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ORIGINAL PAPER

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Distribution and origin of steroid hormones in the yolk of Japanese quail eggs (*Coturnix coturnix japonica*)

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Abstract The yolk of avian eggs contains steroid hormones, which may influence the development and behaviour of hatched birds. The aim of the present study was to investigate the concentration as well as the distribution of various gonadal steroids in the yolk spheres of quail eggs. Steroid concentrations of dissected yolk layers were analysed after alcoholic extraction using enzyme immunoassays (EIAs) for progesterone, androstenedione and testosterone. To monitor the uptake of testosterone into the yolk, radioactive testosterone was injected i.m. into six female quails. The radioactivity of yolk layers of subsequently laid eggs was measured by liquid scintillation counting. Progesterone concentrations were highest in the outer layer (median: 2265 nmol/kg). Androstenedione (median: 453 nmol/kg), as the major androgen, and testosterone (median: 99 nmol/kg) reached their highest concentrations in interior layers, whereas in the centre the concentration of all three hormones was low. No significant variation of steroid levels in yolk layers of subsequently laid eggs was found. The highest radioactivity was detected in the outer yolk layer in those eggs laid 1 day after injection and in subsequently laid eggs was measured nearer to the centre. These results indicated local origin of the steroid hormones especially because of the result that only 0.1% of the radioactivity entered the yolk. We conclude that steroid concentrations in the yolk layers reflected progesterone and androgen production of the cells of the follicular wall at the time.

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Abbreviation *EIA* enzyme immunoassay

Introduction

High concentrations of gonadal steroids are present in the yolk. Average concentrations of steroid hormones decrease down the cascade of hormone synthesis. Hence, cholesterol is highest in concentration, followed by progesterone, androstenedione, testosterone and 17β-oestradiol (Lipar et al. 1999; Möstl et al. 2001). These findings are in parallel to the different steroidogenic activity of follicular cells during maturation of avian follicles (Onagbesan and Peddie 1988, Mori et al. 1985: quail; Bahr et al. 1983: domestic hen). A "three cell" theory for the avian ovarian steroidogenesis has been proposed (Porter et al. 1989), which explained the different steroid production of the follicular tissues (granulosa: progesterone; theca interna: androgens; theca externa: oestrogens). There is an increasing progesterone concentration in granulosa and a decrease in oestrogen production of the theca during follicular maturation. The dominating synthesis of androgens was localised in the theca interna of the immature follicles. The synthesised steroids may be deposited directly into the yolk during oogenesis. In addition, small amounts of lipophilic substances could get there from the systemic circulation simply by diffusion.

Several lines of evidence indicate the production of steroid hormones by theca and granulosa cells, which diffuse into the peripheral circulation. Some of the steroids in the circulation may enter other follicles and therefore contribute to the steroid concentrations in the follicles.

There is a correlation between the androgen concentration in the peripheral blood and the testosterone concentration in the yolk. Schwabl (1997) described that female sparrows in a socially competitive situation

up-regulated systemic testosterone, which, in turn, correlated with the concentration of testosterone in the yolk of the eggs laid by these females.

The transfer of steroid hormones from the peripheral circulation into the yolk is limited. After injecting radioactive 17β-oestradiol intravenously, only 0.04% of the injected dose was found in the eggs laid thereafter (Arcos 1972). In addition, the distribution pattern of steroids in the yolk (Lipar et al. 1999; Möstl et al. 2001) could be indicative for a local process responsible for steroid concentrations in the egg. Yolk is laid down in concentric layers by the cells of the follicular wall. Regular steroid distribution patterns within and between yolks (Lipar et al. 1999; Möstl et al. 2001) and low transfer rates of systemic steroids into the yolk (Arcos 1972: oestradiol 17β; Wilson and McNabb 1997: thyroxine) favour local steroid production by the cells of the respective follicular wall as the main source of the yolk steroid concentrations. This hypothesis in agreement with the increase of yolk androgen concentrations within a single clutch, as found in canaries (Schwabl 1993), sparrows (Schwabl 1997), kestrels (Sockman and Schwabl 2001), lesser black-backed gull (Royle et al. 2001), black-headed gull (Eising et al. 2001) or red-winged blackbird (Lipar and Ketterson 2000), which can also be explained by an increased production in the theca or granulosa cells. But it remains unclear how modulation of yolk androgens by social stimuli (Schwabl 1997) occurs.

The aim of the present study was to investigate the distribution of progesterone, androstenedione and testosterone concentrations in the yolks in successively laid eggs. Furthermore, we investigated how testosterone from the maternal circulation was deposited in the egg yolk.

Material and methods

Tritium labelled (^{3}H) testosterone (964 kBq/quail; specific activity: 3.7–6.6 TBq/mmol) was dissolved in sesame oil and injected into the pectoral muscles of six female Japanese quails (*Coturnix coturnix japonica*). Droppings ($n = 150$) of each individual were collected for 6 days and the eggs for 7 days ($n = 38$) after injection of the radioactive androgen. Twelve eggs taken before the injection of radioactivity were used to measure blank values. All samples were stored frozen at −21 °C until extraction.

Extraction of radioactivity from faeces and measurement of the radioactivity

The extraction of faeces followed the method described for pigs, sheep and ponies by Palme et al. (1996). Only the amount of wet faeces (0.3 g) entering the procedure was lower. For measuring the excreted radioactivity from faecal samples, 0.5 ml methanolic extract was added to 9 ml scintillation fluid. The radioactivity was measured according to the methods described by Palme et al. (1996).

Steroid extraction

As described by Lipar et al. (1999), the frozen albumin was separated from the yolk and an aequatorial disk (3 mm) of the yolk was cut using a knife. The dissection of this yolk disc into different layers, and the subsequent extraction of steroids from these layers with 80% methanol, was carried out similarly to that described by Möstl et al. (2001) for hen eggs; however, due to the smaller size of the quail eggs, only five dissected layers (instead of six) were prepared. For the measurement of steroids from the albumin, 0.2 ml was extracted in the same way as for the yolk.

To analyse the mean radioactivity and the steroid concentrations of the yolk, the remaining part of the yolk (after removal of the central disc) was homogenised and extracted in the same way.

Measurement of radioactivity

For the measurement of the radioactivity in the extracts of the individual yolk layers, in the total yolk and in the albumin, 0.2-ml samples of the methanolic extracts were transferred into vials and 7 ml scintillation fluid added. After a short mixing, the samples were measured using liquid scintillation counting for 10 min (plus quench correction).

Measurement of the steroid concentrations

For measuring the concentrations of steroid hormones we used enzyme immunoassays (EIAs; testosterone and androstenedione: Palme and Möstl 1994; progesterone: Schwarzenberger et al. 1996). Of the extract, 10 μl was used for the measurement of testosterone and androstenedione. For the progesterone quantification, the extracts had to be diluted with assay puffer (1:10) and 20 μl was used for the EIA.

Statistics

The steroid concentrations in the yolk were not normally distributed. Therefore the Mann-Whitney Rank Sum Test was used for statistical analysis.

Results

Steroid hormone concentrations in the yolk layers

All three steroid hormones showed a characteristic distribution pattern between the different layers. There was no significant difference in distribution between the eggs in the sequence of laying.

The progesterone concentration was found to be significantly higher in the superficial layer of the yolk (median: 2265 nmol/kg), compared to the centre (median: 187 nmol/kg; $P < 0.001$, Fig. 1). The distribution pattern of androstenedione (Fig. 2) showed an increase from the superficial layer (median: 188 nmol/kg) to a maximum concentration in the third layer (median: 454 nmol/kg) followed by a decrease towards the centre (median: 237 nmol/kg; $P < 0.001$).

Testosterone distribution within the yolk showed a pattern (Fig. 3) similar to androstenedione; however, the concentrations were lower and reached a maximum in the second layer of the yolk (median: 99 nmol /kg; $P < 0.001$).

The values of gonadal steroids measured in the homogenised yolk were 4 nmol/yolk for progesterone,

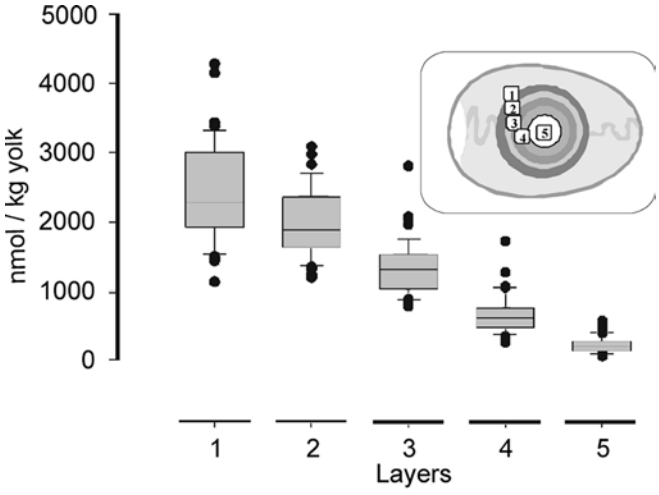

Fig. 1 Boxplot of the progesterone concentrations in the layers of the yolk. *Insert*: Scheme of the layers (outer layer = 1, centre = 5) of the yolk collected for the determination of gonadal steroids

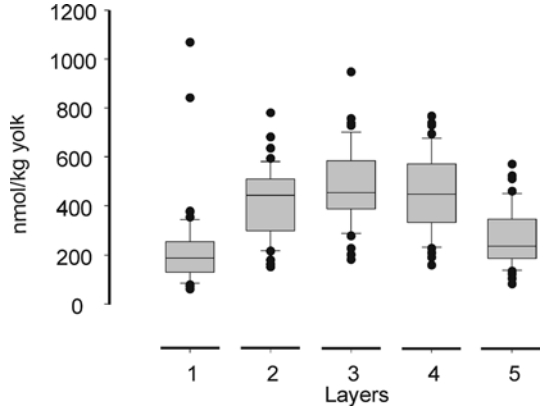

Fig. 2 Boxplot of the androstenedione concentrations in the layers of the yolk

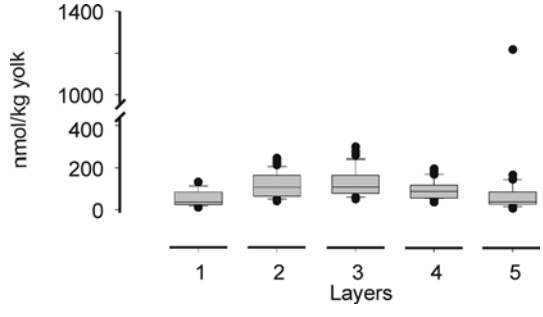

Fig. 3 Boxplot of the testosterone concentrations in the layers of the yolk

1.3 nmol/yolk for androstenedione and 0.36 nmol/yolk for testosterone.

Using the Mann-Whitney Rank Sum Test, no significant differences were measured in the distribution of progesterone and androgens in the homogenised yolk between the eggs in the sequence of laying. There were also no significant differences in steroid hormone concentrations between the eggs of individual quails. Furthermore, statistical calculations of the steroid content of the individual yolk layers showed no significant differences during the sampling period.

Radioactivity in the droppings and in the eggs

The excretion of the radioactivity reached its maximum 2 h after application (mean: 36462.5 KBq/kg faeces) and decreased to background after 2 days (Fig. 4). The recovered radioactivity was about 60% of the injected dose.

In the eggs, radioactivity peaked in the albumin (mean: 25.9 kBq/l) on the first day after injection and declined to background values within a few days (data not shown). The radioactivity of the whole yolk increased to a maximum on the third and fourth day after injection (Fig. 4) and decreased on the following days. The radioactivity excreted via the yolk was 0.12% of the injected amount of ^{3}H-testosterone. On the first day after application, all eggs contained the highest radioactivity in the first layer, whereas on the last day of the sampling period, the maximum radioactivity was found in the centre.

Discussion

Radioactivity

Only a small amount of the administered radioactive testosterone (0.1%) entered the yolks, with no significant differences between the eggs laid by the six individuals used. The injected radioactivity reached the yolk at different stages of development. Thus, follicles destined to ovulate on the day after ^{3}H testosterone injection accumulated the radioactivity in the outermost layers. In contrast, follicles which were hit by the systemic radioactivity peak earlier during their development accumulated highest radioactivity closer to the centre of the yolk. Hence, a change of the yolk layer with the maximum radioactivity occurred in laying order of eggs during the sampling period of 7 days (Fig. 4). High systemic concentrations were present only for 1 day. This was shown by our data on dropping excretion patterns (Fig. 4). Therefore, diffusion into the yolk was possible only during this period. Whether or not radioactive testosterone was metabolised to other substances like androstenedione during the passage through the follicular wall remains to be determined, as only very small amounts of radioactivity were found in the yolk.

The avian follicle consists of theca and granulosa cell layers, which are the major source of gonadal steroid concentrations in the yolk. The transfer of systemic androgens does not play a substantial role, as was shown by the low amount of transferred radioactivity into yolk

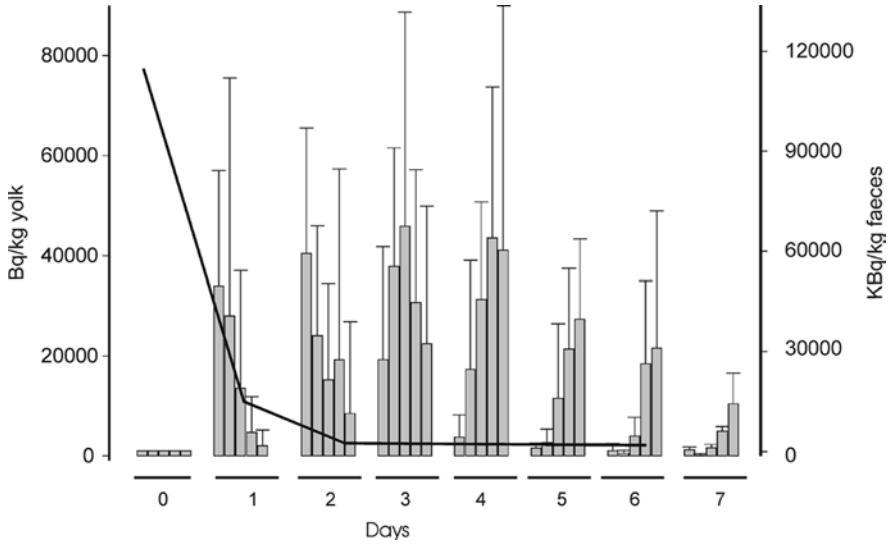

Fig. 4 Deposition of radioactivity in individual yolk spheres after injection of ^{3}H-testosterone (*vertical bars*) during the sampling period of 7 days ($n = 38$). The *solid line* shows the excretion of the radioactivity via the droppings

(0.1%) during the present study. Theca and granulosa cells have different steroidogenic activity during the follicular maturation. Progesterone is mainly produced by the granulosa cells and highest concentrations were measured in the three largest follicles. In contrast, androgens, especially androstenedione, were mainly produced by the theca layer, which showed highest concentrations in the second, third and fourth follicle (Etches and Duke 1984).

A three cell model has been proposed for oestrogen production, which postulates that theca interna cells synthesise androgens and in minor concentrations progesterone, whereas the theca externa is able to convert androgens into oestrogens. The main location for the progesterone synthesis is the granulosa cell of the follicular wall (Porter et al. 1989). This finding was supported by Gomez et al. (1998), investigating the enzymatic activity of granulosa, theca interna and externa. Their results also provided evidence for a multiple cell theory of steroid production, including granulosa, theca interna and theca externa cells, respectively. The highest activity of 3β-hydroxysteroid dehydrogenase was found in the granulosa of the largest follicle, whereas androstenedione synthesis was seen in the theca interna of the smaller follicles. The theca interna had no capacity to aromatise oestrogens but the theca externa of the small follicle converted androgens to oestrogens indicating a loss of aromatase activity in growing follicles (Porter et al. 1989).

In our study, there was no difference in overall hormone concentrations or in the distribution of hormones among successively laid eggs in a clutch, which is in contrast to the hormonal situation described for other species. This discrepancy may be explained by the fact that quails are a precocial species; the differences in successively laid eggs were found in altricial species, where higher androgens may compensate for differences in development caused by hatching asynchrony.

The results demonstrate that the major sources for steroid hormones in the avian yolk are the theca and granulosa cells of the follicular wall. Furthermore they show that the diffusion of testosterone from the peripheral circulation contributes to the androgen concentration in the yolk only in small amounts.

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