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Effects of application of environmental enrichment in kangaroos (*Osphranter rufus*) at Aquário de São Paulo

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Efeito da aplicação do enriquecimento ambiental em cangurus (*Osphranter rufus*) no Aquário de São Paulo

Resumo: Programas de enriquecimento ambiental vêm sendo amplamente realizados em zoológicos e instituições afins para promover a manutenção do bem-estar dos animais. No caso de cangurus, muitos estudos se concentraram no repertório comportamental expresso por alguns grupos de vida livre, mas poucos se referem a esses animais em ambientes controlados. Este estudo objetiva aplicar técnicas de enriquecimento ambiental a um grupo de cangurus vermelhos (*Osphranter rufus*) no Aquário de São Paulo a fim de se avaliar seus efeitos no comportamento desses animais. As observações (amostragem instantânea) foram realizadas ao longo de 60 horas divididas igualmente em três etapas: antes (pré-enriquecimento), durante e depois (pós-enriquecimento) da aplicação de enriquecimentos ambientais, sendo considerados para a análise estatística os 10 comportamentos mais frequentes expressos em todas as etapas. Comportamentos associados à vigilância do ambiente e ao autogerenciamento diminuíram suas frequências, e aqueles relacionados à automanutenção aumentaram suas frequências após a aplicação de enriquecimentos. Os resultados mostraram que o uso de enriquecimentos induziu mudanças na expressão dos comportamentos e ofereceu diversos estímulos positivos aos indivíduos. Portanto, a rotina de enriquecimentos proposta pode ser aplicada a outros grupos de cangurus sob cuidados humanos.

Palavras chave: Bem-estar animal, comportamento, Etologia, zoológicos.

Abstract: Programs of environmental enrichment have been widely conducted in zoos and similar institutions to promote the maintenance of animal welfare. In the case of kangaroos, many studies have focused on the behavioral repertoire expressed by some wild groups, but few regards on these animals in captivity. This study aims at applying environmental enrichment to a group of red kangaroos (*Osphranter rufus*) at Aquário de São Paulo in order to evaluate its behavioral effects in these animals.

Observation (instantaneous sampling) was carried out over 60 hours equally divided into three stages: before (Pre-enrichment), during and after (Post-enrichment) the application of environmental enrichments, with the 10 most frequent behaviors expressed during all stages being considered to the statistical analysis. Behaviors associated to managing the environment and self-managing decreased their frequencies, and those related to self-maintenance increased their frequencies after the application of enrichments. Results showed that enrichments induced changes in the expression of behaviors and offered several positive stimuli to subjects. This routine could thus be applied to other groups of kangaroos in human care.

Key words: Animal welfare, behavior, ethology, zoos.

Introduction

Although the expression of natural behavior in captive animal populations can be hampered by the limits imposed by controlled environments (Hill & Broom 2009), zoos are well known for their importance in both fields of education and conservation (mainly regarding endangered species) (Schulte-Hostede & Mastromonaco 2015). For keeping conservation breeding programs, zoos are currently concerned about animal welfare, which constrains a challenge in the sense of trying to maintain captive species' natural behavioral/adaptive state. One of these tools regards environmental enrichment as "an improvement in the biological functioning of captive animals resulting from modifications to their environment" (Newberry 1995).

Environmental enrichment, a common tool used in zoos worldwide, has been continuously employed together with specific programs for captive species in controlled environments (Quirke & O'Riordan 2012), and if properly performed can prevent welfare problems (Swaisgood 2007). Environmental enrichment programs are fundamental to animal welfare and, once applied by zoos and aquariums, have to deal with high expectations from a critical audience (Shepherdson 2003).

In the case of kangaroos, many studies have focused on the behavioral repertoire expressed by some wild groups (Croft 1980; Priddel 1986; Short 1986; Watson & Dawson 1993; McCullough & McCullough 2000; Munn *et al.* 2010; Munn *et al.* 2013; Munn *et al.* 2014) and even the behavioral adjustments of wild-caught animals when released at larger enclosures to determine the impact of captivity in these animals (Munn *et al.* 2017). Few information has been produced regarding captive-animals, such as the study reporting that kangaroos spend most of their time in vigilance toward visitors and not resting, which can be regarded as curiosity or an anti-predatory behavior (neither being responsible for disturbing the group well-being) (Sherwen *et al.* 2015). It has also been recorded that the red kangaroo, *Osphranter rufus* (Desmarest, 1822), rest at daytime and are active at night, but also can show some activities (e.g., search for food, socialization) during the daytime, resting for short periods (Russell 1970; Calaby & Poole 1971; Croft 1980). Despite this information, little is known about the behavioral repertoire of captive-kangaroos after being submitted to an environmental enrichment routine, which thus corresponds to the aim of this study, as well as evaluating possible gains in their general welfare.

Materials and Methods

This study was conducted at Aquário de São Paulo, state of São Paulo, Brazil, according to internationally accepted standards (Ethic Committee on Animal Use CEUA/UNIFESP, number 4632230317). Subjects were nine *O. rufus* kept at an indoor enclosure (following the Normative Instruction 07 from April 30th, 2015 of IBAMA) with bare soil, dry trees, stone structures, artificial waterfall, feeders and an off-exhibit housing (**Figure 1**). Aquário de São Paulo provides a regular routine of environmental enrichment for the animals, which was interrupted for kangaroos by a month before this study aiming at not to interfere in the results.

Figure 1. Kangaroos' enclosure in left (A) and right (B) lateral views.

Observations were taken during 15 weeks (from August to November 2016), 2 or 3 sessions a week, between 08:00 to 16:00 h for two consecutive hours (120 min) in stages 1 and 3 and for one hour (60 min) in stage 2, adding up to 60 h of observation.

The study was divided into three stages (20 h each): Pre-enrichment (1), to register behavioral repertoire previous to enrichment application; Enrichment application (2); and Post-enrichment (3) as the suspension of enrichment application. During each of these stages all expressed behaviors were recorded at every 5 min (see Martin & Bateson 1993) by observation method of instantaneous sampling (Altmann 1974). The observed behaviors followed the behavioral categories described by Russell (1970, 1974) and Sherwen et al. (2015) (**Table 1**). When the subject was out of the observer's sight (i.e., the choice of the animal of not to be visible to visitors), it was considered "not visible".

Table 1. Ethogram of behaviors for subjects of *O. rufus* based on Russell (1970, 1974) and Sherwen *et al.* (2015).

Behavior	Description	Source consulted
Visitor-Directed Vigilance	Erect posture and fixed gaze toward visitors	Sherwen *et al.* (2015)
General Vigilance	Scanning environment not including visitors	Sherwen *et al.* (2015)
Feed	Manipulation of objects or substrate in search of food and/or put food in the mouth and chew it	Sherwen *et al.* (2015)
Locomotion	Moving in enclosure by walking or hopping	Sherwen *et al.* (2015)
Resting	Lying on side or back with eyes open or closed	Sherwen *et al.* (2015)
Grooming Self	Running mouth or paws through own skin, including licking	Sherwen *et al.* (2015)
Grooming Others	Running mouth or paws through another individual's skin, including licking	Sherwen *et al.* (2015)
Scratch	Pass the paw repeatedly on or bite the skin characterizing the act of scratching	Russell (1970)
Suckling from Mother	Young with head inside mother´s pouch	Russell (1970, 1974)
Play	Hitting with the paw on another individual, between mother and young	Russell (1970, 1974)
Resting Inside the Pouch	Young inside the pouch	Russell (1970, 1974)
Aggression	Chase, cornering, hit/grab with the front paws or stand propped on the tail to hit the hind legs on another individual	Sherwen *et al.* (2015)
Sniffing the Genitalia of Another Individual	Place the muzzle on the genitals of another individual, sniffing it	Russell (1970, 1974)
Chasing the Female	Male moves behind the female and travels the same route as she, and may or may not try to hold her with the front legs during her movement	Russell (1970)
Drink	Water intake	Russell (1970)
Explore the Enclosure	Smell the substrate or elements of the enclosure	Russell (1970)
Stopped Inactive	Individual stopped, erect or not, without keeping a fixed gaze towards the visitors or other members of the group	Russell (1970)
Sniffing Another Individual	Place the muzzle in the body of another individual, excluding the genitals, smelling it	Russell (1970, 1974)

Enrichment application consisted in introducing four types of enrichment of each category (food, cognitive, physical, sensorial and social) (n = 20) simultaneously to the record of subjects' behavior (Sherry 2010). Each item of enrichment was introduced in a different day of observation of stage 2, which were kept for approximately one hour and removed in the end of this time. Enrichments received a code (ER) and were numbered (1-20) according to a previously established application sequence (ER1, as the first applied one, to ER20). The types comprised by each category of enrichment are defined and detailed at **Table 2**. It is worth mentioning that, in both ERs 11 and 16, sound (extracted from Arkive.org) was emitted by a sound box connected by Bluetooth placed at the closed off-exhibit housing.

Table 2. Types of enrichment within each ER category with their description, code and number of items presented to subjects (N). Symbol (-) indicates non-physical (uncountable) itens.

ER Category	Type	Description	Code	N
Food	Diet in vine balls	vine balls with diet inside hung on the branches of a central dry tree in the enclosure	ER1	2
Social	Acrylic mirror	mirrors leaning against the wall and hanging in a feeder	ER2	2
Physical	Tires	car tire hanging from the tree by a rope and tractor tire on the ground, in the front part of the enclosure	ER3	2
Cognitive	Pet ball	pet balls filled with dog food and vegetable leaves	ER4	4
Sensorial	Cinnamon tea	cinnamon tea sprayed in the environment	ER5	-
Food	Food cords	food cords hanging from the branches of a tree and a feeder in front of the enclosure, each of these (cords) made of a series of tied food	ER6	7
Social	Photo	photos of conspecific individuals glued on the glass of the enclosure from the outside, with the figure facing the inner side of the enclosure	ER7	7
Physical	Gymnastic ball	large, red gymnastic ball left in the enclosure	ER8	1
Sensorial	Cloth	clothes soaked in a nutmeg tea hanging from the tree and at the corner of the room in tree stumps	ER9	2
Cognitive	Diet in hay	diet hidden in packs of hay	ER10	2
Social	Vocalization of other species	simultaneous and sequential sounds of Australian birds and dingos (*Canis lupus dingo* Meyer, 1793) emitted for 5 min at 20 min intervals	ER11	-
Food	Diet in PVC pipe	PVC punctured pipes filled with food (access holes of different sizes) hung in feeders and at the central tree	ER12	3
Sensorial	Artificial rain	rain produced through rotating sprinkler placed at the middle of the enclosure	ER13	1 (number of sprinklers)
Physical	Punching bag	punching bags filled with hay hanging from the tree	ER14	2
Cognitive	cardboard box	cardboard punctured boxes with food	ER15	3
Social	Vocalization of conspecific	vocalization of conspecific in sequence for 5 min at 20 min intervals	ER16	-
Physical	Waterfall	a pre-existing waterfall (kept turned off) was turned on	ER17	1
Sensorial	Ice	big piece of ice left at the front part of the enclosure	ER18	1
Cognitive	labyrinth feeder	labyrinth feeder with food	ER19	1
Food	Diet in grass leaves	feeders with food hidden/tied with grass leaves	ER20	2

The 10 most frequent behaviors expressed during all treatments were considered to the statistical analysis. Data of Pre- and Post-enrichment stages (1 and 3) were grouped by behavior and statistically compared by T-test and Mann-Whitney test ($p < 0.05$) performed for parametric and non-parametric groups of behavior, respectively (**Table 3**). Interaction with ERs was considered "Interest" when subject observed by far or attempted to approach enrichment and "Direct interaction" when there was physical contact of subject with enrichment. Shapiro-Wilk test was used to verify if data met the requirements for parametric analysis. Data related to the application of ERs at the moment some behavior was performed were also grouped by behavior at the Enrichment application treatment (2) and submitted to PCA (Principal Component Analysis) to produce predictor variables of most frequent behaviors after the enrichments. Behaviors with loadings > 0.4 on each first three component of PCA were considered on the analyses. All analyses were performed with PAST (version 3.19).

Results

The most frequent behaviors were "Visitor-Directed Vigilance" (VD), "General Vigilance" (GV), "Feed" (FD), "Locomotion" (LC), "Resting" (RT), "Grooming Self" (GS), "Scratch" (SC), "Not Visible" (NV), "Explore the Enclosure" (EX) and "Stopped Inactive" (SI) (Table 3). "Interest" rate was 7.78%, and "Direct interaction" was 14.12%, adding up to 21.90% of the behavioral frequency in stage 2. Environmental enrichment category that obtained the highest frequency of interaction with the group of kangaroos was food (accounting for 25.58% of interactions with enrichments), followed by cognitive (20.08%), sensorial (19.45%), social (17.75%) and physical (17.12%). The enrichment types that provided greater interaction in each category were: diet in PVC pipes (food), diet in hay (cognitive), ice (although it did not generate direct interaction, only interest and curiosity on the part of the group - sensorial), vocalization of other species (social) and tires (arousing greater interest than direct interaction – physical).

Table 3. Frequency (Freq, %), Expression Mean (Mean – stages 1 and 3) and P-value (stages 1 and 3) of each behavior for *O. rufus* group during Pre-enrichment (PRE), Enrichment application (ENR) and Post-enrichment (POST) stages.

BEHAVIOR	PRE	ENR	POST	P-value	T/U-value
VD – Freq	3.10	1.48	1.28		
- Mean	6.70±1.943		2.70±0.715	0.118 (Mann-Whitney)	29
GV – Freq	7.08	2.18	5.04		
- Mean	15.30±3.457		10.60±2.749	0.301 (T test)	1.0639
FD – Freq	27.36	16.39	26.39		
- Mean	58.90±7.051		55.50±11.546	0.804 (T test)	0.25131
LC – Freq	6.38	3.33	5.37		
- Mean	13.80±3.161		11.30±3.238	0.596 (Mann-Whitney)	42.5
RT – Freq	31.71	40.97	48.93		
- Mean	68.50±18.733		102.90±20.881	0.236 (T test)	1.2262
GS – Freq	4.35	4.07	1.85		
- Mean	9.40±1.257		3.90±0.546	<0.01 (Mann-Whitney)	3.5
SC – Freq	6.52	3.56	3.51		
- Mean	14.10±2.818		7.40±1.620	0.054 (T test)	2.0608
NV – Freq	2.87	1.39	2.37		
- Mean	6.20±3.255		5.00±2.654	0.720 (Mann-Whitney)	45
EX – Freq	1.99	0.97	1.14		
- Mean	4.30±1.075		2.40±1.097	0.168 (Mann-Whitney)	31.5
SI – Freq	3.05	1.57	1.28		
- Mean	6.60±1.528		3.00±0.726	0.056 (T test)	2.0497

Three patterns can be outlined regarding frequency of behaviors from stages (**Table 3**): (A) behavior frequency decreases from Pre- to Post-enrichment (1-3), with the lower frequency registered for Post-enrichment (3) (for VD, GS, SC and SI); (B) behavior frequency decreases from Pre-enrichment to the Application of enrichment (1-2), but increases at Post-enrichment (3), although not as much as the frequency rate at Pre-enrichment (1) (for GV, FD, LC, NV and EX); (C) behavior frequency increases from Pre- to Post-enrichment (1-3) (for RT).

PCA analysis related each ER with the abovementioned coded behaviors during the Application of enrichments (**Figure 2**; **Table 4**). The first three axes of the PCA accounted for 65.47% of the data variation. The first component extracted a positive correlation between locomotion (LC) and Scratch (SC), which were negatively correlated to resting (RT), accounting for 33.22% of variation. The second component accounted for 19.40% of variation and was taken to represent a positive correlation between Stopped Inactive (SI) and Explore the Enclosure (EX), which were negatively correlated to Feed (FD). The third component accounted for only 13.85% of variation and extracted a positive correlation between Not Visible (NV) behavior and Visitor-Directed Vigilance (VD), while those variables where negatively correlated to General Vigilance (GV) (**Figure 2**; **Table 5**).

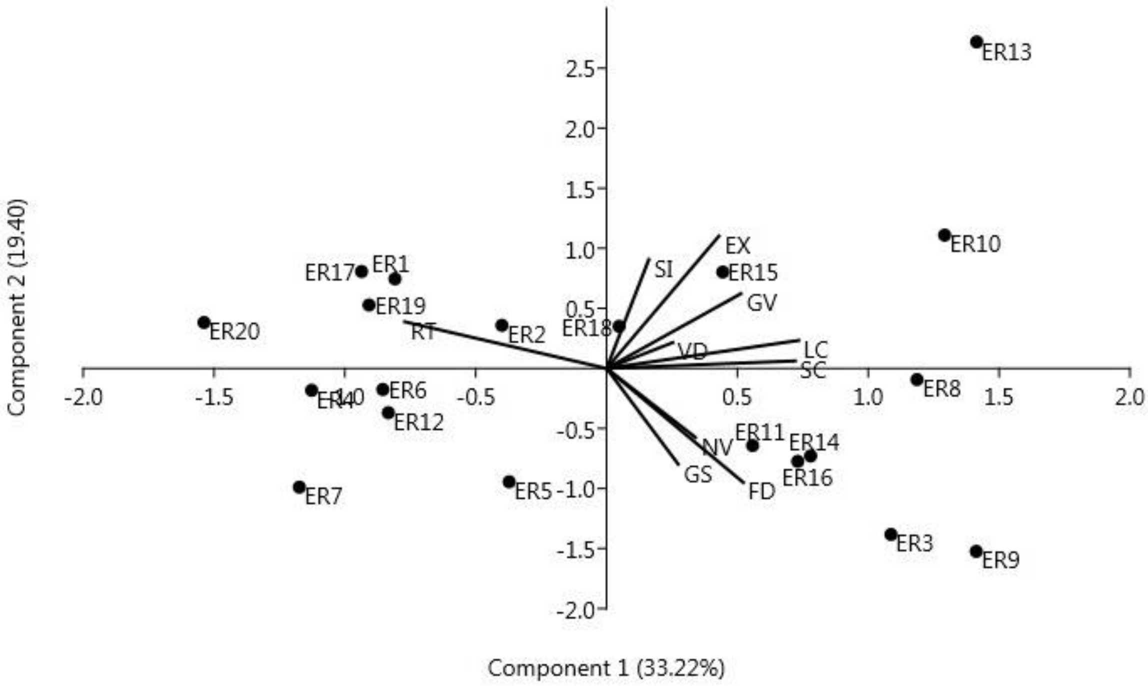

Figure 2. Scatter plot of Principal Component Analysis (PCA) for main behavioral responses of *O. rufus* to several types of enrichment (ERs).

Discussion

Pattern (A) mainly comprised behaviors associated to managing the environment (VD) or self-managing (GS and SC). The first category accounts for Visitor-Directed Vigilance (VD). Exhibiting less vigilance during the Application of enrichment and after the enrichment has been removed from the enclosure (Post-enrichment) could indicate that the focus of subjects changed from the audience (visitors) to the enrichment. In the case of the group here studied the frequency of Visitor-Directed Vigilance (VD) decreased significantly during the interaction of subjects to enrichment (3.10 to 1.48%) and a bit more after it (1.48 to 1.28%). Other studies on the effect of vigilance have already been performed with other captive animals such as birds (Azevedo et al. 2012) and mammals including primates (Birke 2002; Cooke & Schillaci 2007; Carder & Semple 2008; Kuhar 2008; Clark et al. 2012) and non-primates (Shen-Jin *et al.* 2010), and even marsupials such as koalas (Larsen et al. 2014) and kangaroos (Wolf & Croft 2010; Sherwen et al. 2015), and have reported that there was an increase in attention directed towards visitors. Most of these studies have interpreted this behavior as negative since it is currently reported as an antipredator behavior and thus assumed by disturbed animals in the presence of potential danger (Dyck & Baydack 2003), which decreases time spent at other activities such as those of self-maintenance (e.g., feeding and resting) (Lott & McCoy 1995; Roe et al. 1997). However, it can also be a response to visitors (Thompson 1989; Hosey et al. 2013) and thus a positive stimulus to animals (Sherwen et al. 2015).

Table 4. Results of the Principal Component Analysis (PCA) showing each Enrichment category (Code) applied to *O. rufus* and their scores across the first three component of PCA. Values in bold indicate behaviors with scores > 1.0.

ER Category (Code)	PC1	PC2	PC3
ER1	-0,809	0,746	0,109
ER2	-0,399	0,358	1,007
ER3	1,087	-1,383	-0,612
ER4	-1,127	-0,182	-0,468
ER5	-0,372	-0,944	0,098
ER6	-0,855	-0,175	0,690
ER7	-1,174	-0,989	-0,157
ER8	1,186	-0,093	-1,457
ER9	1,412	-1,524	2,518
ER10	1,292	1,110	1,938
ER11	0,558	-0,642	0,137
ER12	-0,834	-0,368	-0,678
ER13	1,414	2,718	-0,655
ER14	0,780	-0,728	-1,121
ER15	0,444	0,803	-0,487
ER16	0,731	-0,774	-1,435
ER17	-0,936	0,807	0,143
ER18	0,048	0,351	-0,129
ER19	-0,908	0,527	0,255
ER20	-1,54	0,382	0,302

Table 5. Results of the Principal Component Analysis (PCA) showing the behaviors of *O. rufus* related with Post-Enrichment application, their loading and the percent variance explained across the first three components of PCA. Values in bold indicate behaviors with loadings > 0.4.

Variables	PC1	PC2	PC3
Visitor-Directed Vigilance (VD)	0.155	0.101	0.539
General Vigilance (GV)	0.315	0.292	-0.427
Locomotion (LC)	0.451	0.109	-0.270
Grooming Self (GS)	0.168	-0.373	-0.048
Feed (FD)	0.320	-0.445	-0.179
Resting (RT)	-0.473	0.182	0.041
Scratch (SC)	0.442	0.029	0.160
Not Visible (NV)	0.208	-0.269	0.583
Stopped Inactive (SI)	0.099	0.425	0.084
Explore the Enclosure (EX)	0.263	0.517	0.214
% Variance Explained	33.22	19.40	13.85
% Accumulated Variance	33.22	51.62	65.47

Regarding vigilance in kangaroos, time spent with visitors (vigilance) increased with an increasing in visitor number simultaneously to a decrease in time spent resting according to Sherwen *et al.* (2015), although the authors argue that these changes are hard to interpret since they can be both related to curiosity or fear without clearly implying in animal welfare. When this approach is made by foot or in a car in natural areas, time spent in maintenance activities were reported higher when approaching comes by car (Wolf & Croft 2010). In this study, subjects are maintained at a glass enclosure from visitors, which accounts for providing more time to subjects to be spent near visitors since glass reduces disturbances that could be made by the latter (Meder 1992). Moreover, the enclosure is designed to simulate the natural environment of kangaroos (see Fig. 1 and the description of enclosure at Materials and Methods), which positively enriches this environment even in the lack of an enrichment routine (not the case of Aquário de São Paulo, which provides enrichment application to their animals regularly). These facts can explain the low frequency of VD behavior in the studied group before the Application of enrichment (Pre-enrichment) and even lower after it, since the conditions (i.e., type and features of enclosure) to which subjects are submitted were previously outlined to reduce stress as many as possible regarding captivity. Nevertheless the use of ERs decreased this behavior, which was positive especially if we regard this behavior as negative.

The second category of Pattern (A) accounts for Grooming self (GS) and Scratch (SC), which are mainly related to self-maintenance. Grooming represents a displacement behavior and is often considered at animal models for stress and anxiety (File *et al.* 1988; Barros *et al.* 1994; Kalueff & Tuohimaa 2005; Leppanen *et al.* 2006) since its excess (overgrooming) is considered as an abnormal behavior that can lead to physical and psychological injuries (Wolfensohn *et al.* 2018). However, since there are reports of grooming activity increasing in both high and low stress conditions (e.g., rodents; Smolinsky *et al.* 2009), this parameter is not reliable to indicate animal anxiety. For kangaroos, despite the prediction of captive animals spending more time grooming than animals in the wild, it was reported that wild-caught *O. rufus* and *M. fuliginosus* (red and western grey kangaroos, respectively) spent more time grooming when initially captured than later (Munn *et al.* 2017), which has already been reported as a behavioral indicator of capture distress (Jackson 2003; Weary *et al.* 2009) even in kangaroos (Dawson 2012). Thus, a reduction in the expression of this behavior after the enrichment application and an even lower record after the removal of ERs can be interpreted as positive for increasing animal welfare, in this case.

Pattern (B) comprised behaviors that had a relative decrease during the Application of Enrichment (regarding the initial frequency of this behavior before subjects are submitted to ERs) and a subsequent increase after the removal of ERs (but not equal to or higher than the first stage), and accounted for General Vigilance (GV), Feed (FD), Locomotion (LC) and Explore the Enclosure (EX). The former (GV) consists of general attentive behavior, and the fact of having decreased during the Application of enrichment could mean that, as abovementioned for VD (Pattern A), the focus has switched from the environment to ERs. Also, Feed (FD) behavior showed a decrease during the enrichment application stage, but it is worth mentioning that subjects fed on the food items during the application of some food and cognitive ERs, which were indeed considered as "direct interaction with the enrichment" instead of "Feed" itself. Thus, it does not mean that subjects would have decreased their food intake during this stage, but can suggest that the group has chosen to feed on food in ERs instead of feeding conventionally in proper recipients. After the Application of ERs this behavior has increased to a similar rate to the Pre-enrichment stage (27.36 to 26.39 %, respectively). Subjects have also been more active regarding moving behaviors (Locomotion and Explore the Enclosure), which means that ERs have fulfilled time spent by kangaroos when applied. This behavior is predicted to decrease when subjects get gradually used to a new place (Munn *et al.* 2017), which can be explained by the fact of animals moving to explore the environment or (in more extreme cases) when they express stress (e.g., pacing, defined as a continuous walking or running on the same path or not, but without clear objective; Camargo & Mendes 2016). However, neither this group is newly in the enclosure, nor they expressed (in any of the stages) excessive movement, which consequently indicate that reduction in moving could be associated to subjects focusing on the ERs during their application (and an increase as they were removed from the enclosure).

Moreover, Not Visible behaviors (NV), also referred as "Out of Sight" (Blumstein & Daniel 2007), has also fallen into this category and has found to be informative regarding animal welfare since it points to the animal's choice of being visible or not to visitors (Carlstead *et al.* 1993; Sellinger & Ha 2005; Davey 2006; Morgan & Tromborg 2007; Hosey *et al.* 2013; Spiezio *et al.* 2017). Thus, this behavior has been time recorded as others once it is also important to assess animal welfare at zoos (Farrand 2007).

The last pattern (C) indicated that frequency increased from Pre- to Post-enrichment (1-3), which was only observed for Resting (RT), a non-alert behavior that tends to decrease as the animal gets used to the environment (signaling that anxiety reduced) (Munn *et al.* 2017). Resting has indeed reached a higher frequency at Post-enrichment stage (from 31.71 to 48.93%), which characterizes a pattern already noticed by Munn *et al.* (2017) for both red and western grey kangaroos. An increase in the frequency of "Resting" in subjects along the experiment can also account for a natural behavior of subjects for the diurnal period (Russell 1970). As statistically demonstrated, this fact seems not to be related to the inclusion of ERs, but possibly to the general increase of temperatures reported for the end of winter, when kangaroos tend to increase rest periods (Croft 1980), although room temperature was not controlled in this study. Another possible factor influencing this increase is the time when ERs were applied (late in the morning; the time of behavioral report at Pre-enrichment stage), once kangaroos tend to be more active early in the morning and to increase their Resting rate after this period (Russell 1970). This behavior mostly increased during the Post-enrichment stage, and may also explain the significant decrease in the expression of Grooming self and Scratch, considered as comfort movements (Croft 1980).

Regarding the frequency of interaction with ERs during their application, food ERs were responsible for providing most of interactions by promoting a different and unpredictable way of supplying food for the group, while favoring an increase in the positive use of the available space by spreading/dispersing the food items. Cognitive ERs, which accounted for the second highest rate of interaction, also promoted a more complex way to access food. Sensorial enrichment has shown to be more efficient than direct interaction in stimulating the sense of smell to explore the scent of cinnamon and nutmeg. On the other hand, social enrichment, although providing less interaction than the previous ERs, led subjects to express different reactions, from marking the territory (mirrors) to attention (vocalization) and staring others (i.e., photos of other individuals). Kaleta & Chudzik (2008) observed that macropodid individuals tended to be cautious in response to new inanimate objects presented; in this way, when physical enrichment was offered subjects stayed away (there was more interest than directly interacting with them) and, even when they approached slowly, they sometimes "frightened" with the inserted element and jumped off quickly. Therefore, the enrichment offered several stimuli (arousing several reactions) to the group of kangaroos.

The PCA analysis showed the most important types of enrichment (ER) that accounted for differences in behaviors of *O. rufus* after the Post-enrichment, which were extracted by the first three axes of PCA. There were no clear pattern of correlation between the types of behaviors extracted at each axis of PCA and the ER categories used in the present study. However, as seen in first axis of PCA, the application of sensorial (cloth soaked in nutmeg tea (ER9) and artificial rain (ER13), cognitive (diet in hay (ER10)) and physical (tires (ER3) and gymnastic balls (ER8)) enrichments resulted in increased frequency of Locomotion (LC) and Scracth (SC) (with tendency to reduce resting behavior), while higher frequency of resting (RT) was associated to the application of food (diet in leave grass (ER20)), social (photo of conspecific (ER7)) and cognitive (pet balls (ER4)) enrichments. In addition, the ER9 (sensorial) and ER3 (physical) also resulted in higher frequency of Feeding (FD) (with tendency of lower Stopped inactive (SI) and Explore the enclosure (EX)), whereas the frequency of SI and EX was in higher frequency after the application of sensorial (ER13) and cognitive (ER10) enrichments, as seen in the second axis of PCA. Finally, as extracted by the third axis of PCA, the frequency of behaviors associated to Not visible (NV) and Visitor-directed vigilance (VC) were higher after sensorial (ER9), cognitive (ER10) and social (acrylic mirror (ER2)) enrichments, whilst the application of physical (ER8 and punching bags (ER14)) and social (vocalization of conspecific (ER16)) enrichments promoted a higher frequency of General Vigilance (GV). Although no explanation can be outlined relating ERs with these behaviors as there was no statistically significant change in the expression of most observed behaviors, ERs related to sensorial (ER9 and ER13), cognitive (ER10) and physical (ER3 and ER8) categories indeed induced an increase in the expression of positive behaviors (e.g. LC, SC, EX, FD) that tended to be kept after their removal from the enclosure, which thus positively accounted for the maintenance of kangaroos' welfare.

Conclusion

The application of this ER routine provided several positive stimuli to kangaroos, increasing the variability and complexity of their environment. Enrichments that stimulated more interaction with kangaroos were those with food items, especially when the form of supply was different (food category) or provided in a more difficult way to assess (cognitive category). This routine could be applied to other groups of kangaroos to substantiate the observed results as positive to be implemented (i.e., regarded positive as to keep animals welfare) when these animals are in human care. It is thus important to continuously provide application of different enrichment as it often manages to avoid stress-inducing behaviors to be performed and stimulate different reactions.

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References

Altmann J. (1974) Observational Study of Behavior: Sampling Methods. *Behaviour*, 49(3/4): 227–267. https://doi.org/10.1163/156853974x00534

Azevedo C.S.D., Lima M.F.F., Silva V.C.A.D., Young R.J. & Rodrigues M. (2012) Visitor influence on the behavior of captive greater rheas *Rhea americana*. *Journal of Applied Animal Welfare Science*, 152: 113–125. https://doi.org/10.1080/10888705.2012.624895

Barros H.M., Tannhauser S.L. & Tannhauser M.A. (1994) The effects of GABAergic drugs on grooming behaviour in the open field. *Pharmacology and Toxicology*, 74: 339–344. https://doi.org/10.1111/j.1600-0773.1994.tb01370.x

Birke L. (2002) Effects of browse human visitors and noise on the behaviour of captive orangutans. *Animal Welfare*, 11: 189–202.

Blumstein D.T. & Daniel J.C. (2007) Quantifying Behavior the JWatcher way. Sunderland, Massachusetts: Sinauer Associates. 198 p.

Calaby J.H. & Poole W.E. (1971) Keeping kangaroos in captivity. *International Zoo Yearbook*, 11: 5–12. https://doi.org/10.1111/j.1748-1090.1971.tb01825.x

Camargo M.R. & Mendes F.D.C. (2016) Induced Tool Use as Environmental Enrichment for Captive Capuchin Monkeys (*Sapajus libidinosus*). *Psicologia*: *Teoria e Pesquisa*, 32: 1–8. http://dx.doi.org/10.1590/0102-3772e32ne21

Carder G. & Semple S. (2008) Visitors effects on anxiety in two captive groups of western lowland gorillas. *Applied Animal Behaviour Science*, 115: 211–220. https://doi.org/10.1016/j.applanim.2008.06.001

Carlstead K., Brown J.L. & Strawn W. (1993) Behavioral and physiological correlates of stress in laboratory cats. *Applied Animal Behaviour Science*, 38: 43–158. https://doi.org/10.1016/0168-1591(93)90062-T

Clark F.E., Fitzpatrick M., Hartley A., King A.J., Lee T., Routh A., Walker S.L. & George K. (2012) Relationship between behavior adrenal activity and environment in zoo-housed western lowland gorillas (*Gorilla gorilla gorilla*). *Zoo Biology*, 31: 306–321. https://doi.org/10.1002/zoo.20396

Cooke C.M. & Schillaci M.A. (2007) Behavioral responses to the zoo environment by white handed gibbons. *Applied Animal Behaviour Science*, 106: 125–133. https://doi.org/10.1016/j.applanim.2006.06.016

Croft D.B. (1980) Behaviour of red kangaroos, *Macropus rufus* (Desmarest, 1822) in northwestern New South Wales, Australia. *Australian Mammalogy*, 4: 5–58.

Davey G. (2006) Relationships between exhibit naturalism, animal visibility and visitor interest in a Chinese Zoo. *Applied Animal Behaviour Science*, 96: 93–102. https://doi.org/10.1016/j.applanim.2005.04.018

Dawson T.J. (2012) Kangaroos. 2nd edition. Melbourne, Australia: CSIRO Publishing. 208 p.

Dyck M.G. & Baydack R.K. (2003) Vigilance behaviour of Polar Bears (Ursus maritimus) in the context of wildlife-viewing activities at Churchill, Manitoba, Canada. *Biological Conservation*, 116: 343–350. https://doi.org/10.1016/S0006-3207(03)00204-0

Farrand A. (2007) The effect of zoo visitors on the behaviour and welfare of zoo mammals. Doctoral Thesis. University of Stirling, Scotland, UK.

File S.E., Mabbutt P.S. & Walker J.H. (1988) Comparison of adaptive responses in familiar and novel environments: modulatory factors. *Annals of the New York Academy of Sciences*, 525: 69–79. https://doi.org/10.1111/j.1749-6632.1988.tb38596.x

Hill S.P. & Broom D.M. (2009) Measuring Zoo Animal Welfare: Theory and Practice. *Zoo Biology*, 28: 531–544. https://doi.org/10.1002/zoo.20276

Hosey G.R., Melfi V. & Pankhurst S. (2013) Zoo animals: behaviour, management, and welfare. 2° edition. Oxford, UK: Oxford University Press. 643 p.

Jackson S.M. (2003) Australian Mammals: Biology and Captive Management. 1st edition. Melbourne, Australia: CSIRO Publishing. 524 p.

Kaleta T. & Chudzik P. (2008) The comparison of behaviour in two macropodid marsupials: red kangaroo *Macropus rufus* and red-necked wallaby *Macropus rufogriseus* kept in adjacent enclosures at Warsaw ZOO. *Annals of Warsaw University of Life Sciences – SGGW, Animal Science*, 45: 53–58.

Kalueff A.V. & Tuohimaa P. (2005) Contrasting grooming phenotypes in three mouse strains markedly different in anxiety and activity (129S1, BALB/c and NMRI). *Behavioral Brain Research*, 160: 1–10. https://doi.org/10.1016/j.bbr.2004.11.010

Kuhar C.W. (2008) Group differences in captive gorillas' reaction to large crowds. *Applied Animal Behaviour Science*, 110: 377–385. https://doi.org/10.1016/j.applanim.2007.04.011

Larsen M.J., Sherwen S.L. & Rault J.L. (2014) Number of nearby visitors and noise level affect vigilance in captive koalas. *Applied Animal Behavioral Science*, 154: 76–82. https://doi.org/10.1016/j.applanim.2014.02.005

Leppanen P.K., Ravaja N. & Ewalds-Kvist S.B. (2006) Twenty-three generations of mice bidirectionally selected for open-field thigmotaxis: selection response and repeated exposure to the open field. *Behavioral Processes*, 72: 23–31.

Lott D.F. & McCoy M. (1995) Asian Rhinos Rhinoceros unicornis on the run? Impact of tourist visits on one population. *Biological Conservation*, 73: 23–26. https://doi.org/10.1016/0006-3207(95)90053-5

Martin P. & Bateson P. (1993) Measuring Behaviour: An introductory guide. 2nd edition. Cambridge, UK: Cambridge University Press. 222 p.

McCullough D.R. & McCullough Y. (2000) Kangaroos of Outback Australia: Comparative Ecology and Behavior of Three Coexisting Species. New York: Columbia University Press. 308 p.

Meder A. (1992) Effects of the environment on the behaviour of lowland gorillas in zoo. *Primate Report*, 32: 167–183.

Morgan K.N. & Tromborg C.T. (2007) Sources of stress in captivity. *Applied Animal Behaviour Science*, 102: 262–302. https://doi.org/10.1016/j.applanim.2006.05.032

Munn A.J., Dawson T.J. & McLeod S.R. (2010) Feeding biology of two functionally different foregut-fermenting mammals, the marsupial red kangaroo and the ruminant sheep: how physiological ecology can inform land management. *Journal of Zoology*, 282: 226–237.

https://doi.org/10.1111/j.1469-7998.2010.00740.x

Munn A.J., Dawson T.J., McLeod S.R., Dennis T. & Maloney S.K. (2013) Energy, water and space use by free-living red kangaroos *Macropus rufus* and domestic sheep *Ovis aries* in an Australian rangeland. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, 183: 843–858. https://doi.org/10.1007/s00360-013-0741-8

Munn A., Skeers P., Kalkman L., Mcleod S.R. & Dawson T. (2014) Water use and feeding patterns of the marsupial western grey kangaroo (*Macropus fuliginosus melanops*) grazing at the edge of its range in arid Australia, as Compared with the dominant local livestock, the Merino sheep (*Ovis aries*). *Mammalian Biology - Zeitschrift fur Saugetierkunde*, 79: 1–8. https://doi.org/10.1016/j.mambio.2013.03.003

Munn A.J., Phelan S., Rigby M. & Roberts J.A. (2017) Behavioral adjustments of wild-caught kangaroos to captivity. *Australian Mammalogy*, 39: 213–218. https://doi.org/10.1071/AM16019

Newberry R.C. (1995) Environmental enrichment: Increasing the biological relevance of captive environments. *Applied Animal Behaviour Science*, 44: 229–243.

Priddel D. (1986) The diurnal and seasonal patterns of grazing of the red kangaroo, Macropus rufus, and the western gray-kangaroo, *Macropus fuliginosus*. *Wildlife Research*, 13: 113–120.

Quirke T. & O'Riordan R.M. (2012) Evaluation and Interpretation of the Effects of Environmental Enrichment Utilizing Varying Degrees of Sampling Effort. *Zoo Biology*, 32: 262–268. https://doi.org/10.1002/zoo.21003

Roe D., Leader-Williams N. & Dalal-Clayton B. (1997) Take only Photographs, Leave only Footprints: The Environmental Impacts of Wildlife Tourism. London, UK: International Institute for Environment and Development. 83 p.

Russell E.M. (1970) Observations on the behaviour of the red Kangaroo (*Megaleia rufa*) in captivity. *Ethology*, 27: 385–404. https://doi.org/10.1111/j.1439-0310.1970.tb01880.x

Russell E.M. (1974) The biology of kangaroos (Marsupialia-Macropodidae). *Mammal Review*, 4: 1–59.

Schulte-Hostede A.I. & Mastromonaco G.F. (2015) Integrating evolution in the management of captive zoo populations. *Evolutionary Applications*, 8: 413–422. https://doi.org/10.1111/eva.12258

Sellinger R.L. & Ha J.C. (2005) The effects of visitor density and intensity on the behavior of two captive jaguars (*Panthera onca*). *Journal of Applied Animal Welfare Science*, 8: 233–244. https://doi.org/10.1207/s15327604jaws0804_1

Shen-Jin L., Todd P.A., Yan Y., Lin Y., Hongmei F. & Wan-Hong W. (2010) The effects of visitor density on sika deer *Cervus nippon* behaviour in Zhu-Yu-Wan Park, China. *Animal Welfare*, 19: 61-65.

Short J. (1986) The effect of pasture availability on food intake, species selection and grazing behaviour of kangaroos. *Journal of Applied Ecology*, 23: 559–571.

Shepherdson D.J. (2003) Environmental enrichment: past, present and future. *International Zoo Yearbook*, 38: 118–124.

Sherry E.V. (2010) FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching. *Journal of the American Association for Laboratory Animal Science*, 51: 30–44.

Sherwen S.L., Hemsworht P.H., Butler K.L., Fanson K.V. & Magrath M.J.L. (2015) Impacts of Visitor Number on Kangaroos Housed in Free-Range Exhibits. *Zoo Biology*, 34: 287–295. https://doi.org/10.1002/zoo.21226

Smolinsky A.N., Bergner C.L., Laporte J.L. & Kalueff A.V. (2009) Analysis of grooming behavior and its utility in studying animal stress, anxiety, and depression (p. 21–36). *In*: Gould, T. D., Mood and Anxiety Related Phenotypes in Mice. Neuromethods 42, Humana Press.

Spiezio C., Vaglio S., Scala C. & Regaiolli B. (2017) Does positive reinforcement training affect the behaviour and welfare of zoo animals? The case of the ringtailed lemur (*Lemur catta*). *Applied Animal Behaviour Science*, 196: 91–99. https://doi.org/10.1016/j.applanim.2017.07.007

Swaisgood R.R. (2007) Current status and future directions of applied behavioral research for animal welfare and conservation. *Applied Animal Behaviour Science*, 102: 139–162. https://doi.org/10.1016/j.applanim.2006.05.027

Thompson V. (1989) Behavioral response of 12 ungulate species in captivity to the presence of humans. *Zoo Biology*, 8: 275–297. https://doi.org/10.1002/zoo.1430080308

Watson D. & Dawson T. (1993) The effects of age, sex, reproductive status and temporal factors on the time-use of free-ranging red kangaroos. *Wildlife Research*, 20: 785–801. https://doi.org/10.1071/WR9930785

Weary D.M., Huzzey J.M. & von Keyserlingk M.A.G. (2009) Using behavior to predict and identify ill health in animals. *Journal of Animal Science*, 87: 770–777. https://doi.org/10.2527/jas.2008-1297

Wolf I.D. & Croft D.B. (2010) Minimizing disturbance to wildlife by tourists approaching on foot or in a car: A study of kangaroos in the australian rangelands. *Applied Animal Behaviour Science*, 126: 75–84. https://doi.org/10.1016/j.applanim.2010.06.001

Wolfensohn S., Shotton J., Bowley H., Davies S., Thompson S. & Justice W.S.M. (2018) Assessment to welfare in zoo animals: towards optimum quality of life. *Animals*, 8: 110. https://doi.org/10.3390/ani8070110