Received: 14 November 2016 | Revised: 4 April 2017 | Accepted: 5 April 2017

DOI: 10.1002/jez.2073

RAPID COMMUNICATION

WILEY JEZ-A ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY

Distribution and concentration of maternal progesterone in the yolk of Greater Rhea eggs (*Rhea americana*)

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Grant sponsor: Secretaría de Ciencia y Tecnología of the Universidad Nacional de Córdoba (SECyT-UNC); Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica de Argentina; Grant numbers: Proyecto 1170 and PICT 2012.

Abstract

Progesterone is the most concentrated maternal yolk steroid characterized to date in birds; however, no information about it is available in ratite eggs. We collected freshly laid eggs from zoo-housed Greater Rhea females (*Rhea americana*) bred under similar rearing conditions during two breeding seasons to characterize concentration and distribution of maternal yolk progesterone. After high-performance liquid chromatography analysis, yolk hormone was measured using a commercial electrochemiluminescence immunoassay. Progesterone concentrations were found to vary significantly among the yolk layers, supporting a follicular origin for this steroid in Greater Rhea eggs. Additionally, highly similar mean absolute yolk progesterone concentrations were detected between 2013 and 2015 breeding seasons (1,332.98 ± 82.59 and 1,313.59 ± 85.19 ng/g, respectively). These values are also comparable to those found in some domestic carinate species. Findings suggest that at population level, when rearing conditions are similar, mean absolute yolk maternal progesterone concentrations also appear bounded. Future research on the factors and mechanisms that regulate progesterone deposition in Greater Rhea eggs is needed to better understand whether its levels depend on different rearing conditions.

KEYWORDS

ratite, Greater Rhea, steroid hormone, yolk progesterone

1 | INTRODUCTION

Freshly laid eggs, especially their yolks, contain substantial levels of maternal hormones, which influence the offspring development (see Groothuis & Schwabl, 2008, for a review). Several factors modulate the yolk hormone levels, such as the genetic background, the female environment and quality, social interactions (Schwabl, 1997; Whittingham & Schwabl, 2002; Pilz & Smith, 2004), and the male quality (Gil, Graves, Hazon, & Wells, 1999; Gwinner, Yohannes, & Schwabl, 2013). Furthermore, yolk hormone levels could also vary among clutches (Gil, 2008), and even across the laying sequence within a clutch (Eising, Eikenaar, Schwabl, & Groothuis, 2001; Schwabl, 1993, 1997). According to that, hormones in bird eggs are interesting as a potential mechanism by which information experienced in one generation can be transmitted to the next generation and thereby allow flexible adjustment of development to prevailing environmental conditions (reviewed by von Engelhardt & Groothuis, 2011).

Progesterone is the most concentrated yolk steroid characterized to date in carinate species (Hackl, Bromundt, Daisley, Kotrschal, & Möstl, 2003; Möstl, Spendier, & Kotrschal, 2001), and serves as a precursor in the production of other steroids such as testosterone, estradiol, and corticosterone (reviewed in Payne & Hales, 2004). As both testosterone and progesterone can be metabolized by some of the same steroidogenic enzymes, the amount of progesterone in the yolk may influence testosterone metabolism due to enzyme kinetics or competition for cofactors involved in steroid metabolism (Paitz & Casto, 2012). Progesterone is produced in the follicle granulose cell layers (Bahr, Wang, Huang, & Calvo, 1983) and because the steroidogenic activity of the follicle wall changes with follicle maturation, progesterone concentrations are highly variable between the different yolk layers (Lipar, Ketterson, Nolan, & Casto, 1999).

Although it is the principal hormone surrounding avian embryos in the early stage of development, progesterone has not received much research attention to date and little is known about its role in the

development of the embryo. Recent studies have shown that steroid metabolism takes place in avian eggs during the early part of development (von Engelhardt, Henriksen, & Groothuis, 2009), and it has been hypothesized that progesterone plays a role in regulating that process (Paitz & Bowden, 2010). Moreover, in European starlings, it has been demonstrated that yolk progesterone concentration during incubation depends on embryonic development (Paitz & Casto, 2012). Additionally, there is some evidence that progesterone affects the growth of chick embryo (Ahmad & Zamenhof, 1979; Schär, 1967), influences embryonic heart rate and prenatal perceptual learning, as well as emotional reactivity after hatching (Bertin et al., 2015; Herrington, Vallin, & Lickliter, 2015, 2016). Metabolites of progesterone, such as pregnanediol and pregnanolone, have also been shown to produce biological effects such as increased porphyrin and hemoglobin synthesis in bird embryos (Granick & Kappas, 1967; Irving, Mainwaring, & Spooner, 1976; Levere, Kappas, & Granick, 1967).

To our knowledge, corticosterone is the only maternal yolk steroid quantified to date in ratite eggs (Della Costa et al., 2016), and no information about yolk progesterone is available in this group of birds; therefore, this study aims to characterize the distribution and concentration of maternal yolk progesterone in freshly eggs laid by captive Greater Rhea females bred under similar rearing conditions during two breeding seasons. First, for this purpose, we validated a commercial electrochemiluminescence immunoassay kit for progesterone concentration measurement.

2 | MATERIALS AND METHODS

2.1 | Animals and egg collection

We studied a population of Greater Rheas (*Rhea americana*) hatched in captivity at the experimental farm at Córdoba Zoo, Argentina (31°S, 64°W). Greater Rhea is a South American ratite bird included in the "Near threatened" IUCN category (IUCN Red List of Threatened Species, 2017) that is usually reared in captivity for both commercial and conservation purposes (Navarro, & Martella, 2011). During 3 years (2013–2015), 14 adult birds (10 females and 4 males above 2 years of age) were maintained under similar rearing conditions: all birds were housed in 0.02-ha pens with bare ground, received Vasquetto® processed feed for chicken, chopped alfalfa and water *ad libitum*, and they were also exposed to natural light and temperature conditions and had access to a 10-m^2 roofed shelter. The quality, quantity, and variety of diet did not change during these years, nor did the group size, sex ratio, protocols of cleaning of pens, veterinary controls, and handling.

During the 2013 and 2015 breeding seasons, freshly laid eggs from all clutches were collected (27 and 16 eggs, respectively) by the same experienced farmer. None of the eggs stayed in the nest for more than 24 hs to prevent the metabolism of the hormone by the developing embryo (Groothuis & von Engelhardt, 2005). Egg sample size was restricted to the maximum amount authorized in the Zoo, as the birds were also included in a reintroduction program (Lèche et al., 2016; Vera Cortez, Valdez, Navarro, & Martella, 2015). All eggs were collected during the intermediate laying period of each season, when the peak of egg production occurs (Lábaque, Martella, Maestri, Hoyos, & Navarro, 2010). Although the identity of the laying females could not be discerned, we estimated that 7 of the 10 females had laid the eggs collected during this study, considering the total number of eggs laid during each season, and the average number of eggs laid per female in this Zoo as reference (21 ± 3.5 eggs per season; Lábaque et al., 2010). The proposal of this work was approved by the ethics committee of the CONICET (Resolution 1047 Annex II, 2005) before its implementation as part of the author's fellowship project.

2.2 | High-performance liquid chromatography analysis

We extracted steroids from egg yolk according to Rettenbacher, Henriksen, Groothuids, and Lepschy (2013). Briefly, we homogenized three egg yolks, and 5 g was mixed with 10 mL of double-distilled water and stirred for 30 min. Then, 30 mL methanol (high-performance liquid chromatography [HPLC] grade) dropwise was added in each sample and stirred for 30 min. Samples were then centrifuged and 30 mL of the supernatant was diluted with 45 mL of double-distilled water and then filtered through a C-18 matrix column (Supelclean, LC-18 Cartridges, Supelco) via air flow. Elution was dried at 60°C and suspended in 0.1 mL methanol, centrifuged, and supernatant (20 μL) was injected into 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 progesterone in Greater Rhea yolk. We also performed these procedures adding steroid standards to the yolk before extraction to monitor the recovery and possible interferences during HPLC analysis.

Because standards showed maximum absorbance at 223 nm for progesterone and corticosterone, and 238 nm for cortisol and cortisone, we operated the UV spectrometer at these wavelengths for the simultaneous determination of steroid concentrations. Then, 20 μL was eluted during 80 min at a flow rate of 1 mL/min using a methanol–water gradient (20–100%:80–0%). The retention time of the peak coincident with the respective standard steroid was used to identify the steroid peak (Busso, Ponzio, Fiol de Cuneo, & Daniel Ruiz, 2007). Additionally, to assess immunoreactivity, 2 min fractions were separately collected during the elution and dried for further hormonal analysis. Finally, all fractions were reconstituted in immunoassay buffer to assess immunoreactivity by electrochemiluminescence immunoassay.

2.3 | Quantification of yolk progesterone

As described by Lipar et al. (1999), the frozen yolk from the albumin was separated by taking advantage of the fact that albumen thaws more quickly than yolk. The yolk was then weighed and its diameter was measured. Eggshells were also separated, washed with water to remove the albumin, dried and weighed. We determined the weight of the albumin by subtracting the weight of the eggshell and the yolk from the total mass of the egg. We considered yolk, albumen, and shell weight as indicators of egg quality. Following Lipar et al. (1999), we prepared three layers from each yolk to assess the hormone concentrations in the exterior, intermediate, and interior layers of the

yolk sphere: the frozen yolk was dissected with a knife, and a disk of approximately 1-cm thick covering the core of the yolk was first removed, the disk was then cut to a three-dimensional rectangular structure, and yolk samples (0.30 g) were then excised from the interior, intermediate, and exterior portions of the yolk. According to Henriksen, Groothuis, and Rettenbacher (2011), each layer sample was extracted by mixing the 0.30 g of yolk with 1.2 mL of double-distilled water, homogenized with a hand vortex, and frozen overnight. On the next day, samples were shaken for 15 min before 6 mL of methanol (100%) was added. The solution was shaken for 30 min and centrifuged at 2,500 *g* for 15 min.

For the progesterone immunoassay analysis, the methanolic suspensions were simply diluted with assay buffer (1:10). Progesterone concentrations were measured using a commercial electrochemiluminescence immunoassay kit (Elecsys Progesterone II from ROCHE). Samples were analyzed in COBAS 6001 equipment, with a module for immunoassay e 601 (HITACHI High Technology Corporation-ROCHE Diagnostic GmbH). Three samples were serially diluted in the assay buffer, and their displacement curves were parallel to the standard curve. Presence of progesterone was detected in samples of known concentrations starting as low as 1.2 ng/g, whereas the minimum quantifiable concentration was 4.8 ng/g. Mean intra- and interassay coefficients of variation were 4.9% and 6.5%, respectively.

For each egg, we calculated the absolute progesterone concentration of whole yolk by multiplying the measured concentration of each layer with the calculated weight of the respective layer, as described by Rettenbacher, Möstl, Hackl, and Palme (2005). Progesterone concentration was expressed as nanograms of hormone per gram of yolk (ng/g).

Avian follicle cells have the enzymatic capacity to synthesize a plethora of steroid hormones and hormone metabolites (Porter, Hargis, Silsby, & Halawani, 1989), and it is therefore likely that yolk contains steroids that have not yet been identified. Because the measured signal in the yolk could be caused by two or more hormones or hormone metabolites (other progestogens, in our case) that bind to the antibody (Rettenbacher, Möstl, & Groothuis, 2009), we will refer to the immunoreactive substances detected by our progesterone antibody as "yolk progesterone."

2.4 | Statistical analyses

We used the Infostat statistical software package (Di Rienzo et al., 2012). A mixed general linear model was used to compare progesterone concentrations among exterior, intermediate, and interior yolk layers, including egg as random factor. Hormonal concentration data were transformed to natural logarithm for normality of residuals. We also used mixed models to evaluate the effect of breeding season on absolute yolk progesterone concentrations, and on yolk, albumen, and shell weight, also including egg as random factor. Albumen weight data were transformed to sequential ranks (Shirley, 1987) for normality of residuals. We tested data for normality of residuals using the Shapiro–Wilks modified test. Means of main effects were compared using Fisher's least significant difference post hoc test. Values are expressed as the mean ± SEM and the significance level was set at 0.05.

3 | RESULTS AND DISCUSSION

HPLC analysis of yolk extracts from Greater Rhea eggs revealed a peak that coeluted with the reference standard progesterone (Fig. 1). The immunoreactivity of the fractions obtained after HPLC showed that the fraction 80 had the maximum immunoreactivity value and the same retention time as the external standard progesterone (Fig. 1). These findings validated the electrochemiluminescence immunoassay procedure as an accurate method for measuring maternal progesterone in Greater Rhea eggs and that could (prior validation) also be used on other bird species.

We observed significant variations in the concentration of progesterone among the yolk layers of Greater Rhea eggs ($F_{2,94}$ = 418.31, $P < 0.01$). Progesterone concentration in the exterior layer was significantly higher than those in both the intermediate and interior layers, which also differed from one another ($P < 0.05$ in all cases, Fig. 2). This pattern of hormone deposition observed in Greater Rhea eggs is consistent with the steroidogenic activity of the follicle wall that depends on follicle maturation (Bahr et al., 1983), and agrees with the pattern

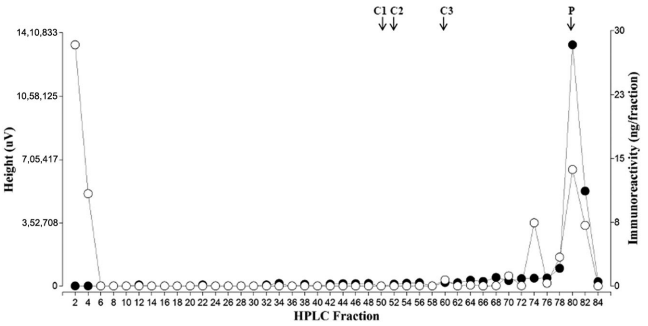

FIGURE 1 High-pressure liquid chromatography (HPLC) separation of yolk extract obtained from Greater Rhea eggs (open circle) and immunoreactivity (filled circle) of each HPLC 2-min fraction measured by an electrochemiluminescence immunoassay

Notes: Arrows indicate elution position of cortisone (C1), cortisol (C2), corticosterone (C3), and progesterone (P) standards.

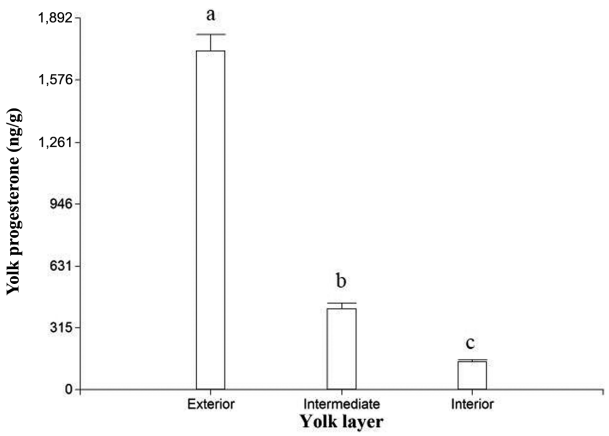

FIGURE 2 Mean (±SEM) progesterone concentration in the exterior, intermediate, and interior layers of Greater Rhea egg yolk ($n = 43$ eggs)

Notes: Levels not connected by the same letter (a, b, c) are significantly different ($P < 0.05$).

TABLE 1 Absolute yolk progesterone concentration and weight of greater rhea egg components (yolk, albumen, and shell), in two breeding seasons

	2013 Breeding Season ($n = 27$)	2015 Breeding Season ($n = 16$)	Statistic Values
Absolute yolk progesterone concentration (ng/g)	1,332.98 ± 82.59	1,313.59 ± 85.19	$F_{1,9} = 0.02, P = 0.88$
Yolk weight (g)	151.91 ± 3.19	158.93 ± 4.37	$F_{1,9} = 1.73, P = 0.22$
Albumen weight (g)	326.5 ± 9.26	328.74 ± 8.8	$F_{1,9} = 0.02, P = 0.87$
Shell weight (g)	81.95 ± 2.4	88.41 ± 2.23	$F_{1,9} = 3.29, P = 0.10$

In neither case, there were significant statistical differences.
n, number of eggs measured.

described in carinate species (Hackl et al., 2003; Lipar et al., 1999; Möstl et al., 2001). As reported by Lipar et al. (1999), the presence of variation in the concentrations of progesterone has implications for the reliability of yolk sampling techniques, considering that the different layers would contribute with highly different amounts of yolk if it was homogenized prior to analysis.

Mean absolute yolk progesterone concentrations were the same during the two breeding seasons (Table 1) suggesting that at a population level, when rearing conditions are similar, mean absolute yolk progesterone concentrations also appear bounded. Moreover, our results showed that egg quality (measured in this study as yolk, albumen, and shell weight) did not change between seasons (Table 1). In Greater Rheas, diet composition and traits of laying females have been found to influence egg characteristics (Lábaque et al., 2010, 2013); therefore, the stability that we found in egg quality during the breeding seasons is in accordance with the idea that rearing conditions did not change substantially across the years.

The mean absolute yolk progesterone concentration, considering all eggs collected during the two breeding seasons, was 1,325.77 ± 60.15 ng/g ($n = 43$), and it was comparable to those found in other domestic bird species (Bertin et al., 2013; Okuliarova, Kostal, & Zeman, 2011). According to the reported information revised in Groothuis and von Engelhardt (2005), yolk progesterone levels quantified in this study should have derived from the laying female, because the hormone was detected in the yolk of freshly laid eggs, before the embryo was able to start its own synthesis. Additionally, absolute yolk progesterone concentrations in all eggs collected ranged from 651.85 to 2,287.32 ng/g, and this variation was not associated with the yolk weight (Fig. 3). In other bird species, it has been shown that yolk hormone levels vary with the egg´s composition such as yolk mass or carotenoid levels (von Engelhardt & Groothuis, 2011). Moreover, yolk hormone levels could vary between females and both between and within clutches, according to the position in the laying order (Carere & Balthazart, 2007; Schwabl, 1993). In this study, we could not include female traits in the analysis, because we were not able to identify the female that laid each egg; therefore, future studies are needed to explain these hormonal variations in Greater Rhea eggs.

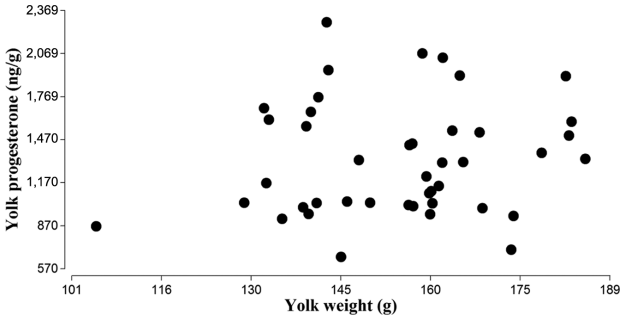

FIGURE 3 Absolute yolk progesterone concentration (ng/g) versus yolk weight (g) in Greater Rhea eggs (n = 43)

Many studies report variations in the yolk hormone levels when environmental conditions change, such as temperature (Bertin et al., 2013), food quality (Gasparini et al., 2007; Verboven et al., 2003), breeding density (Groothuis & Schwabl, 2002), and social interactions (Gil et al., 2006; Navara, Siefferman, Hill, & Mendonça, 2006). These variable amounts of hormones deposited into egg yolk that could potentially affect embryonic growth and development (von Engelhardt & Groothuis, 2011), could be explained as the result of context and dose-dependent optimal strategies to maximize offspring fitness, in which the balance between costs and benefits depends on the prevailing conditions (Muriel et al., 2015). Consistently, the variation in yolk corticosterone levels observed among eggs of Greater Rhea females bred under different rearing conditions, such as size of pens and presence or lack of natural pastures in the pens (Della Costa et al., 2016), suggests that yolk steroids in this species could also function as indicators of relevant environmental conditions for the offspring development. Future experimental studies are needed to know whether yolk progesterone levels in Greater Rheas are also regulated under different rearing conditions. Nevertheless, our findings provide the basis for future research on the regulation of progesterone deposition in Greater Rhea eggs to contribute to enlighten its influence on the offspring development.

4 | CONCLUSIONS

Overall, in this study an electrochemiluminescence immunoassay was validated as an accurate method for measuring maternal yolk progesterone in Greater Rhea eggs. The distribution of progesterone in the yolk spheres supported a follicular origin for this hormone. Finally, our finding of similar yolk progesterone levels in two nonconsecutive breeding seasons suggests that when rearing conditions are similar, yolk progesterone concentrations also appear bounded.

ACKNOWLEDGMENTS

We are grateful to the Jardín Zoológico de Córdoba (Argentina) for allowing us to conduct this research. This work was made possible

because of the generous collaboration of L. Giojalas. G. Giacone helped in English language. We are grateful to the anonymous reviewers for their comments that helped improve a previous version of this manuscript. N.S.D.C. is a student of the Doctorado en Ciencias Biológicas, FCEFyN—UNC, and fellow from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). J.L.N., J.M.B., R.H.M., and M.B.M. are researchers of CONICET. This work was supported by grants from the Secretaría de Ciencia y Tecnología of the Universidad Nacional de Córdoba (SECyT-UNC) and the Agencia Nacional de Promoción Científica y Tecnológica de Argentina (FONCyT, Proyecto 1170, PICT 2012), to M.B.M.

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How to cite this article: Della Costa NS, Navarro JL, Busso JM, Marin RH, Martella MB. Distribution and concentration of maternal progesterone in the yolk of Greater Rhea eggs (*Rhea americana*). *J. Exp. Zool.* 2017;327:143–148. https://doi.org/10.1002/jez.2073