuscular injection of 0.5 mg/kg (Harvey et al., 1980) of synthetic ACTH (Synacthen® Depot, Novartis Pharma, United Kingdom) into the pectoral muscle of four adult female blue-and-yellow macaws at the FAG Conservation Center. Excreta collection was performed by slightly modifying the method used for golden parakeet (*Guaruba guarouba*; Sinhorini, 2013). Samples were collected 72 h, 48 h, 24 h and 2 h before the ACTH administration. On the day of administration, physical restraint and administration of the drug were performed at 11:00 am and samples were collected 2 h, 4 h, 6 h, 8 h, 22 h, 24 h, 48 h and 72 h afterwards. The same macaws were part of the control group 15 days after treatment in which no experimental manipulation was performed (Puvadolpirod and Thaxton, 2000), and the same sample collection procedure was applied. A total of 96 samples were collected (48 samples in treatment and 48 samples in control groups), 12 for each macaw in each experiment.

2.6. Analysis of corticosterone metabolites

Extraction was performed following Palme et al. (2013). In brief, 5 mL of 60% methanol was added to 0.5 g of homogenized wet droppings (or an aliquot in case of smaller samples; e.g.: 0.4 g and 4 mL), which were vortexed for 15 min and centrifuged at 727 G for 10 min. Corticosterone metabolites (CM) were quantified by an 11-oxoetiocholanolone enzyme immunoassay (EIA), as previously described in detail (Möstl et al., 2002). A cortisone EIA (Rettenbacher et al., 2004) seemed equally suited, but as it was less robust, we restricted the analysis of all samples to the former EIA only. Results are expressed in ng/g dropping. Small samples (< 0.05 g) were excluded since results might be biased (Tempel and Guitiérrez, 2004).

2.7. Statistical analysis

Statistical analyses were performed using R Statistical Software (R Core Team, 2013). Data were checked for normality and homoscedasticity using Shapiro-Wilk and Bartlett tests respectively. The level of significance (α) was set to 0.05. Relative frequency of behaviors was calculated by dividing the number of times each macaw displayed determined behavior by the total number of recorded behaviors in each phase. The relative frequency of behaviors showed non-normal distribution and data could not be normalized, thus comparisons between phases were performed using Friedman test followed by Nemenyi posthoc test. The preference of macaws for an EE item was tested using the chi-squared test to compare the relative frequencies of interaction with the objects. The one-way ANOVA with repeated measures and the Tukey posthoc test were used to examine differences in means of corticosterone metabolites levels among macaws, sex, enclosures and study sites, considering the three phases. Data were normalized using logarithmic transformation (base 10). Baseline CM concentrations were calculated by an iterative approach described in detail before (Fanson et al., 2017). The Spearman rank correlation test was performed to assess a possible association between behavioral data and CM levels in droppings using only data of macaws whose excreta were collected. Clinical examination values and CM levels from physiological validation are presented as mean ± SEM. Behavioral data are given as median and CM levels are presented as mean (in Table 3, we presented mean and transformed data).

2.8. Ethics statement

Relevant authorizations to carry out the research were obtained from the Biodiversity Authorization and Information System (SISBIO/protocol No. 44745-1), and from the Animals Use Ethic Committee (CEUA, protocol # 52/2014) from Palotina Sector of the Federal University of Paraná.

3. Results

3.1. Clinical examination

Macaws showed good nutritional and health status. Based on Faust

Fig. 1. Environmental enrichment items offered to blue-and-yellow macaws (*Ara ararauna*) from Cascavel Municipal Zoo and Conservation Center of Assis Gurgacz Faculty. (a) Sisal rope; (b) stuffed pine cone; (c) egg box; (d) surprise box; (e) sunflower roll; (f) dried corncob; (g) fruit popsicle.

method, we did not find helminths eggs, larvae or protozoan cysts. In general, macaws weight (1228 ± 37 g) was within the range reported for this species (995–1380 g) according to Mikich and Bérnils (2004), except for macaws: FAG 150 (1500 g – in preparation for the breeding season), N4 (862 g) and ZOO CVEL 147 (1700 g).

3.2. Physiological validation

In the physiological validation experiment baseline CM levels ranged between 7 and 74 ng/g (40 ± 16 ng/g dropping). ACTH administration resulted in a pronounced increase in CM levels (Fig. 2) starting after 2 h–4 h (lag time). Peaks occurred between 8 h and 24 h (median: 22 h) after ACTH administration (350–702 ng/g). Individual peak concentrations were 9–18 times (median: 12) higher than baseline concentrations.

3.3. Behavior

Behavioral data collection resulted in a behavioral catalog, which includes behaviors presented during the three phases of the study (Table 2). The most frequent behaviors in all phases were "Vocalization" (21.2%, 16.7% and 10.0%, respectively; $Chi^2_{(2)} = 13.45$; $p < 0.001$), "Movement" (16.8%, 24.3% and 33.2%, respectively; $Chi^2_{(2)} = 38.27$; $p < 0.001$) and "Resting" (8.8%, 8.8% and 9.2%, respectively; $Chi^2_{(2)} = 3.06$; $p = 0.217$). "Locomotion" and "Movement" increased significantly ($p = 0.018$; $p < 0.003$, respectively) from pre-enrichment to enrichment, but only "Locomotion" decreased ($p < 0.001$) from enrichment to post-enrichment (Fig. 3). "Vocalization", "Interaction with object" and "Preening" decreased significantly from pre-enrichment to enrichment phase ($p = 0.007$; $p < 0.001$; $p < 0.001$, respectively), but "Preening" increased significantly from enrichment to post-enrichment ($p < 0.001$; Fig. 3). "Interaction with another individual" (6.1%, 5.2% and 4.8%, respectively; $Chi^2_{(2)} = 3.36$, $p = 0.186$), "Foraging" (3.8%, 4.9% and 4.2%, respectively; $Chi^2_{(2)} = 4.46$, $p = 0.108$), "Resting" (8.8%, 8.8% and 9.2%, respectively; $Chi^2_{(2)} = 3.06$, $p = 0.217$), "Abnormal behaviors" (3.8%, 4.8% and 3.3%, respectively; $Chi^2_{(2)} = 1.91$, $p = 0.385$), "Parental care" (0.0% in all phases; $Chi^2_{(2)} = 2.00$, $p = 0.368$) and "Other behaviors" (1.8%, 1.5%, 2.2%, respectively; $Chi^2_{(2)} = 4.36$, $p = 0.113$) showed no significant difference between phases.

3.3.1. Abnormal behaviors

Abnormal behaviors did not differ between phases (pre-enrichment: 3.8%, enrichment: 4.8%, post enrichment: 3.3%; $Chi^2_{(2)} = 1.91$, $p = 0.385$). The most frequent behaviors in all phases were "Standing on the vertical grid" (68.9%, 69.5% and 68.7%, respectively), "Head shaking" (11.34%, 7.3% and 8.3%, respectively) and "Bending and shaking" (0.0%, 0.8% and 1.4%, respectively), which showed no significant difference between phases ($Chi^2_{(2)} = 0.71$, $p = 0.700$; $Chi^2_{(2)} = 4.30$, $p = 0.117$; $Chi^2_{(2)} = 0.26$, $p = 0.878$, respectively). "Standing on the grid ceiling" behavior showed a significant difference between phases ($Chi^2_{(2)} = 12.20$, $p = 0.002$), increasing from pre-enrichment to enrichment and decreasing to post-enrichment phase. Other behaviors which were significantly different were "Biting itself" ($Chi^2_{(2)} = 6.09$, $p = 0.048$) and "Pacing" ($Chi^2_{(2)} = 8.40$, $p = 0.012$), decreasing from pre-enrichment to enrichment (Fig. 4). "Standing on the vertical grid and stretching the right leg repeatedly" was a variation of "Standing on the vertical grid" behavior that was observed only in macaw N1. This behavior appeared in the enrichment phase and persisted in post-enrichment phase, but at low frequencies (0.9% and 0.8%, respectively; no difference between phases: $Chi^2_{(2)} = 2.00$, $p = 0.368$).

3.3.2. Interaction with EE items

The average frequency of interaction with EE items was 5.1%, varying from 0.4% to 17.4% ($Chi^2_{(21)} = 72.70$, $p < 0.001$). Macaws showed greater interaction with "Sunflower rolls" (24.4%), "Stuffed pinecones" (21.1%), "Dried corncobs" (17.0%) and "Egg boxes"

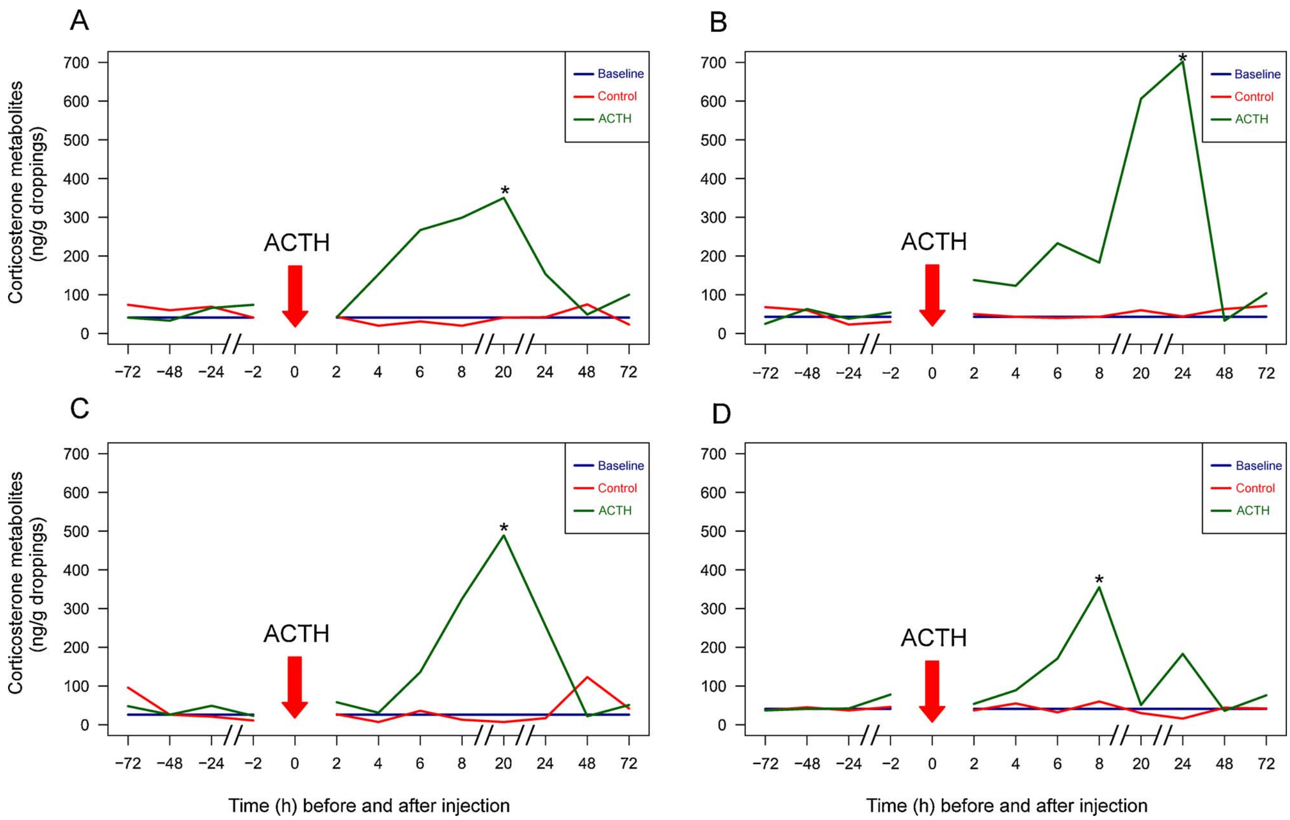

Fig. 2. Baseline concentrations of corticosterone metabolites (ng/g droppings) during control (control group) and ACTH challenge (treatment group) in blue-and-yellow macaws (*Ara ararauna*). A: macaw J1; B: macaw J2; C: macaw FAG 141; D: macaw FAG 150. Asterisks (*) indicate peaks (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(13.3%), and less interaction with "Fruit popsicles" (7.9%), "Surprise boxes" (6.6%), "Sisal ropes" (6.3%) and "Non-stuffed pinecones" (3.2%). However, when the frequencies in each enclosure were compared, macaws from enclosures D4 and D5 interacted more with "Fruit popsicles" (for both 9.4%) than with "Dried corncobs" (4.7% and 0%, respectively). Macaws from enclosure 22A interacted more with "Surprise boxes" (21.4%) than with "Stuffed pinecones" (20.9%), and the macaw from enclosure BC interacted only with "Dried corncobs" (this macaw was in the reproductive period during the enrichment phase and rarely left the nest).

Analysis of similarity of interaction with EE items between birds showed that macaws did not interact with the same frequency with each EE item (stuffed pine cones: $Chi^2_{(21)} = 181.62$, $p < 0.001$; surprise boxes: $Chi^2_{(21)} = 576.0.3$, $p < 0.001$; sisal ropes: $Chi^2_{(21)} = 326.34$, $p < 0.001$; sunflower rolls: $Chi^2_{(21)} = 118.55$, $p < 0.001$; non-stuffed pine cones: $Chi^2_{(21)} = 119.91$, $p < 0.001$; fruit popsicles: $Chi^2_{(21)} = 147.11$, $p < 0.001$; dried corncobs: $Chi^2_{(21)} = 562.35$, $p < 0.001$; egg boxes: $Chi^2_{(21)} = 149.23$, $p < 0.001$).

Table 2
Behavioral catalog developed for blue-and-yellow macaws (*Ara ararauna*) from Cascavel Municipal Zoo and Conservation Center of Assis Gurgacz Faculty.

Initials	Behavior	Description
R	Resting	Standing on perch and observing activities inside or outside the enclosure, or sleeping.
L	Locomotion	Displacement: walk, flight, jump from perch to grid.
M	Movement	Posture change without displacement.
V	Vocalization	Typical sound emitted by macaws, without distinction between low and high frequencies.
F	Foraging	Search for or ingest regular food or water.
IO	Interaction with object	Chew grids and perches.
IE	Interaction with EE	Chew and hold EE items, and/or ingest nutritional EE items.
P	Preening	Straighten and clean feathers.
II	Interaction with another individual	Intra or interspecific interactions.
II+	Positiveinteraction	Alopreening, feed another individual of the same or different species.
II-	Negative interaction	Agonistic interactions, stealing food from another individual of the same or different species.
FB	Feather bristling	Erect feathers in response to negative social interaction and other stressful stimuli.
PC	Parental care	Incubation and interaction with eggs (no chicks were present during the study).
AB	Abnormal behaviors	1) Head shaking (constantly head moving from side to side or backwards); 2) Standing on the vertical grid; 3) Standing on grid ceiling (by paws and/or the beak, in horizontal or vertical position, and shaking wings or moving wings shortly and quickly); 4) Bending and shaking (bend forwards and downwards, and shaking the body and/or the wings); 5) Flutter wings and move the head up and down; 6) Biting itself; 7) Hitting the beak (hit the beak on grid); 8) Pacing (walking repeatedly from one side to the other on grid or on perch); 9) Standing on the vertical grid and stretching the right leg repeatedly; 10) Body movements (up and down, from side to side, or in a circular motion); 11) Hold the wing under the leg and scratch the head.
OB	Other behaviors	Scratching, defecating, digging, yawning, sneezing, pecking leg rings.

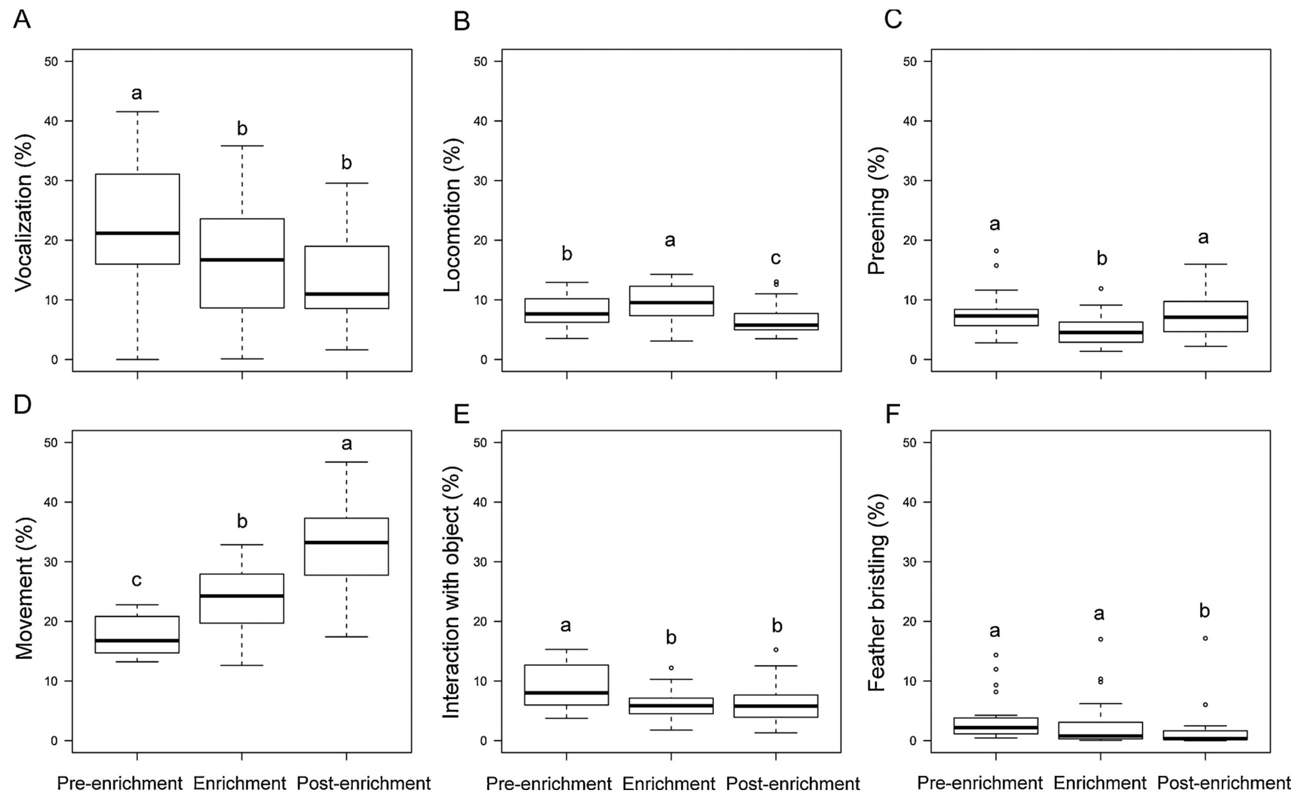

Fig. 3. Relative frequencies (%) of behaviors (median, interquartile range, min and max) with significant differences between phases in blue-and-yellow macaws (*Ara ararauna*) from Cascavel Municipal Zoo and Conservation Center of Assis Gurgacz Faculty. Different letters indicate significant differences ($p < 0.05$).

3.4. Corticosterone metabolites

CM levels (Table 3) did neither differ significantly between pre-enrichment and enrichment phase, nor between enrichment and post-enrichment phase ($F_{(1;13)} = 0.07$, $p = 0.798$). A significant effect of individual macaws ($F_{(1;13)} = 15.43$, $p < 0.001$), enclosure ($F_{(1;3)} = 22.23$, $p < 0.001$) and study site ($F_{(1;1)} = 64.81$, $p < 0.001$) was found. A significant effect of sex ($F_{(1;1)} = 11.85$, $p = 0.001$) was found, but the Cascavel Municipal Zoo was the only study site housing males of blue-and-yellow macaws. No sex difference in CM levels was found there ($F_{(1;1)} = 0.31$, $p = 0.581$). Females from FAG Conservation Center showed significantly lower concentrations than females from Cascavel Municipal Zoo ($F_{(1;1)} = 57.19$, $p < 0.001$).

3.5. Correlation between behaviors and CM levels

In the pre-enrichment phase, “Locomotion”, “Interaction with object” and “Negative interaction” showed a significant, negative correlation with CM levels ($r = -0.58$, $p = 0.033$; $r = -0.55$, 0.046; $r = -0.65$, $p = 0.014$, respectively), while “Vocalization” showed a moderate (but not significant) negative correlation with CM levels ($r = -0.41$, $p = 0.151$). In the enrichment phase, there was a negative correlation between “Negative interaction” and CM levels ($r = -0.57$, $p = 0.035$), and positive correlation between “Foraging” and CM levels ($r = 0.62$, $p = 0.021$). No correlation between behaviors and CM levels was observed in post-enrichment phase.

4. Discussion

4.1. Physiological validation

Results of the ACTH challenge test indicate that the 11-oxoetiocholanolone EIA (Möstl et al., 2002) can be used for measuring CM in droppings of blue-and-yellow macaws to monitor HPA axis activity. Studies in great tits (*Parus major*; Carere et al., 2003; Stöwe et al., 2010), ravens (*Corvus corax*; Stocker et al., 2016) and upland geese (*Chloephaga picta*; Koch et al., 2009) also succeeded using this assay, but Goymann et al. (2002) showed that the same EIA was not suited for European stonechats (*Saxicola torquata rubicola*). Studies in black grouse (*Tetrao tetrix*; Baltic et al., 2005), capercaillies (*Tetrao urogallus*; Thiel et al., 2005), golden parakeets (*Guaruba guarouba*; Sinhorini, 2013), gull-billed terns (*Gelochelidon nilotica*; Albano et al., 2015) and blue-fronted parrots (*Amazona aestiva*; Ferreira et al., 2015) demonstrated that a cortisone EIA (Rettenbacher et al., 2004) showed the best results for measuring glucocorticoid metabolites in droppings. We found this cortisone EIA equally suited for blue-and-yellow macaws, but for practical reasons we limited the analysis to the 11-oxoetiocholanolone EIA. These studies underline the presence of species differences in metabolism and excretion of corticosterone and thus the importance of a proper validation of an EIA before it can be successfully applied (Palme, 2005; Sheriff et al., 2011).

It was not possible to collect samples between 8 h and 20 h after ACTH administration because of the Assis Gurgacz Faculty logistics. Thus, the highest CM peak may have occurred soon after 8 h post ACTH administration. In blue-fronted parrots, Ferreira et al. (2015) verified that peaks occurred 3–9 h after ACTH challenge. In golden parakeets, Sinhorini (2013) detected individual peaks occurring between 4 h and 8 h, and between 11 h and 15 h after ACTH administration. In blue-and-yellow macaws, higher levels occurring between 6 h and 8 h after the ACTH challenge could represent the first peak, and peaks occurring after 20 h could be the second peak.

4.2. Behavior

“Vocalization” was the most frequent behavior in the pre-enrichment phase, also observed by Pimenta et al. (2009) in captive hyacinth macaws (*Anodorhynchus hyacinthinus*). Despite being vocal animals due to socialization (Graham et al., 2006), psittacids show a low relative

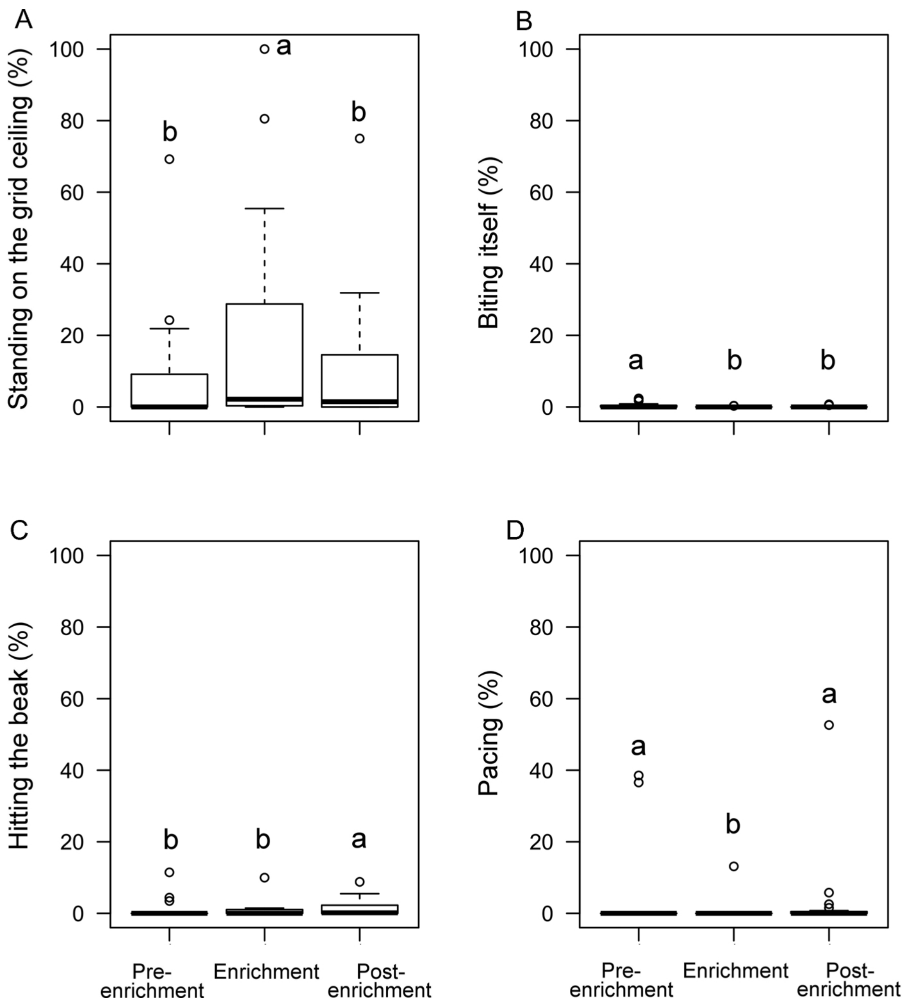

Fig. 4. Relative frequencies (%) of abnormal behaviors (median, interquartile range, min and max) with significant differences between phases in blue-and-yellow macaws (*Ara ararauna*) from Cascavel Municipal Zoo and Conservation Center of Assis Gurgacz Faculty. Different letters indicate significant differences ($p < 0.05$).

frequency (2–5%) of vocalization in wildlife (Lightfoot and Nacewicz, 2006). The decrease in "Vocalization" seemed to be related to offering EE items, which was also associated with an increase in birds' activity ("Locomotion" and "Movement" behaviors) towards the enrichment, also observed in crimson-bellied conures (*Phyrrhura perlata*) by van Hoek and King (1997).

We observed an increase in "Locomotion" and "Movement" behaviors by offering EE in the enrichment phase. An increase in activity and/or mobility behaviors during enrichment phase was also recorded in crimson-bellied conures (van Hoek and King, 1997), hyacinth macaws (Pimenta et al., 2009), blue-fronted parrots (Andrade and Azevedo, 2011; Melo et al., 2014), and white-eyed parakeets (*Psittacara leucophthalmus*; Telles et al., 2015). These results support the idea of the importance of EE to increase activity since activity is good for health and improves quality of life.

"Interaction with another individual" showed no significant

Table 3
Mean and (transformed data), baseline and peak concentrations of corticosterone metabolites (ng/g droppings) in blue-and-yellow macaws (*Ara ararauna*) from Cascavel Municipal Zoo and Conservation Center of Assis Gurgacz Faculty.

Individual	Pre-enrichment			Enrichment			Post-enrichment		
	Mean and (transformed data)	Baseline	Peak	Mean and (transformed data)	Baseline	Peak	Mean and (transformed data)	Baseline	Peak
FAG 141	37 (3.60)	32	94	43 (3.76)	36	136	70 (4.25)	49	348
J1	63 (4.14)	53	116	70 (4.25)	48	415	64 (4.16)	49	220
J2	39 (3.67)	32	89	69 (4.23)	36	451	64 (4.16)	45	331
FAG 150	70 (4.24)	42	204	65 (4.17)	65	–	48 (3.87)	49	109
ZOO CVEL 146	86 (4.45)	86	–	81 (4.39)	54	289	107 (4.67)	48	501
ZOO CVEL 147	137 (4.91)	137	–	110 (4.70)	83	374	113 (4.73)	113	–
ZOO CVEL 148	112 (4.72)	75	330	123 (4.81)	123	–	91 (4.52)	78	280
ZOO CVEL 159	268 (5.59)	268	–	173 (5.15)	81	567	143 (4.96)	96	657
ZOO CVEL 160	139 (4.94)	117	323	272 (5.61)	193	982	216 (5.38)	158	698
ZOO CVEL 164	102 (4.62)	102	–	108 (4.68)	61	409	160 (5.07)	96	782
ZOO CVEL 165	215 (5.37)	126	930	235 (5.46)	96	1620	191 (5.25)	127	582
ZOO CVEL 012	231 (5.44)	141	821	171 (5.14)	67	669	125 (4.83)	76	416
ZOO CVEL 141	162 (5.09)	69	1180	102 (4.63)	71	233	103 (4.64)	88	285
ZOO CVEL 143	252 (5.53)	98	1006	237 (5.47)	237	–	251 (5.52)	157	782

difference between phases. However, enclosures D5 and D12 contained just one blue-and-yellow macaw each, which rarely interacted with birds of other species. In most cases, these interactions were agonistic. These birds also showed higher frequencies of abnormal behaviors than other macaws (macaw N3: 10.6% in pre-enrichment phase, 16.0% in enrichment phase and 12.7% in post-enrichment phase; macaw N4: 5.9% in pre-enrichment phase, 8.0% in enrichment phase and 12.4% in post-enrichment phase). Because psittacids are extremely social birds, flying, foraging and sleeping in large groups (Engebretson, 2006; Sick, 1997), deprivation of social interactions with the same species in captivity can result in low quality of life (Engebretson, 2006), causing physical abnormalities and abnormal behaviors. In enclosure 20B, despite the presence of three macaws, there were low interactions between the couple (macaws ZOO CVEL 012 and ZOO CVEL 143) and the macaw ZOO CVEL 141, which were, in most cases, agonistic. Maintenance of birds that are not accepted socially or that have dominant and subordinate relationships in the same enclosure triggers stress among those individuals. According to our results, the EE implemented in this study did not ameliorate potential psycho-social stress between these birds.

“Interaction with object” behavior decreased from pre-enrichment to enrichment phase, which brings benefits both to birds and the institution that maintains them. Less interaction with grids and perches reduces the need for their continuous replacement and, consequently, avoids that employees enter the enclosures, preventing stress in birds.

Psittacids spend much time on preening, but this behavior can increase in an enclosure that has few resources to reduce idleness and boredom, which causes self-mutilation by feather picking (Assis et al., 2016). “Preening” decreased significantly from pre-enrichment to enrichment and increased significantly to post-enrichment. A positive effect of EE in relation to “Preening” is shown in enclosure D12: during the pre-enrichment phase, the macaw N4 showed tail feather picking; in enrichment phase, there was a decrease (from 10.0% to 4.7%) in “Preening” and tail feathers started growing. However, during the post-enrichment, this macaw plucked its feathers again. Further studies are necessary to elucidate if this negative response was to boredom or frustration after removal of the EE.

4.2.1. Abnormal behaviors

Abnormal behaviors associated with the grid in pre-enrichment phase totalized 63.3% (“Standing on the vertical grid”, 55.9%, and “Standing on the grid ceiling”, 7.4%). High frequencies of these behaviors were also found in Hyacinth macaws by Pimenta et al. (2009). According to the authors, behaviors associated with the grid along with high frequencies of vocalization are related to territorial demarcation in captivity. When visitors approach the enclosure, macaws move to the grid and start to vocalize. “Pacing” decreased significantly from pre-enrichment to enrichment phase, the same observed in Japanese quails (Laurence et al., 2015) and orange-winged amazon parrots (Meehan et al., 2004). In our study, increased time spent in “Locomotion”, “Movement” and “Interaction with EE” prevented macaws from showing “Pacing”. All these results demonstrate that EE can significantly decrease these types of stereotypies.

In this study, some abnormal behaviors did not decrease from pre-enrichment to enrichment phase and other behaviors increased like “Hitting the beak”, “Head shaking”, “Bending and shaking”, “Flutter wings and move the head up and down”, “Body movements”, “Standing on the vertical grid” and “Hold the wing under the leg and scratch the head”. These results can be explained by the factors below:

a) Presence of a great number of visitors (Azevedo et al., 2012) in enrichment phase. Despite it was not actually measured, there was a perceptive increase in number of visitors in both study sites due to the summer vacation in January and February. Noise, active crowds and interactions with animals are stressors for many captive species (Fernandez et al., 2009; Morgan and Tromborg, 2007; Quadros et al., 2014);
b) Presence of unfamiliar people into enclosures for perches and grid maintenance and rodent control. The inability to deal with new stimuli can be a factor that prevents habituation of birds to human proximity (Mason, 2010);
c) Introduction of novelty in enclosures may trigger fear and stress responses (Mason et al., 2007; Melleu et al., 2015). No prior knowledge of a situation potentially stimulates the HPA axis (Romero and Sapolsky, 1996). Psittacids are preys and tend to be neophobic (Wilson and Luescher, 2006), and when EE is a novelty for them, it may increase fear in birds that show high level of neophobia (Fox and Millam, 2007). These factors delay the onset of a positive interaction with EE on behavior as observed in some macaws in this study. Furthermore, two months may not have been time enough for macaws overcome their fears and show fewer frequencies of abnormal behaviors;
d) Introduction of EE in enclosures caused an increase in activity, which may increase the frequency of abnormal behaviors, some of them not performed by macaws in the pre-enrichment phase;
e) Individuals with different personalities do not respond similarly to the same stimulus or stressor (Stöwe et al., 2010; Fox and Millam, 2007) and may show abnormal behaviors in response to EE.

4.2.2. Interaction with EE items

In this study, a high variation of the frequency of “Interaction with EE” was observed between macaws, also observed by Telles et al. (2015) in parakeets, which may be associated with the hierarchy within the group (not measured in this study), the degree of interest for EE items (Telles et al., 2015) or the degree of neophobia. Further studies are needed to clarify these issues about hierarchy and neophobia. Additionally, some macaws showed a preference for specific items like sunflower rolls, stuffed pinecones and dried corncobs, and other macaws preferred surprise boxes or sisal ropes. A study on blue-fronted parrots showed that boxes containing fruits and fruit popsicles were less accepted by birds, while dried corncobs and green corncobs were the items with greater interaction (Melo et al., 2014), similar as in the present study. Fox and Millam (2007) tested some unfamiliar EE items of different sizes to orange-winged Amazon parrots. The authors verified that birds avoided certain types of objects, indicating that they can perceive the difference between items and show a preference for particular objects.

Preference assessment is an essential step to identify the best items to offer to animals in both individual and species levels (Mehrkam and Dorey, 2015), enabling to plan better housing conditions and maintenance of animals in captivity. For example, in crimson-bellied conures, van Hoek and King (1997) found that birds had a preference for natural and edible materials like items that contain fruits because they are familiar to birds and are part of the diet. Meehan et al. (2003) also verified the preference for food items in Orange-winged Amazon parrots. In our study, macaws interacted more with sunflower rolls, stuffed pinecones, dried corncobs and egg boxes; all these EE contained food, but they interacted with the inedible parts too. When EE without food was applied, they did not interact so much. Furthermore, foraging behavior is an activity that occupies 40% to 60% of the time of the psittacids (Lightfoot and Nacewicz, 2006). So, food attracts macaws and this enrichment is a good alternative for captive blue-and-yellow macaws since allows physical activity. This information can be used for other psittacids because different species of this group share similar behaviors. The daily observation of birds is very important to understand their behavior and how they interact with EE items, evaluating which items should be used.

4.3. Corticosterone metabolites

In this study, there were significant differences in CM levels between study sites and individuals, which may be explained by several factors. Both behavioral and hormonal responses depend on personality, age, sex, genetics, social status, adrenal responsiveness and experience with the different stressors, which influence strategies used by each individual to deal with stressful situations (Carere et al., 2003; Moreira et al., 2007; Parnell et al., 2014; Shepherdson and Carlstead, 2001; Stocker et al., 2016; Touma and Palme, 2005). Diet, metabolic rate, bacterial composition in the gut and route of excretion of metabolites (urine or feces) also may differ between individuals and influence the concentration of excreted metabolites (Goymann, 2005, 2012; Palme et al., 2005).

Regarding the study sites, despite the apparent habituation of macaws to visitors, the number of visitors was higher in Cascavel Municipal Zoo than in FAG Conservation Center, which may have increased CM levels in droppings of macaws from Cascavel Municipal Zoo. In free-living little bustards (*Tetrax tetrax*), Tarjuelo et al. (2015) found high CM levels in droppings in response to increased number of visitors during weekends in the study area. Size, composition and degree of kinship among birds in each enclosure, differences in management, interaction between keepers and animals, and characteristics of enclosures are also factors that may also influence the physiological responses (Parnell et al., 2014), but further studies need to be developed to associate these factors with physiological responses in blue-and-yellow macaws.

Macaws FAG 150 and ZOO CVEL 012 were incubating their eggs during the enrichment phase and the incubation period is associated with higher corticosterone secretion, as verified by Adams et al. (2005) in grey-faced petrels (*Pterodroma macroptera goudi*). However, these macaws had similar levels as the other macaws of their respective study sites, indicating no relation between incubation and high CM levels.

Despite sex differences in excreted CM in some bird species (e.g. chicken: Rettenbacher et al., 2004; black grouse: Baltic et al., 2005; golden conure: Sinhorini, 2013), no sex differences were found in this study (the same observed in greylag geese: Scheiber et al., 2015 and mourning doves: Washburn et al., 2003). Males (resident only in Cascavel Municipal Zoo) showed higher CM levels in droppings than females just when data of females from both study sites were in the analysis. When females from the FAG Conservation Center were excluded, no difference was found between males and females from Cascavel Municipal Zoo. The difference in concentrations between females from FAG Conservation Center and females from Cascavel Municipal Zoo may be explained by personality, age, genetics, social status, adrenal responsiveness, number of visitors in each study site, and diet. Future studies are necessary to elucidate these issues.

4.4. Correlation between behaviors and CM levels

Combining physiological and behavioral data helps in understanding the responses to stress. In the pre-enrichment phase, we found that macaws which showed higher frequencies of "Locomotion" and lower frequencies of "Abnormal behaviors" (moderate positive correlation between this behavior and CM levels) excreted lower CM concentrations. On the other hand, macaws which showed low frequencies of "Locomotion" associated with higher frequencies of "Abnormal behaviors", excreted higher CM concentrations, indicating a higher degree of stress. Exercise is good to reduce stress and improve the quality of life, as discussed before.

In the pre-enrichment phase, we also found that macaws which interacted more with perches and grids excreted lower CM concentrations. This behavior may be a way to deal with stress in captivity. In the enrichment phase, there was no correlation between "Abnormal behaviors" and CM levels. This may indicate individual variations in coping with stress and neophobia, and in physiological responses and excretion of metabolites (urine or feces), as discussed on previous topics.

Both in the pre-enrichment and enrichment phases, macaws which showed higher frequencies of "Negative interaction" with other birds excreted lower CM concentrations. Because these phases were performed in the reproductive period, aggression may be related to the defense of resources and partners, and dominance and subordination among individuals. Testosterone is associated with these behaviors, and testosterone levels decrease with increasing corticosterone levels (Deviche et al., 2014). By contrast, dominance condition and aggressive or agonistic interactions are associated with an increase in glucocorticoid secretion (Creel et al., 2013; Stocker et al., 2016). Thus, further studies are needed to clarify those relations in macaws.

In the post-enrichment phase, macaws that showed more frequency of "Resting" had higher CM levels (a positive correlation with CM levels). It does not necessarily indicate a reduction in quality of life because resting is required for maintenance of biological functions. Furthermore, there was no correlation between "Locomotion" and CM levels in this phase. So, it is not possible to affirm that macaws which showed low frequency of locomotion had higher CM levels, as observed in the pre-enrichment phase. In addition, in the post-enrichment phase, EE could still be having effects on birds, which is associated with different behavioral and physiological responses to several environmental factors acting on them.

Comparing corticosterone levels in feathers and behavior of Clark's nutcrackers (*Nucifraga Columbiana*) during "short-term enrichment" and "long-term enrichment", Fairhurst et al. (2011) found that corticosterone levels were higher in short-term enrichment than pre-enrichment and post-enrichment. In long-term enrichment, the corticosterone levels did not differ from pre and post-enrichment. Pecking behavior was higher in short-term enrichment than other phases. According to the authors, birds showed neophobia to EE when it was offered for the first time (short-term enrichment), and during long-term enrichment, birds could adapt to environmental change, which resulted in lower corticosterone levels and harmful behaviors. In this study, the time needed for adaptation to enrichment phase by blue-and-yellow macaws may have been too short, reflecting in non-significant decreasing in CM levels and abnormal behaviors. Curiosity and neophobia may be associated with increases in CM levels when birds interact with enrichment. Elevated CM levels for short periods are not bad, and can be related to excitement.

5. Conclusions

The present study demonstrated that an 11-oxoetiocholanolone EIA (Möstl et al., 2002) is suited to measure CM in droppings of blue-and-yellow macaws, and thus enables non-invasive monitoring of the HPA axis activity in this species. EE had a positive influence on the quality of life of blue-and-yellow macaws by reducing passiveness and some stress behaviors, but did not significantly decrease abnormal behaviors and CM levels. Providing EE items to animals may improve the quality of life by stimulating cognitive and motor activities, but some issues like enrichment duration, neophobia, suited intra- and interspecific combination of birds, preference for specific items and management should be further evaluated to reduce efficiently the stress in captivity and increase natural behaviors. These results are valuable to increase the quality of life of parrots and other captive birds.

Conflict of interest

The authors declare there is no any conflict of interest.

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