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BRIEF REPORT

Influence of the Rearing System on Yolk Corticosterone Concentration in Captive Greater Rheas (*Rhea americana*)

Natalia S. Della Costa,[1] Raul H. Marin,[2] Juan M. Busso,[2] Cristian Hansen,[3] Joaquín L. Navarro,[1] and Mónica B. Martella[1]*

[1]*Instituto de Diversidad y Ecología Animal (IDEA, CONICET-UNC) and Centro de Zoología Aplicada, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Rondeau 798, (X5000AVP) Córdoba, Argentina*
[2]*Instituto de Investigaciones Biológicas y Tecnológicas, Instituto de Ciencia y Tecnología de los Alimentos, (IIBYT, CONICET-UNC), and Cátedra de Química Biológica, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sársfield 1611, (X5000AVP) Córdoba, Argentina*
[3]*Laboratorio de Análisis Clínicos Especializados (LACE), Av. Vélez Sársfield 562, (X5000AVP) Córdoba, Argentina*

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Many environmental conditions elevate plasma corticosterone in laying birds, leading to elevated hormone accumulation in the egg. We investigated whether maternal yolk corticosterone levels in Greater Rheas differ between fresh eggs collected from an intensive (IRS) and a semi-extensive (SRS) rearing system. After HPLC validation, yolk corticosterone was measured using a corticosterone ^{125}I radio-immunoassay kit. Results (mean ± SE) showed that eggs collected from the IRS exhibited a significantly higher corticosterone concentration than eggs from SRS (89.88 ± 8.93 vs. 45.41 ± 5.48 ng/g yolk, respectively). Our findings suggest that rearing conditions under an intensive scheme (e.g., small pens with bare ground, no direct foraging and handling) might be perceived as more stressful for Greater Rhea females than semi-extensive rearing conditions (e.g., low animal density distributed in extensive areas and direct foraging), which would result in the transfer of higher yolk corticosterone levels. A better understanding of environmental conditions and female traits that affect yolk corticosterone deposition provides a background for future studies concerning the roles of maternal corticosterone on offspring development. Zoo Biol. 35:246–250, 2016.

INTRODUCTION

Avian eggs contain a variety of maternal hormones, including steroid (Schwabl, 1993), amino acid-derived (Wilson and Mcnabb, 1997), and peptide hormones (De Pablo et al., 1982). Steroid hormones received great attention from researchers because they are considered one of epigenetic factors that can modify genetic expression in response to the maternal environment (reviewed by Von Engelhardt and Groothuis, 2011). Several research works have evaluated the consequences of maternal stress on the offspring, assuming that those effects are due to hormone transfer from the mother to the egg (see Henriksen et al., 2011, for a review): stress in laying birds induces elevated plasma corticosterone concentrations, leading to elevated hormone accumulation in the egg (Almasi et al., 2012) and therefore influencing embryo development and offspring phenotype (Hayward and Wingfield, 2004).

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*Correspondence to: Mónica Beatriz Martella; Rondeau 798, (X5000AVP) Córdoba, Argentina
E-mail: martemo@efn.uncor.edu

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The Greater Rhea (*Rhea americana*) is a flightless bird endemic to South America that has been classified as "Near Threatened" by the International Union for Conservation of Nature and Natural Resources (IUCN, 2016), due to drastic declines of their free-ranging populations in recent years. This ratite is reared in captivity for conservation purposes, as a reservoir of genetic resources and a source of individuals for reintroduction into the wild, as well as for commercialization of meat, skin, feathers, fat, and eggs (Navarro and Martella, 2011). Captive breeding of Greater Rheas can be performed in intensive rearing systems (IRS) or semi-extensive ones (SRS). In IRS, the animals are kept in pens (which range in size from 1.000 to $<2.000\ m^2$) and fed a diet composed solely of processed feed and chopped alfalfa, whereas in SRS, Greater Rheas are housed in paddocks greater than 1 ha, where they graze on alfalfa and clover (Martella and Navarro, 2006). Captive rearing systems involve management activities that expose Greater Rhea females to stressful situations, increasing their plasma corticosterone concentrations (Lèche et al., 2013) and altering their behaviors (Della Costa et al., 2013); however, it is still unknown whether these high corticosterone levels are transferred to the offspring. Therefore, here we validated an immunoassay to determine the presence of maternal corticosterone in eggs of Greater Rheas aiming to assess whether maternal yolk corticosterone concentrations differ between fresh eggs collected from an intensive and a semi-extensive rearing system.

METHODS

Egg Collection

During the egg laying season (September–December), Greater Rhea eggs were collected from an SRS located at the YPF Monte Cristo plant in Córdoba city, Argentina (31° 21′ S, 63° 58′ W), and from an IRS located at the Córdoba Zoo, Argentina (31° 25′ S, 64° 10′ W). Most individuals of the IRS population come from the SRS; therefore, the two populations are closely genetically related (Lábaque et al., 2004).

In the SRS, 14 adult males and 13 adult females were housed in a 30 ha enclosure; feeding involved direct foraging on vegetation present in the farm, composed mainly of grasses and dicot herbs, and processed feed (Vasquetto®, Córdoba, Argentina) and water ad libitum. Greater Rheas had occasional visual and auditory contact with the farmers. Since the SRS was located in a rural environment, animals were not exposed to urban or vehicular noise nor where they, under any circumstance, manipulated by humans.

In the IRS, 4 adult males and 10 adult females were housed in a 0.02 ha pen with bare ground, and fed ad libitum on processed feed (Vasquetto®) and chopped alfalfa. Water was provided ad libitum. Visual and auditory contact of animals with humans (farmers and Zoo visitors) and with other animal species housed in the Zoo was permanent and animals were continually exposed to urban and traffic noise. Moreover, Greater Rheas were regularly manipulated during veterinary controls.

We measured yolk corticosterone from four eggs collected from the SRS and nine eggs from the IRS. Sample size was restricted to the amount authorized at each site, since the animals are included in reintroduction programs (Lèche et al., 2015). We kept sampled eggs in the nest for a maximum of 24 h to avoid hormone production by the developing embryo (Groothuis and Von Engelhardt, 2005). Upon collection, we individually marked them with a pencil, determined their fresh weight to the nearest 0.01 g and froze them at −20°C until steroid extraction. We used the egg weight as it is the easiest most accurate and measurable direct indicator of egg size or volume, as it was verified by Lábaque et al. (2007).

High-Pressure Liquid Chromatography With Ultraviolet Detector (HPLC-UV) Analysis

We conducted reverse-phase HPLC (Column Syncronis C18, 5 μm, 250 × 4,6; Perkin Elmer, serie 200, UV/VIS detector) to separate and determine the presence of corticosterone in Greater Rhea yolk. Since standards showed maximum absorbance at 223 nm for corticosterone, 238 nm for cortisol and cortisone, we operated the UV spectrometer at these wavelengths for the simultaneous determination of steroidal concentrations. Additionally, since progestagen concentrations in the yolk may yield measurement errors of yolk corticosterone (Quillfeldt et al., 2011), we also considered progesterone as standard at 223 nm of maximum absorbance. Testosterone was also determined at 223 nm. We extracted steroids from yolk according to Rettenbacher et al. (2013). Briefly, we homogenized yolk of each egg and took a portion of yolk (0.714 g per egg) and mixed it in order to obtain 5 g (0.714 g per egg × 7 eggs), which we mixed with 10 mL of double-distilled water and stirred for 30 min. We repeated this procedure three times to obtain three samples of 5 g each (21 eggs were sampled in total for three HPLC analyses). Then, we added of 30 mL methanol (HPLC grade) dropwise in each sample and stirred it for 30 min. We centrifuged the sample and diluted 30 mL of the supernatant with 45 mL of double-distilled water; we filtered the sample through a C-18 matrix column (Supelclean, LC-18 Cartridges, SUPELCO, Belletonte, PA, USA) via air flow. Elution was dried (60°C) and suspended in 0.1 mL methanol, centrifuged and supernatant (20 μL) was injected into HPLC. We also performed these procedures adding steroid standards to yolk before extraction in order to monitor recovery and possible interferences in HPLC-UV analysis. We eluted 20 μL by HPLC using methanol–water gradient (20–100%:80–0%) for 80 min, at a flow rate of 1 mL/min. We used the retention time of the peak coincident with the respective standard steroid to identify a steroid peak (Busso et al., 2007). Additionally, to assess immunoreactivity, we separately collected two minute-fractions during elutions

from each HPLC analysis, and then took fractions to dryness (60°C). Finally, we reconstituted all two minute-fractions in immunoassay buffer to test immunoreactivity by RIA.

Quantification of Yolk Corticosterone

We defrosted the eggs collected for hormone analysis until the albumin could be scraped off from the yolk with a spatula. We extracted each yolk as described by Almasi et al. (2012). We homogenized the yolk by swirling with a spatula. From each egg, we diluted 0.15 g of yolk in 600 μL of distilled water, vortexed for 30 sec and froze overnight. On the next day, we added 3 mL of 100% methanol. We shook samples for 30 min and froze them overnight. After centrifugation, 1 mL of the supernatant was evaporated under a stream of nitrogen and then resuspended in 250 μL of assay buffer. We determined corticosterone concentrations in 50 μL using a commercially available ^{125}I corticosterone-radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA), which we validated to assess corticosterone and corticosterone metabolites in this species (Lèche et al., 2009, 2011).

Avian follicle cells have the enzymatic capacity to synthesize a plethora of steroid hormones and hormone metabolites (Porter et al., 1989) and it is therefore likely that yolk contains various steroids that have not yet been identified. Because the measured signal in the yolk was caused by two or more hormones or hormone metabolites that bind to the antibody (Rettenbacher et al., 2009), we will refer to immunoreactive substances detected by our corticosterone antibody as "yolk corticosterone." Mean intra and inter-assay coefficients of variation were 11.89% and 11.18%, respectively. The immunoreactivity of the fractions was expressed as percentages of the corticosterone determined.

Statistical Analyses

We performed all statistical analyses using the Infostat (Córdoba, Argentina) statistical software package (Di Rienzo et al., 2012). We performed a mix model to evaluate the effect of rearing system (SRS and IRS) on yolk corticosterone concentration, including Identity variance function to rearing system, and egg as random factor. We tested data for normality of residuals using the Shapiro–Wilks modified test. Means of main effects were compared using Fisher's LSD. Values are expressed as the mean ± standard error (SEM) and the significance level was set at 0.05.

RESULTS

High liquid chromatography analysis of the steroid extract from Greater Rhea egg yolks indicated the presence of corticosterone in the 57–58 fractions (Fig. 1). Neither cortisol nor cortisone was detected in the same yolk extract. The immunoreactivity test using the 57–58 fractions showed the highest reaction of the antibody to corticosterone confirming RIA detection of this glucocorticoid. We observed less important reactions in the fractions where other steroids were identified (cortisol 0%, cortisone 0%, testosterone 1.4%, and progesterone 2.5%). Crossed reactions were also detected on fractions immediately before and after corticosterone elusion (10.9% and 15.9%, respectively), and on fractions 63–64 (21.2%) and 79–80 (5.0%).

The eggs collected from the IRS exhibited higher yolk corticosterone concentrations than those collected from the SRS ($F_{1,11} = 18.01$, $P < 0.01$; 89.88 ± 8.93 vs. 45.41 ± 5.48 ng/g, respectively). We observed this difference even in eggs of the same weight (Fig. 2).

DISCUSSION

In this study, the antibody used in the immunoassay reacted mainly with corticosterone and crossed reaction with progesterone was found low compared with the percentage of the crossed reaction reported elsewhere using other immunoassays (Rettenbacher et al., 2009, 2013; Quillfeldt et al., 2011). This is particularly relevant in Rheas where progesterone concentration in eggs is about 20 times higher than corticosterone (PhD thesis in prep). Cross reaction with an unknown substance was also observed in the fractions 63–64. It affected the total corticosterone determination in a higher percentage than progesterone (21.2% vs. 2.5%). It is interesting to note that the antibody present in this corticosterone kit was also able to detect corticosterone reacting metabolites in fecal samples (Lèche et al., 2011).

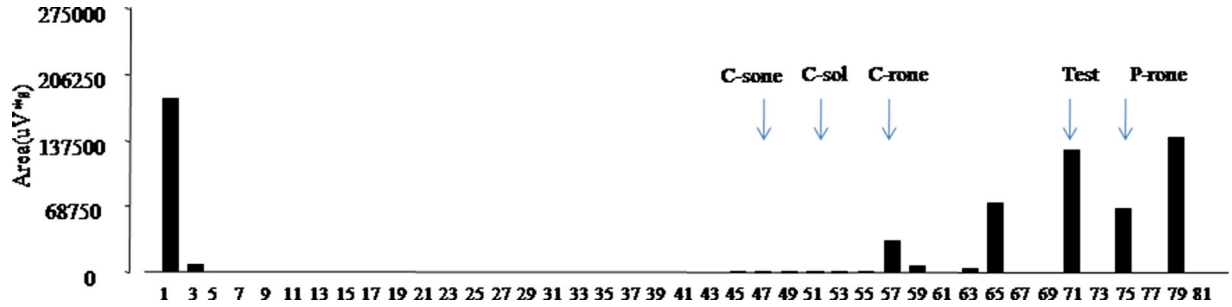

Fig. 1. High-pressure liquid chromatography separation of three yolk extracts obtained from Greater Rhea eggs ($n = 7$ each extract). Elution position of standards is indicated by arrows: C-sone, cortisone; C-sol, cortisol; C-rone, corticosterone; Test, testosterone; and P-rone, progesterone.

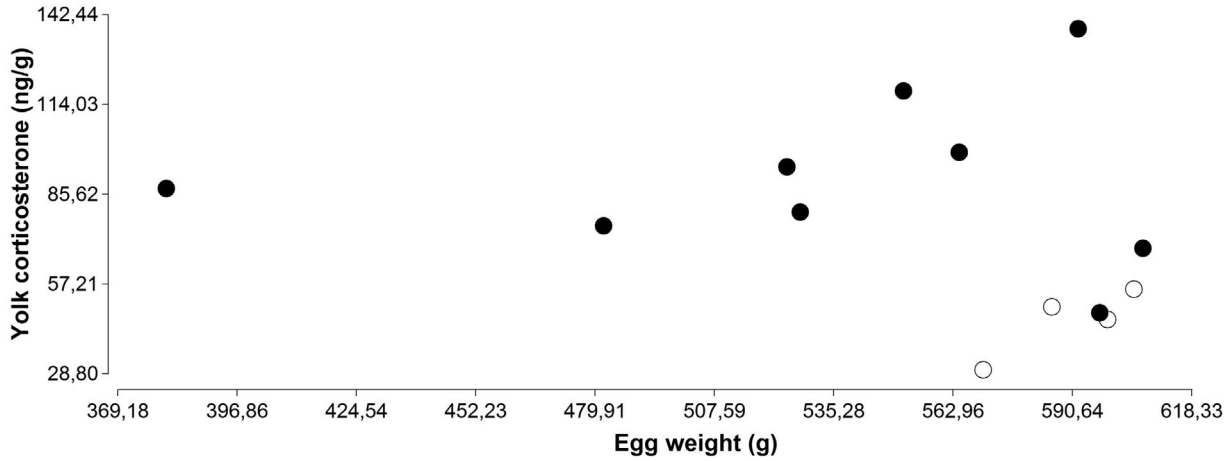

Fig. 2. Yolk corticosterone (ng/g) versus egg weight (g), in Greater Rhea eggs collected from the IRS (solid dots, $n = 9$) and SRS (open dots, $n = 4$).

Thus, it is conceivable to think that the unknown substance reacting in fractions 63–64 is also a corticosterone derived metabolite. However, more experimentation is needed to determine the exact chemical composition and potential implication of this particular substance.

Greater Rhea females bred under intensive captive conditions transferred higher corticosterone levels to the eggs than females housed in a semi-extensive rearing system. Hence, environmental conditions at the IRS may have been perceived as more stressful by Greater Rhea females than conditions at the SRS, which caused an increase in females' plasma corticosterone concentrations and, therefore, a greater deposition of this hormone in the eggs.

In farmed species, it has been shown that different environmental conditions and husbandry practices induce elevated plasma corticosterone concentrations in females (Fraisse and Cockrem, 2006; Lèche et al., 2013), leading to elevated hormone accumulation in the egg (Hayward et al., 2005; Downing and Bryden, 2008; Okuliarová et al., 2010). In this study, for example, the lack of natural pastures in the pen with bare ground of IRS could be perceived by Greater Rhea females as a stressful condition that affected their natural foraging behavior. It has been shown that environmental enrichment (fruits and vegetables scattered around the enclosure) reduced the abnormal behaviors in captive Greater Rheas and increased their time allocated to foraging and walking (Azevedo et al., 2013). The Greater Rhea is a highly selective feeder both in captivity (Bazzano et al., 2011) and in the wild (Martella et al., 1996); therefore, a rearing system that facilitates the development of the normal foraging behavior in females might reduce stress and optimize their welfare.

In Greater Rheas, diet composition and traits of laying females have been found to influence egg characteristics (Lábaque et al., 2010, 2013). Here, we may assume that there were no nutritional differences between the diets offered in the two rearing systems, since females from both IRS and SRS had grasses and balanced feed available ad libitum. However, we could not include the female phenotypic variables in the analysis because we did not identify the females that laid each egg. The set of environmental factors of each rearing system and their interaction with females' phenotypic traits may have determined the corticosterone levels that they transferred to the yolk. Therefore, future experimental studies would be needed to know the factors and mechanisms that influence corticosterone deposition in Greater Rhea eggs and the implications of embryo exposure to different levels of maternal corticosterone in the offspring development and performance.

CONCLUSIONS

We evaluated an available corticosterone ^{125}I radioimmunoassay kit for yolk corticosterone measurement. Greater Rhea eggs collected from an intensive rearing system exhibited significantly higher maternal corticosterone concentration than those from a semi-extensive rearing system. Rearing conditions under an intensive scheme (e.g., small pens with bare ground, no direct foraging and handling) might be perceived as more stressful for Greater Rhea females than semi-extensive rearing conditions (e.g., low animal density distributed in extensive areas and direct foraging), which would result in the transfer of higher yolk corticosterone levels in the former system. This work is the first contribution of its type in ratites and, although not conclusive in some aspects, it uses a working protocol that adds to the understanding of the roles of maternal corticosterone in offspring development.

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