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The Old School, Brewhouse Hill, Wheathampstead,
Hertfordshire AL4 8AN, UK
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Seasonal pattern of salivary cortisol secretion in the greater one-horned rhino (Rhinoceros unicornis)

A Menargues*[†‡§], V Urios[‡] and R Limiñana[‡§#]

[†] Universidad de Alicante, Campus San Vicente del Raspeig, Facultad de Educación I, E-03080 Alicante, Spain
[‡] Estación Biológica Terra Natura, Instituto Universitario de Investigación CIBIO, Universidad de Alicante, Apdo correos 99, E-03080, Alicante, Spain
[§] Didáctica de las Ciencias Experimentales, Facultad de Educación, Universidad de Alicante, Apdo correos 99, E-03080, Alicante, Spain
[#] Instituto de Investigación en Recursos Cinegéticos (IREC), CSIC-UCLM-JCCM, Ronda de Toledo, s/n E-13071, Ciudad Real, Spain
* Contact for correspondence and requests for reprints: a.menargues@ua.es

Abstract

*The Indian rhinoceros or greater one-horned rhino (*Rhinoceros unicornis*) is listed as vulnerable by the IUCN Red List of Threatened Species and, therefore, captive individuals have been subject to the European Endangered Species Programme since 1990. Enhancement of welfare is key in ensuring the breeding success of this species in captivity. Salivary cortisol has been recently used to assess welfare of captive and free-ranging animals. Nevertheless, rhythms of cortisol secretion may fluctuate throughout the year and therefore, knowledge of the circannual pattern of cortisol secretion is essential to evaluate the physiological significance of seasonal variations of cortisol levels as an indicator of stress in animals. Here, we analyse monthly differences in cortisol secretion in two Indian rhinoceros. Saliva samples of two rhinoceros were collected and analysed by radioimmunoassay for the period of one year to determine cortisol concentrations. We found a seasonal pattern of salivary cortisol secretion. The highest cortisol concentrations were found in August and decreased until reaching a nadir in January. Cortisol concentrations in these two animals showed a correlation with temperature and visitor numbers but it is not possible to draw conclusions from this study as to whether the variation in cortisol was due to these or other factors.*

Keywords: *animal welfare, Indian rhinoceros, salivary cortisol, seasonality, stress, zoo visitors*

Introduction

There have been rhinoceros for more than 50 million years although, in the past, species tended to be much more diverse and widespread (occurring in North America and Europe, as well as Africa and Asia). Today, however, only five species of rhinoceros survive. The Indian rhinoceros or greater one-horned rhino (*Rhinoceros unicornis*) population, with strict protection from Indian and Nepalese wildlife authorities, has recovered from less than 200 individuals in the early 1900s to an estimated 2,575 individuals, with 375 occurring in Nepal and 2,200 in India (Talukdar *et al* 2008; Milliken *et al* 2009). However, the distribution is largely fragmented and more than 70% of the overall population are found in Kaziranga National Park. Since 1990, Indian rhinoceros living in European zoological parks have been subject to the European Endangered Species Programme (EEP) (http://www.eaza.net/activities/cp/Pages/EEPs.aspx) and assessment of welfare in these captive individuals is key to helping ensure success in breeding programmes.

When animals are exposed to a stressor, the hypothalamic pituitary-adrenal (HPA) axis is activated, inducing cortisol secretion by the adrenal cortex (Munck *et al* 1984; Sheriff *et al* 2011). Prolonged physiological stress can result in suppressed immune function (Moberg 1990; McEwen 2006; Dhabhar 2009), increased disease susceptibility, abnormal behaviour (Moberg 1990) and poor reproductive success (Carragher *et al* 1989; Moberg 1990; Hardy *et al* 2005). Salivary cortisol concentration has been used to assess captive animal welfare in several species of mammal species (Fuchs *et al* 1997; Pedernera-Romano *et al* 2006; Powell *et al* 2006; Menargues *et al* 2008). There is a strong correlation between levels of salivary glucocorticoids and levels of free-unbound serum glucocorticoids (Sheriff *et al* 2011) and the salivary cortisol peak in concentration occurs 20–30 min after stressor exposure (Duncko *et al* 2007). Hence, this is a non-invasive technique that reflects the free glucocorticoids' concentration in blood (Kirschbaum & Hellhammer 1994; Gómez *et al* 2004; Gröschl 2007; Wood 2009).

Cortisol secretion exhibits fluctuations in ultradian and circadian rhythms (Ekkel *et al* 1996; Ruis *et al* 1997; de Jong *et al* 2000; Brown *et al* 2010, Menargues *et al* 2012b), and can change seasonally or circannually (Monfort *et al* 1993; Ingram *et al* 1999; Suzuki *et al* 2003; Vera *et al* 2011; Menargues *et al* 2012a). Moreover, in several species, glucocorticoid levels have been shown to be influenced by temperature or inclement weather (Wingfield *et al* 1983; Millspaugh *et al* 2001; Huber *et al* 2003; Brown *et al* 2010). And, finally, the well-being of zoo animals can be affected detrimentally by visitors' desire to observe and interact with the animals in close proximity (Fernández *et al* 2009).

Knowledge of cortisol's seasonal secretory rhythms is a necessity when the physiological significance of fluctuations are being evaluated as a stress indicator in animals. Circannual rhythms of cortisol secretion have not been described in the greater one-horned rhinoceros. The aim of this study was to determine whether any evidence existed for seasonal variation in cortisol levels from samples from two animals, using a saliva analysis method, to help interpret data of cortisol levels in welfare assessment. A further aim was to evaluate whether external factors, such as temperature or visitors' attendance have any influence on cortisol secretion.

Materials and methods

Location and study animals

The study was conducted at Terra Natura Zoological Park, Alicante, Spain (38°32'N, 0°08'W). Daily salivary cortisol concentration was recorded for two female, non-pregnant, three- and four-year old Indian rhinoceros (Shiwa and Nisha, respectively), both weighing approximately 1,500 kg. Rhinoceros arrived at this zoological park in March 2003: Nisha came from Stuttgart Zoo and Shiwa from Munich Zoo. Rhinoceros were housed at night in individual cages of 20 m^2 and (from 0900 to 1900h in summer and 0900 to 1800h in winter) ranged freely in a 2,000 m^2 meadow equipped with an artificial lake. Animals were fed on a diet of oats, branches, fruit and vegetables, and provided with water *ad libitum*.

Sample collection

Saliva samples were taken each day at 0730h for a year from the start of August 2005 to the end of July 2006, prior to feeding and cleaning, in order to avoid circadian diurnal variations, given that significantly large variations in cortisol concentrations can occur during the early morning within relatively short time intervals (Brown *et al* 2010). From January 2005, rhinoceros were trained and habituated to saliva sampling by keepers with whom they became familiarised, hence samples were able to be obtained within 2 min. They were collected using the Salivette® kit (Sarstedt, AG & Co, Numbrecht, Germany) and centrifuged at 2,000 rpm for 2 min at 15ºC. The eluted saliva was stored at –20ºC until assaying, which was carried out on a monthly basis during the sampling period (ie samples were frozen for a maximum of one month).

Hormonal analysis

Salivary cortisol was measured in duplicate, using a solid-phase radioimmunoassay (RIA) (Coat-A-Count®, Siemens Medical Solutions Diagnostics, Barcelona, Spain) and the tubes were counted on a Packard Cobra Auto Gamma Counter (Auto-Gamma® 5000 series, Cobra 5005, Packard Instruments Company, Meridien, CT, USA). A minimum of 0.4 ml of saliva was required for the duplicate assay (Menargues *et al* 2008). Modifications were made in the form of increasing the volume of the sample to 200 μL, in the standard curve, through 1:10 dilutions of the standards and by increasing the incubation period to 24 h to increase assay sensitivity (López-Mondéjar *et al* 2006). This assay was previously validated (Menargues *et al* 2008). Sensitivity was 0.82 nmol L^{-1}, inter- and intra-assay coefficients of variation were 6.1 and 6.2%, respectively.

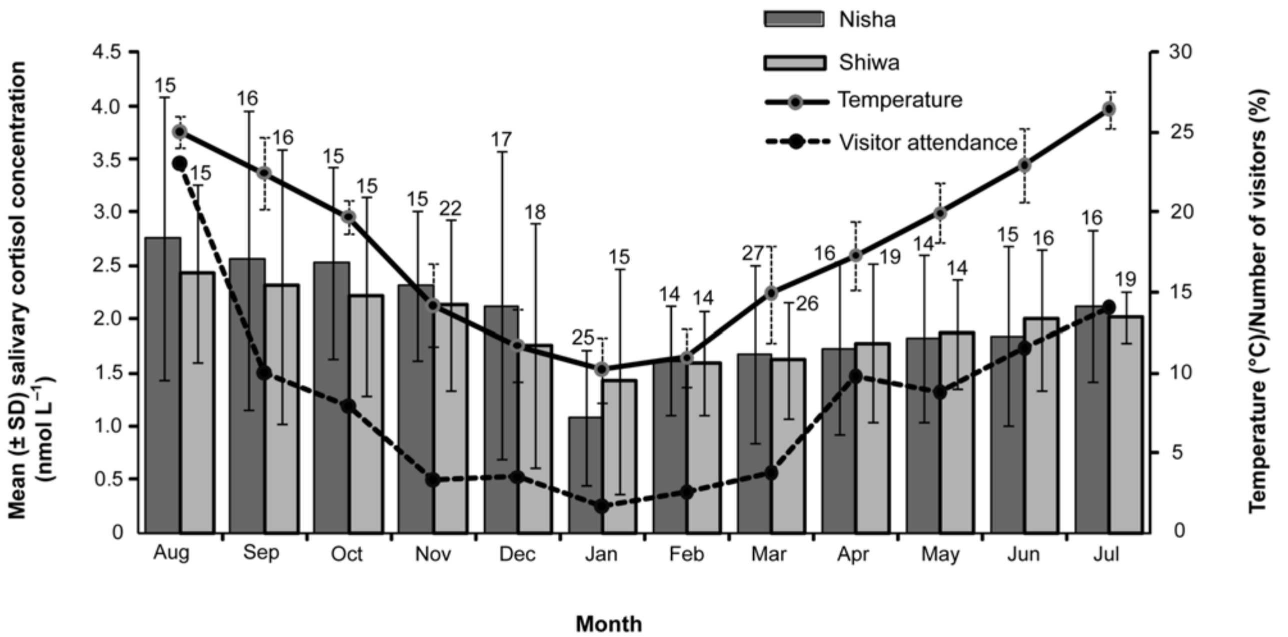
Statistical analysis

Differences in cortisol concentrations between months and individuals (also considering the interaction between both variables) were analysed using a Generalised Linear Model (GLM), with a normal error distribution and an identity log function; pair-wise *post hoc* comparisons were conducted using the Games-Howell test. Pearson's correlation coefficient was used to test whether cortisol profiles of both rhinos (mean monthly values) were correlated and also to test for correlations between mean monthly temperature and visitors' attendance and mean monthly temperature or visitors' attendance and cortisol concentration of both individuals. Temperature data were provided by the Villajoyosa Weather Station (located a distance of 7 km from the Terra Natura Zoological Park) from the Irrigation Technology Service, Valencian Institute of Agrarian Research. Numbers of visitors in attendance during the study period (percentage of the total number of visitors attending the park every month throughout the study period) was provided by the Terra Natura Zoological Park's sales department. We were not supplied with actual visitor numbers each month since this was deemed to be confidential information by the zoological park. Statistical analyses were conducted using SPSS, version 15.0. All values are reported as means (± SD) and statistical significance levels were set at $P < 0.05$.

Results

Mean monthly salivary cortisol concentration ranged between 1.08 (± 0.63) (Nisha, January) and 2.76 (± 1.32) nmol L^{-1} (Nisha, August), with concentrations of both individuals being positively correlated, showing similar values and changing similarly throughout the whole year ($r = 0.940$, $P < 0.001$). There were significant differences in mean salivary cortisol concentration between months ($\chi^2_{11} = 63.93$, $P < 0.001$; Figure 1), due especially to significant differences between the warmest and coldest months (Table 1). Mean monthly salivary cortisol concentration increased between February and August, reaching a peak in August. From August onwards, hormone levels decreased, reaching their lowest values in January (August: Nisha 2.76 [± 1.32], Shiwa 2.43 [± 0.83] nmol L^{-1}; January: Nisha 1.08 [± 0.63], Shiwa 1.42 [± 1.05] nmol L^{-1}). The greatest numbers of visitors were seen throughout the summer months, with the peak coming in August (23% of the annual total). In contrast, attendances were lowest in winter, with January showing only 2% of the total of the annual visitors (Figure 1). On the other hand, there were neither differences between individuals ($\chi^2_1 = 0.73$, $P = 0.392$), nor for the interaction between individuals and months ($\chi^2_{11} = 5.16$, $P = 0.923$). Additionally, mean monthly temperature was

Figure 1



Mean (± SD) monthly salivary cortisol concentration (vertical bars) of two Indian rhinoceros during the year (from August 2005 to July 2006). N-values for each individual and month appear above the errors bars. Solid line represents mean monthly temperature in this same period, with error bars representing standard deviation. Dotted line represents visitor numbers (monthly percentages of the total number of visitors during the study period).

Table 1 Games-Howell *post hoc* multiple comparisons test between months of mean salivary cortisol concentration of two Indian rhinoceros.

Month	January	February	March	April	May	June	July	August	September	October	November	December
January												
February	ns											
March	ns	ns										
April	ns	ns	ns									
May	*	ns	ns	ns								
June	ns	ns	ns	ns	ns							
July	**	ns	ns	ns	ns	ns						
August	**	*	ns	ns	ns	ns	ns					
September	**	ns	ns	ns	ns	ns	ns	ns				
October	**	ns	ns	ns	ns	ns	ns	ns	ns			
November	***	**	*	ns	ns	ns	ns	ns	ns	ns	ns	
December	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

* $P < 0.05$;
** $P < 0.01$;
*** $P < 0.001$;
ns: non-significant.

positively correlated with mean monthly cortisol concentration in both rhinos during the study period (Nisha: $r = 0.597$, $P = 0.04$; Shiwa: $r = 0.751$, $P = 0.005$), also attendance levels were also positively correlated with cortisol concentration in both individuals during the same period (Nisha: $r = 0.596$, $P = 0.041$; Shiwa: $r = 0.716$, $P = 0.009$) and temperature was positively correlated with attendance levels ($r = 0.875$, $P < 0.001$) (Figure 1).

Discussion

A number of studies in mammals have revealed a circannual pattern of cortisol secretion in different species, such as red deer (*Cervus elaphus*) (Ingram *et al* 1999), bottlenose nose dolphins (*Tursiops aduncus*), killer whales (*Orcinus orca*) (Suzuki *et al* 2003) and tuco-tuco (*Ctenomys talarum*) (Vera *et al* 2011). In free-living animals, hormone concentration can fluctuate in response to variables, such as ambient temperature, diet or sex (Goyman 2012). This study reveals that both Indian rhinoceros studied here show a seasonal pattern of salivary cortisol secretion, with the highest levels occurring in August and the lowest in January.

The annual pattern of cortisol concentration in these two animals showed a tendency to increase between February and August, before decreasing until January. This pattern follows annual shifts in monthly temperatures; high levels of salivary cortisol coincided with elevated annual temperatures, whereas low hormone concentrations coincided with the lower temperatures. During the study period, both individuals were maintained with the same feeding conditions and remained indoors for only one extra hour in winter due to insufficient daylight. Romero (2002) proposed that annual changes in glucocorticoid concentrations serve to modulate the priming of stress pathways during periods with different potential exposures to adverse conditions and it could be possible that hot or cold temperatures may represent adverse conditions for this species, thereby influencing cortisol secretion. High cortisol levels have been found in undisturbed red deer in the northern hemisphere in December and January, probably related to low temperatures (Huber *et al* 2003). There are also studies showing that inclement weather can lead to an increase in glucocorticoid levels in white-crowned sparrows (*Zonotrichia leucophrys pugetensis*) (Wingfield *et al* 1983) or arctic ground squirrels (*Urocitellus parryii*) (Sheriff *et al* 2012). However, studies on Asian Elephants (*Elephas maximus*) (whose individuals can reproduce throughout the year) revealed that this species demonstrated a seasonal pattern of salivary cortisol, although no correlation between temperature and annual cortisol secretion was found (Menargues *et al* 2012a).

On the other hand, the annual pattern of cortisol secretion of these two animals described in the present study correlated positively with higher visitor numbers. Creel *et al* (2002) showed that glucocorticoid levels in wolves (*Canis lupus*) and red deer at Yellowstone National Park increased when both species were exposed to snowmobile traffic. In summer, zoological parks receive greater numbers of visitors, which could impact negatively on animal welfare, increasing cortisol levels of individuals (Menargues *et al* 2008). A critical issue regarding exhibited zoo animals is the impact on the behaviour and well-being from visitors (Fernandez *et al* 2009). A number of studies suggest that zoo visitors induce stress in animals, and these effects vary relative to the species (Anderson *et al* 2002; Mallapur *et al* 2005). In Indian blackbuck (*Antelope cervicapra*), zoo visitor density was shown to affect adrenocortical secretion, with levels peaking during the busiest times (Rajagopal *et al* 2011). In black rhinoceros (*Diceros bicornis*), Carlstead and Brown (2005) found that corticoid concentrations were positively correlated with exposure to zoo visitors and several studies also reveal that zoo visitors are a source of stress for primates (eg Hosey 2000). The present study shows that although temperature levels were higher in July 2006 than in August 2005, the number of visitors was higher in the August, however, with our data, we cannot discard the effect of temperature or other possible influences we are yet to consider, such as endogenous mechanisms, diet or period of housing related to daylength (sunlight). Therefore, it would be of great interest to take the aforementioned variables with individuals from other zoos with free-ranging individuals to assess whether an endogenous seasonal pattern of cortisol secretion might exist or if there are other external stressors contributing which could enhance this endogenous rhythm. This would be helpful in determining whether attendance levels is actually an important factor to consider as regards the captive welfare of this endangered species. Knowledge of these circannual rhythms in cortisol secretion is essential in helping establish assessment criteria to interpret data obtained for the assessment of well-being in captive individuals of this species, through salivary cortisol.

Animal welfare implications

This study represents the first attempt at defining the seasonal pattern of cortisol secretion in the Indian rhinoceros. Improved knowledge of this pattern is essential in helping evaluate the physiological significance of fluctuations in cortisol concentrations as stress indicator in animals.

Conclusion

The two Indian rhinoceros studied here display a seasonal pattern of salivary cortisol secretion. The highest cortisol concentration was recorded in August and the lowest in January, coinciding with increased visitor numbers. The cause of this is yet to established, and factors such as attendance numbers, temperature, diet or housing could be considered in future studies to determine their effect on the seasonal pattern of cortisol secretion in these animals.

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