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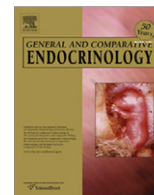

Short Communication

Measuring corticosterone in seabird egg yolk and the presence of high yolk gestagen concentrations

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ABSTRACT

Large inter-species differences have been found in yolk corticosterone amounts in avian eggs. While some studies have failed to detect significant amounts of corticosterone, in other species high amounts have been recorded, such as in a recent study of southern rockhopper penguins *Eudyptes chrysocome chrysocome*. However, attention has been drawn recently to the fact that many antibodies for corticosterone measurement cross-react with other steroids present in the yolk. In particular, progesterone and related substances can occur in yolk in high concentrations, such that also low cross-reactions of corticosterone assays may lead to measurement errors. We thus performed high-performance liquid chromatographic (HPLC) analyses of yolk extracts and determined the concentration of immunoreactive corticosterone, as well as cross-reacting progesterone and cortisol in egg yolks of southern rockhopper penguins and imperial shags *Phalacrocorax atriceps albiventer*. We found that high gestagen concentrations in the yolk result in large measurement errors for yolk corticosterone, even when the cross-reactivity seems small. This was observed for both species. We further found species-specific differences in the actual corticosterone amounts present in the egg yolks.

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1. Introduction

Corticosterone is the dominant plasma glucocorticoid in birds and is present in avian eggs. Behavioral ecologists are interested in the role of this hormone in adaptive maternal effects. Immunoassays are used to determine egg levels of hormones. It has been noted, however, that studies of maternal hormones rely heavily on the proper use and validation of hormone quantification methods used, and that these methods have not yet received the necessary attention (e.g. [20]).

While several studies measured high yolk corticosterone amounts in avian eggs (e.g. [10,7,12]), some studies using chromatographic separation of different hormones for immunoassays have failed to detect significant amounts of corticosterone [17,15].

Egg yolks contain substances such as lipids and proteins that interfere with the binding of the hormone to the antibody. These are removed by extraction techniques (e.g. [17,6]). However, avian eggs also contain a variety of steroid hormones, and many antibodies for corticosterone measurement cross-react with other steroids present in the yolk. The avian follicle wall cells produce androgens, estrogens and progesterone which are incorporated into the yolk. Ovulability is gained as the ability of the follicle to produce androgens and estrogens declines and the ability to produce progesterone increases during the final 24 h of follicular maturation (e.g. [3]). This pattern leads to a high concentration of progesterone in the outer yolk layers (e.g. [5]). Some evidence suggests that progesterone can be down-regulated, thus preventing ovulation, by stress [18]. Furthermore, a role of progesterone in sex determination has been suggested [2].

As progesterone and related substances have been found in yolk in high concentrations (e.g. [5]), also low cross-reactions of the corticosterone assay used with those gestagens have to be considered. Therefore, reported corticosterone concentrations of samples not separated appropriately by chromatography may result in part from cross-reactions with other steroids that are found in the yolk in abundant concentrations [16], even though cross-reactions of tests used are typically low (e.g. 1.4% for progesterone and pregnenolone and 1.3% for 17α-hydroxyprogesterone, [9]).

Poisbleau et al. [12] stated that female southern rockhopper penguins *Eudyptes chrysocome chrysocome* deposited "the highest yolk corticosterone concentrations of any bird species studied so far" (54.5 ± 7.3 ng/g and 62.4 ± 6.3 ng/g for A-and B-eggs, respectively). In light of the recent findings in Rettenbacher et al. [16],

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these previous results need validation [1]. We thus performed high-performance liquid chromatographic (HPLC) analyses of yolk extracts and determined the concentration of immunoreactive corticosterone, as well as cross-reacting progesterone and cortisol in egg yolks of southern rockhopper penguins. We completed these tests with the same analysis on another species of seabird for which we already performed yolk corticosterone measurement according to the same method we used in Poisbleau et al. [12,13], the imperial shag *Phalacrocorax* (*atriceps*) *albiventer* (the Falkland population is also called king shag or king cormorant). We further compared the results of two corticosterone immunoassays (one enzyme immunoassay and one radio immunoassay) on the fractions obtained from the HPLC separation.

2. Materials and methods

2.1. Sampling

Eggs were collected in November and December 2008 in the Settlement colony on New Island, Falkland/Malvinas Islands (51°43′S, 61°17′W) under license from the Falkland Islands government.

Rockhopper penguin eggs were collected as described previously in Poisbleau et al. [12,13]. Around 5000 pairs of southern rockhopper penguins breed in the Settlement colony. They lay two eggs, but usually rear only one chick [11]. Thus, we assume that we did not significantly affect the breeding success of the colony by collecting the clutch and replacing the eggs with eggs found outside their own nest (that we considered as lost by their original parents). For further details, see Poisbleau et al. [12,13].

Imperial shag eggs were sampled as described in Quillfeldt et al. (2009) [13]. About 3000 pairs breed in the Settlement colony. Imperial shags arrived at the colony during early October, when courtship and nest building commenced. Egg laying took place between early November and the end of December 2008. Imperial shags experience strong brood reduction, and as we left one egg per clutch in the nest, we assume we did not significantly affect the breeding success of the colony (for further details, see Quillfeldt et al. [14]).

After collection, the eggs were frozen whole at −20 °C for at least four days. They were afterwards all prepared according to the same method Poisbleau et al. [12,13]. We removed the shell while the egg was still frozen. Then, we separated the yolk from the albumen by taking advantage of the fact that albumen thaws more quickly than yolk. A small quantity of yolk was transferred to a 2-ml Eppendorf tube and stored at −20 °C for transport. We included three eggs of each species in the present analysis.

2.2. Extraction and HPLC

Extraction and HPLC protocols followed Rettenbacher et al. [16]: Subsamples of 5 g of each yolk were mixed with 10 ml of double distilled water and tritiated progesterone (Perkin Elmer, MA, USA) to calculate recoveries as well as to have a standard elution pattern for the HPLC. The mass of the spike was 1.4 ng. After stirring the mixture for 30 min, 30 ml of 100% MeOH were added dropwise with stirring. After centrifugation, 30 ml of the supernatant were diluted with 45 ml double distilled water and loaded onto a primed Sep-Pak Classic C18 Cartridge (Fa. WATERS; Part No WAT051910) via airflow. Cartridges were washed with double distilled water and 30% MeOH and then left to dry overnight. On the next day, elution was performed with 5 ml of 100% MeOH. After evaporation of the solvent, the eluate was resuspended in 1 ml MeOH. An aliquot of 50 μl was used to determine recoveries of radioactivity, while the rest was evaporated, resuspended in the starting solvent and injected onto an HPLC column. In total, six HPLC runs (one for each yolk sample) were performed. We performed straight-phase HPLC with a 70:30 (*n*-hexane:chloroform) eluate and a gradient from 0% to 6% MeOH. Flow was 2 ml/min and 76 fractions were collected in 30 s intervals. The solvent of the eluting fractions was evaporated and the samples were reconstituted in assay buffer. The elution positions for the different steroids (Figs. 1 and 2) were determined previously in HPLC runs with identical setup [16]. Recovery from the extraction was 87 ± 4% for progesterone and 90 ± 4% for corticosterone, respectively, while losses during the HPLC procedure were 23 ± 15% for progesterone and 28 ± 16% for corticosterone [16].

2.3. Vienna enzyme immunoassay (EIA)

Concentrations of immunoreactive steroids were determined in aliquots of the fractions with antibodies against corticosterone [9]. Detection limit was 0.3 ng/g yolk [16]. Cross-reactivity of the antibody, assessed at 50% binding was 1.4% for progesterone and pregnenolone and 1.3% for 17α-hydroxyprogesterone [9].

2.4. Chizé radio immunoassay (RIA)

Material remaining from the EIA measurements was freeze dried and the HPLC fractions were transferred to the laboratory at Chizé for further analysis. Here, all fractions of two rockhopper penguin eggs and the relevant fractions of the four remaining eggs were re-suspended and measured, using the RIA as in Poisbleau

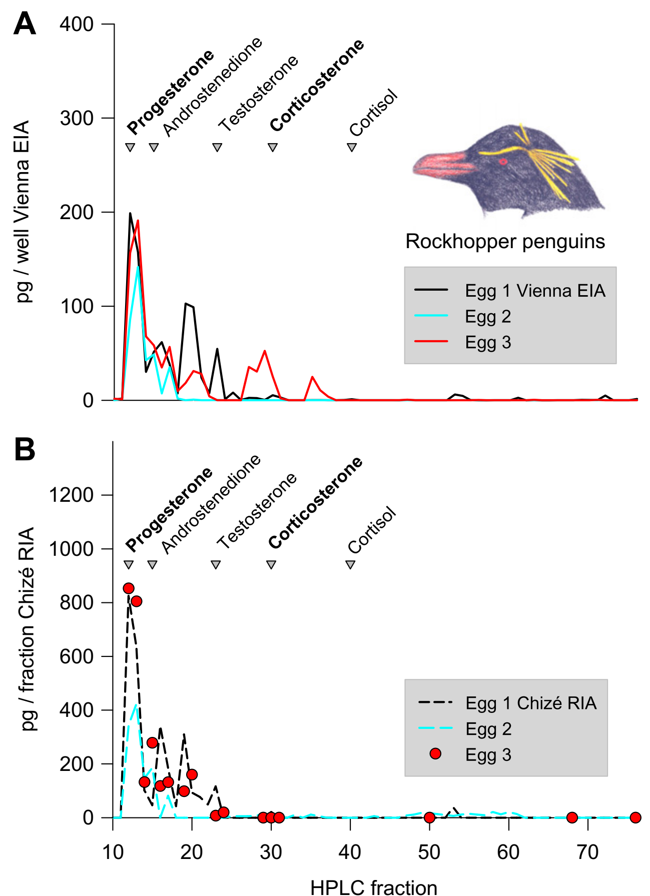

Fig. 1. Concentrations of immunoreactive steroids in aliquots of HPLC fractions of extracted Rockhopper penguin egg yolks, determined with two different antibodies against corticosterone. Lines are drawn where all HPLC fractions were measured (eggs 1–3 in Vienna EIA, and eggs 1 and 2 in Chizé RIA), while dots indicate measurements of relevant fractions (egg 3 in Chizé RIA).

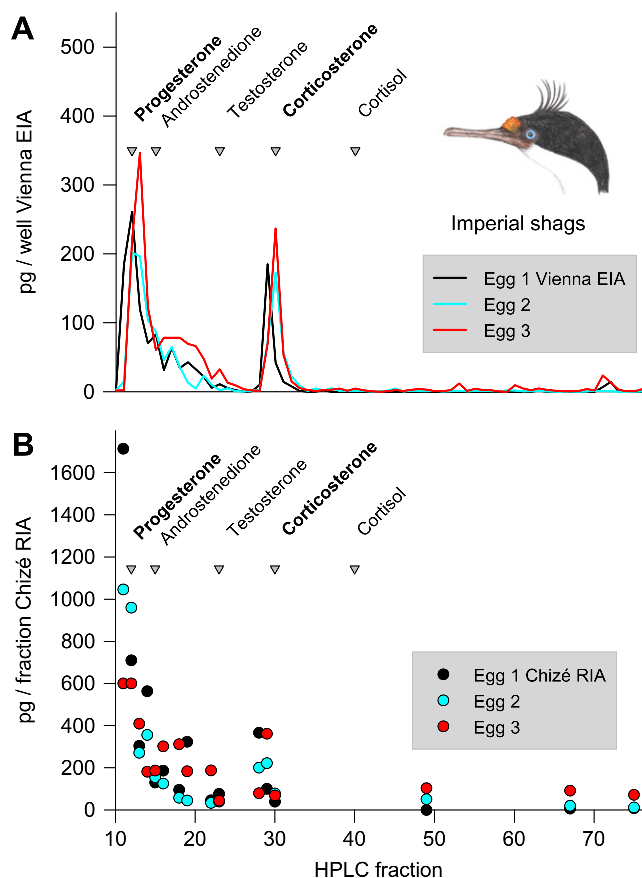

Fig. 2. Concentrations of immunoreactive steroids in aliquots of HPLC fractions of extracted Imperial shag egg yolks, determined with two different antibodies against corticosterone. Lines are drawn where all HPLC fractions were measured (eggs 1–3 in Vienna EIA), while dots indicate measurements of relevant fractions (eggs 1–3 in Chizé RIA).

et al. [12,13]. Corticosterone radioimmunoassay was performed using a polyclonal rabbit antiserum supplied by Sigma (USA). This antibody cross reactions were as follows: progesterone (15.7%), 11-deoxycorticosterone (20%), 20α-hydroxyprogesterone (8.8%), cortisol (4.5%), 20β- hydroxyprogesterone (5.2%), testosterone (7.9%), 17-hydroxyprogesterone (1.8%), androstenedione (2.6%), aldosterone (4.4%), 5α-dehydrotestosterone (1.4%), androsterone (<0.1%), cortisone (3.2%), estrone (<0.1%), estriol (<0.1%).

3. Results

Both corticosterone immunoassays (Vienna EIA and Chizé RIA) detected the highest amounts of immunoreactive substances which were more apolar than corticosterone. The dominant peak showed an elution pattern like progesterone and only a small peak at the elution position of corticosterone was detected, both for southern rockhopper penguins (Fig. 1A) and imperial shags (Fig. 1B). In the imperial shags, the corticosterone peak was more pronounced compared to the rockhopper penguins.

The estimated total concentration of immunoreactive steroids in the Vienna EIA was 20–65 ng/g in rockhopper penguins, but of these only <0.1–6.8 ng/g were located in the corticosterone fractions. Moreover, only one egg (egg 3) had a moderate peak in this position, and this was not repeatable in the Chizé RIA.

For imperial shags, the estimated total concentration of immunoreactive steroids in the Vienna EIA was 52–85 ng/g, of which 10–18 ng/g were found in the corticosterone fractions.

4. Discussion

The present findings clearly demonstrate that the high corticosterone measurements reported on homogenized yolk extracts in rockhopper penguins with a previous double extraction but without previous chromatography separation (Poisbleau et al.) [12] in reality reflect high concentrations of yolk progesterone and its precursors (Fig. 1a), such as pregnenolone [16]. In this species, corticosterone is hardly detectable in egg yolk, as there was no peak in immunoreactivity at the elution position of corticosterone. In imperial shags, in contrast, a clear peak was visible at the elution position of corticosterone but progesterone was also present in high quantity. After chromatographic separation, yolk corticosterone levels could therefore be determined in this species. The same tests still have now to be performed on the albumen in order to test whether the levels of albumen corticosterone measured in rockhopper penguin [13] and imperial shag eggs also mostly represent the levels of progesterone and its precursor present in albumen.

The use of chromatography in steroid analyses has been proven efficient and necessary in other contexts such as the analysis of urinary cortisol for clinical purposes (e.g. [8,19]) since the specificity can be greatly improved by the chromatographic step.

In conclusion, we found that high gestagen concentrations in the yolk result in large measurement errors for yolk corticosterone, even when the cross-reactivity seems small. This was observed for both species. We further found species-specific differences in the actual corticosterone amounts present in the egg yolks. The same methodological issues have also to be addressed for egg corticosterone analysis on other species (see review in Groothuis and Schwabl [4].

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