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BRIEF REPORT

Influence of Breeding Season on Fecal Glucocorticoid Levels in Captive Greater Rhea (*Rhea americana*)

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Sex hormones and stress-related changes can be seasonally influenced. We investigate whether fecal glucocorticoid metabolite (FGM) levels can differ between male and female captive Greater Rheas during the breeding and non-breeding seasons. Over a 3-year-period, fresh fecal samples from 10 individuals (five of each sex) were collected during the breeding months (October, November, and December) and non-breeding months (April and June). A total of 960 samples were assayed using a commercial radioimmunoassay. Results showed that FGM levels (mean ± SE) were affected by the breeding season in a sex-dependent way. Male Greater Rheas showed significantly higher FGM levels in the breeding months than in the non-breeding months (13.44 ± 0.37 vs. 7.92 ± 0.1 ng/g feces, respectively). By contrast, females did not show FGM seasonal changes throughout the same sampling periods (7.55 ± 0.14 vs. 7.26 ± 0.73 ng/g feces). Moreover, during the breeding season months, males showed higher average FGM levels than females (13.44 ± 0.37 vs. 7.55 ± 0.14 ng/g feces, respectively), and no differences were found between sexes during the non-breeding season (7.92 ± 0.1 vs. 7.26 ± 0.73 ng/g feces, respectively). Our findings suggest that male Greater Rheas have a higher adrenocortical activity during the breeding season, which is probably indirectly related to the increased testosterone levels and agonist interactions that are also observed during that phase. Studies aimed to determine the appropriate sex *ratio* for captive rearing should be developed to minimize male agonist encounters and therefore improve welfare of the captive group. Zoo Biol. 34:71–75, 2015.

Keywords: ratite; adrenocortical response; non-invasive monitoring; captivity; welfare

INTRODUCTION

The Greater Rhea (*Rhea americana*) is a large, flightless bird endemic to South America that inhabits grasslands and open areas. Free-ranging populations of this ratite have declined drastically in the last years due to several anthropogenic factors [Bellis et al., 2004; Martella and Navarro, 2006; Giordano et al., 2008]. As a consequence, the species has been categorized as "Near Threatened" by the International Union for Conservation of Nature and Natural Resources [IUCN, 2013]. Predictive models generated for Greater Rheas [Giordano et al., 2010] indicate that the high risk of extinction exhibited under the current rate of agricultural expansion can be reduced by supplementing wild populations with captive-bred individuals.

The endangered situation of wild Greater Rhea populations and the importance of raising animals in captivity as a potential contribution to conservation and commercial activity [Navarro and Martella, 2008; Alonso Roldán et al., 2011] have led us to initiate lines of research on stress assessment. Our investigations are aimed to understand factors that may affect wild or captive Greater Rhea populations, thereby contributing to the development of management practices and/or tools for conservation and welfare purposes. These studies showed that adrenocortical response (corticosterone levels) in this species are very sensitive to stressors such as handling and transport [Lèche

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et al., 2013]. Indeed, corticosterone response to an ACTH challenge was found to be considerably higher than that observed in other bird species, which was associated with the flightless condition of this ratite and their use of fast running as antipredator strategy [Lèche et al., 2009]. Greater Rheas also showed sensitive behavioral responses to transport and differences between sexes in the post-transport time-activity budget [Della Costa et al., 2013].

Despite the knowledge that has been gained on the stress physiology of this ratite in captivity, further scientific research related to its reproductive responses is still needed to contribute to welfare and conservation of this species. The mating system of Greater Rhea combines harem polygyny and sequential polyandry [Handford and Mares, 1985] in which several females lay eggs in communal nests and males incubate and care for the precocial chicks. Harem formation in Greater Rheas during the breeding season is associated with a more aggressive behavior among males [Sales et al., 2000; Codenotti and Alvarez, 2001]. Female reproductive behavior has no distinguishable characteristics except for egg laying [Fernández and Mermoz, 2003]. Accordingly, we hypothesized that Greater Rhea males would have significantly higher levels of fecal glucocorticoid metabolites (FGM) during the breeding period, whereas females would not show significant seasonal changes. A recent study investigating the annual variation of FGM levels in free ranging Greater Rheas showed that the highest annual increases coincided with the reproductive period [Lèche et al., 2014]. However, male and female samples were not individualized in that work and, therefore, it was not possible to know if the observed increases were due to changes in males, females, or both. Therefore, the aim of this study was to investigate, under controlled captive conditions, whether FGM levels differ between male and female Greater Rheas during the breeding and non-breeding season. We used captive Rheas as a first approximation to avoid potential uncontrolled environmental and/or human-related variables that could affect/confound he results. Additionally, we used a non-invasive technique for monitoring stress because blood sample collection for hormonal studies is complicated in this species due to their stressful nature, and their large size and muscle strength that make them extremely difficult to capture and manipulate. This method based on measuring FGM has been successfully used in captive [Lèche et al., 2011] and wild populations of Greater Rhea [Lèche et al., 2014].

MATERIALS AND METHODS

Animals and Housing

This study was conducted over a 3-year period (April 2009–June 2011) on a Greater Rhea breeding stock housed at the Córdoba Zoo (31°12, 320S; 64°16, 840W; Argentina). Five adult males and five adult females born in captivity were randomly selected. The group of Greater Rheas was maintained in a large outdoor pen (about 1,000 m^2) with natural soil floor, where food (Vasquetto® processed feed for chicken and Lucerne) and water were provided *ad libitum*.

Fecal Sample Collection

Fresh fecal samples from the selected Greater Rheas were collected monthly during the breeding season (October, November, and December) and non-breeding season (April and June). Each sampling period lasted 4 consecutive days. Feces were collected from birds immediately after deposition, twice a day between 10:00 and 12:00 in the morning and between 16:00 and 18:00 in the afternoon. Fecal samples were frozen immediately and stored in labeled, sealed plastic bags at −20°C until steroid analysis. A total of 480 samples were collected from males and the same number from females. It is important to recall that unlike most avian species, ratite urine is stored and excreted separately from feces [Stewart, 1994] and, therefore, it is not mixed in dropping material.

Steroid Analysis

Fecal glucocorticoid metabolites were extracted using a simple method that was previously applied in this species [Lèche et al., 2011]. Briefly, 5.0 ml of 60% methanol (100% methanol/distillated water) was added to 0.5 g of each well-homogenized fecal sample and stirred with a shaker for 30 min. The tubes were then centrifuged (10 min, 1,200 g), an aliquot of the supernatant (1 ml) was transferred into a new tube and evaporated in a warm water bath (40°C). The extracts were reconstituted in 5.0 μl of methanol 100% + 195 μl of radioimmunoassay (RIA) buffer and briefly vortexed. All FGM measurements were run in duplicate using the commercial ^{125}I corticosterone RIA kit (MP Biomedicals, Costa Mesa, CA, USA) successfully validated for this species [Lèche et al., 2011]. The procedure used was the one provided by the manufacturer. FGM concentrations are expressed as nanograms per gram of wet fecal matter (ng/g). The intra-assay coefficient of variation for randomly selected samples was 2.6% ($n = 10$). The inter-assay coefficient of variation was 7.3% for pooled fecal samples run with each assay ($n = 4$).

Data Analysis

Data from each animal and each sampling period were averaged. A repeated-measures ANOVA that evaluated the effects of the sex (male and female) and the month of the year (the repeated factor) on FGM levels was assessed. Normality and homogeneity of variance were tested. The LSD Fisher test was used for post hoc analysis. Data were expressed as mean ± standard error (SE). Values of $P < 0.05$ were considered significant.

RESULTS

Fecal glucocorticoid metabolite levels varied throughout the year and between sexes ($F_{[11,88]} = 15.60$; $P = 0.0001$; Fig. 1). Greater Rhea males showed seasonal changes in FGM. On average, FGM levels obtained in months of the breeding season were significantly higher than those in months of the non-breeding season (13.44 ± 0.37 vs. 7.92 ± 0.1 ng/g feces, respectively). By contrast, females did not show seasonal changes in FGM levels, which remained the same throughout the sampling period. Average FGM levels observed during months of the breeding season showed similar values to those of the non-breeding season (7.55 ± 0.14 vs. 7.26 ± 0.73 ng/g feces). Moreover, during the months of the breeding season, males showed higher average FGM levels than females. On the other hand, during the non-breeding season, no differences between males and females were observed (7.92 ± 0.1 vs. 7.26 ± 0.73 ng/g feces, respectively). A detailed analysis of the FGM profile of each male during the breeding season revealed that some individuals consistently (four out of the six sampled months) showed higher FGM levels than others (Fig. 2).

DISCUSSION

This is the first study that evaluates indicators of adrenocortical function during the breeding and the non-breeding seasons in captive Greater Rheas. FGM levels were affected by the breeding season in a sex-dependent way, that is, only male glucocorticoid secretion was affected by the reproductive phase and females did not show any significant change throughout the year. In the present study, neither males nor females were intentionally stressed; therefore, the FGM samples were expected to reflect either bird's basal levels or the responses to natural non-forced stressors

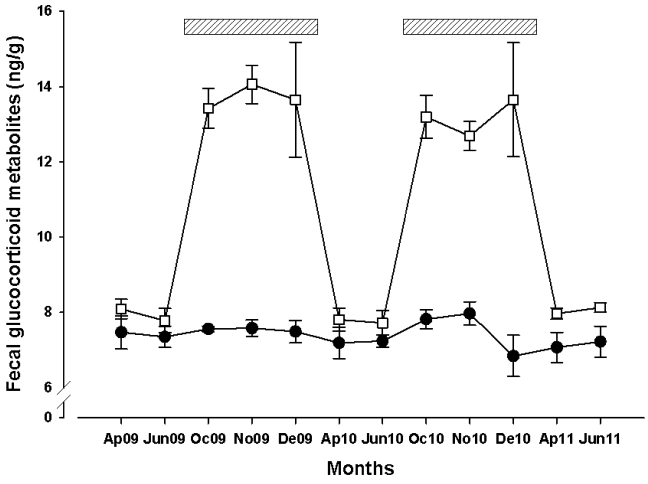

Fig. 1. Mean (±SE) fecal glucocorticoid metabolite levels of Greater Rheas (females: solid dots; males: open squares) during breeding and nonbreeding season months. Timing of reproductive season is depicted by horizontal bars on the top of the graph.

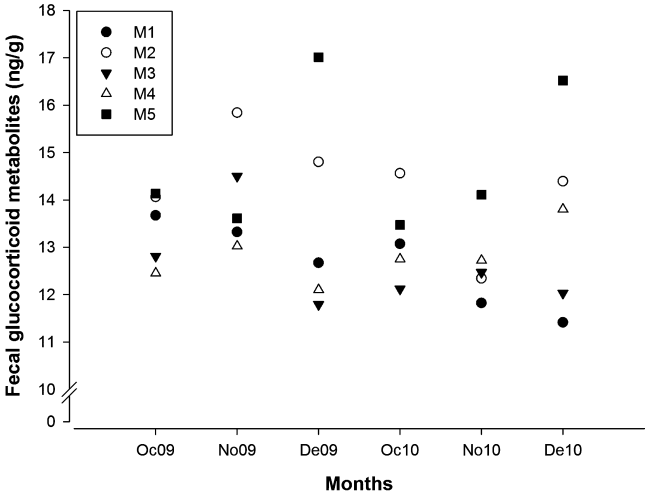

Fig. 2. Individual profiles of fecal glucocorticoid metabolite levels (mean ± SE) of five male Greater Rheas in the breeding season months.

that are concomitant with their behavioral interactions or environmental copying responses. A similar fluctuation in glucocorticoid levels associated with mating/breeding seasonality has already been reported for males of other vertebrate species, such as the Pampas deer (*Ozotoceros bezoarticus*) [Garcia Pereira et al., 2006], the bison (Bison bison) [Mooring et al., 2006], the sifakas (*Propithecus verreauxi*) [Fichtel et al., 2007] and the passerine European nuthatch (*Sitta europaea*) [Landys et al., 2010]. Elevations in glucocorticoid output have been generally linked to increased levels of aggressive interactions [Sapolsky, 1992; Mooring et al., 2006; Landys et al., 2010; Schoof and Jack, 2013]. For example, Garcia Pereira et al. [2006] showed that in male deer, grouping may also influence glucocorticoid secretion, and proposed that groups with multiple males would be more stressed because of the increased competition among males and the resulting increase in agonistic behavior (aggression and subordination). Accordingly, it is reasonable to assume that the higher FGM levels observed in this study during the breeding season are related to the high rate of male-male fighting observed during this time of the year. High testosterone levels have also been recorded throughout the entire breeding period in Greater Rheas [Valdez et al., 2014], which would explain the increased agonist interactions between males during that period and the increased stress levels. Increased fecal glucocorticoid and androgen metabolite levels associated with greater rates of interaction and higher frequency of aggressive and courtship behaviors were observed by Corlatti et al. [2012, 2013] in chamois territorial males, during the rut.

Alternatively, the observed elevated FGM levels might be a consequence of the seasonal modulation of glucocorticoid levels, as it has been shown in many other birds [Romero, 2002; Romero et al., 2006]. Thus, not only animal encounters during reproductive phase as a consequence of

the establishment of social status, but also the consequent increased metabolic rate might have caused the observed increased glucocorticoids. On the other hand, the recorded higher FGM levels in the breeding season should be related to higher male metabolic constraints of reduced food intake, not associated with food shortage but rather with a mate-guarding behavior, as was recorded in other species [Alberts et al., 1996; Komdeur, 2001]. Therefore, the observed male adrenocortical stress response may be due to a combination of repeated aggressive interactions, endurance competition and reduced nutritional intake over several weeks of intense mating activity.

Glucocorticoid secretion appears to be affected by the reproductive rank or status of males (Creel, 2001). In our study, during the breeding season, some males showed higher FGM values than others. Unfortunately, because it was not possible to record individual behavior at the time of this study, we can not rule out whether the males with higher FGM values were those better adapted to the energy demanding social interactions during the breeding season, or the ones that were under a strong stress response. However, regardless of the underlying behavioral mechanism that modulates FGM response, it is clear that some captive males go through the breeding season in better health conditions.

CONCLUSIONS

Our data show that breeding season has a significant effect on glucocorticoid output in captive male Greater Rheas and that this variation is presumably related to the higher levels of testosterone and increased agonist interactions observed in this season. High rates of stressors (i.e., high levels of aggression associated with reproductive competition and reduced food intake due to mating activity) can be associated with higher glucocorticoid levels in Greater rheas. Finally, although the increase in the level of stress response that would enable males to maintain the activities inherent to the breeding season should be considered a "positive stress" (eustress), it may be detrimental for some of the males. Future studies aimed to determine the sex ratio that minimizes male agonist encounters and/or male levels of stress would be important to help improve the welfare of the whole group.

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