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Research paper

# Accumulation of steroid hormones in the eggshells of Japanese quail (*Coturnix coturnix japonica*)

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ABSTRACT

Oviparous mother transfer significant amounts of steroid to egg yolk during oviposition and the amounts may vary throughout the embryonic development. Eggshell may contain steroid hormones and the amounts could be different during embryonic development inside the egg. This study was designed to quantify the steroid concentrations in the eggshells of Japanese quail. We hypothesized that the steroids would be accumulated in the eggshells in a sex-dependent manner. Eggshells were obtained from three different stages (after laying, 15 days of incubation, and after hatching). The internal contents of the shells were carefully removed, completely dried and pulverized. The steroid contents of the eggshells were then measured by RIA. Physiologic variations in steroids were analyzed according to the amounts accumulated in the eggshells with the different embryonic stages. Results indicate that eggshell testosterone concentrations were high after laying. However, the concentrations were decreased during embryonic development and hatching and no difference was found in eggshell testosterone levels between male and female. However, eggshell estradiol concentrations were undetectable at laying time and the amounts were significantly increased at 15 days of incubation and slightly after hatching. Eggshell estradiol levels were significantly high in female eggshells than male during embryonic development. In contrast, eggshell corticosterone levels were significantly higher in males than in females after hatching. These results clearly demonstrated that eggshells accumulated steroid hormones, and the amounts varied during embryonic development concomitant with changes the internal contents of the eggs.


## 1. Introduction

The eggshell is a hard membrane that encloses the embryo, and mainly consists of outer (calcareous crust) and inner (mammillary) membranes (Schaafsma et al., 2000; Makkar et al., 2016). The eggshell plays crucial roles in avian reproduction, including (a) protecting the egg contents from the surrounding physical environment, (b) controlling water and gas exchange during embryonic development in the egg, and (c) providing calcium for embryonic development once the yolk stores are depleted (Nys et al., 2004). The outer layer of the eggshell is made of calcite and calcium carbonate crystals and the inner shell membrane consists of organic matters that include collagen, protein, peptides, and lipids (Nys et al., 2004; Makkar et al., 2016).

Steroids are lipophilic hormones that, possess small molecular weight (ranging from 270 to 370 daltons), and are mainly produced by the adrenal cortex and gonads and transferred *via* the circulation to peripheral target tissues (Kawata 1995). In addition, non-reproductive tissues also produce physiologically active steroids (Inoue et al., 2012). The lipophilic and highly yolk-soluble structure of steroids facilitated easily diffusing of these hormones from the mother into the egg yolk during vitellogenesis in oviparous animals (Schwabl, 1993; Conely et al., 1997). Previous studies showed that elevating plasma steroid concentrations in the female birds resulted an elevated level of steroids in her egg yolk (Adkins-Regan et al., 1995). In avian species, because the embryo develops outside the mother's body in the egg, the mother transfers nutrients and hormones to the egg yolk within the developing follicles (Groothuis et al., 2005, Hsu et al., 2016). Egg yolk contains high levels of androstenedione, testosterone, and 5α-dihydrotestosterone; while 17β-estradiol and corticosterone are at low levels (Elf and Fivizzani 2002; Groothuis et al., 2005). Additionally, the avian embryo is not only exposed to maternal steroids, but also to endogenously produced steroids which play key roles in

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sexual differentiation and development (Tanabe et al., 1979; Abdelnabi et al., 2000; Ottinger et al., 2001). In the chicken embryo, the receptor of ESTRADIOL has been detected immunohistochemically as early as day 3.5 of incubation in undifferentiated gonads, which reflect the crucial roles of endogenous steroid productions on developments and sex differentiation (Woods and Erton 1978). During embryonic development, the chorioallantoic membrane (CAM) of the chicken and turtle (*Pseudemys nelsoni*) was also shown to display steroidogenic function, ability to synthesize progesterone and sensitivity to progesterone signaling (Albergotti et al., 2009; Cruze et al., 2013).

It is currently unclear which steroid hormones could be accumulated in the eggshell of avian species or not, and the sources and physiologic status of the steroids are not clear. Previous report in loggerhead sea turtle *Caretta caretta* showed that embryonic sex steroids accumulate in the eggshell (Kobayashi et al., (2015). In the present study, we hypothesized that the steroids could be accumulated in the eggshells of Japanese quail. As the incubation progresses, the yolk steroids could be diffused through the albumen and CAM by polarization (conjugated steroids) and accumulated in the eggshell. Moreover, in late embryonic development, the embryo is enclosed by the CAM, thus affecting hormonal levels in the eggshell. Thus, the current study was aimed at 1) quantifying steroid accumulation in the eggshells of Japanese quail, and 2) finding the steroid variation of the eggshell at three crucial incubation points, after laying, 15 days of incubation and post hatching, in both sexes.

## 2. Materials and methods

### 2.1. Sampling and eggshell processing

The Japanese quail is known as an excellent avian model for studying neuroendocrine, reproduction and behaviors (Ball and Balthazart 2010). A total of 100 fertilized eggs were collected from our laboratory quail stock during 3 consecutive days. Before egg collection, quails were arranged in their cages in order to determine the sex-ratio and three females and one male were placed in one cage. Stock quails were housed a controlled environment (lights on, 05:00–19:00 h; temperature, 23 ± 2 °C; humidity, 50 ± 10%; air exchanged 20 times hourly). Animals had free access to food (Cosmos Company, Aichi, Japan) and water. Stock animals were 10 weeks old and from the same laying clutch. The inner and the outer shell membranes were obtained from the three different stages; after laying (non-incubated eggshells), 15 days of incubation and after hatching and all eggs were processed and cleaned in the same way at some point in the following paragraph.

#### 2.1.1. After laying

Collected eggs were processed for their shells within 24 h after laying. The egg was broken into half and the yolk and albumen were gently removed from the eggshells. The eggshells then individually placed into 50-ml tubes and dried in an incubator at 100 °C for 24 h as previously described (Kobayashi et al., 2015). Dried eggshells were pulverized using a glass stick, and then 5 ml of 80% methanol was added, vortexed for 30 min, and centrifuged at 1700*g* for 10 min at 4 °C. The supernatant was transferred to another tube and stored for hormonal analysis.

#### 2.1.2. Days of incubation

To evaluate the variation in steroids in the eggshell during incubation and hatching, the remaining eggs were incubated at 37 ± 5 °C in a humidified incubator (Showa Furanki, Tokyo, Japan). After 15 days of incubation, eggshells were obtained from the eggs which the embryo was developing inside (confirmed by candling). Euthanasia of developing embryos was accomplished by freezing with ice for 30 min, a methodology approved by the Animal Care Committee of the Tokyo University of Agriculture and Technology. Collected eggshells were processed as above. To identify the sex-related eggshells at this stage, embryos were fixed in 4% paraformaldehyde (Wako Co., Osaka, Japan) for 24 h. Morphologic identification of sexes was performed by macroscopic by naked eyes and microscopic with hematoxylin-eosin staining (Li et al., 2006).

#### 2.1.3. Hatching time

Before hatchling, the eggs were individually placed inside compartments made from carton box in the hatchery space. On the hatching day, eggshells containing the CAM were collected and cleaned residue left by the chicks. Day-old chicks were tagged on their legs and kept for sexual identification through feather colors after 20 days.

### 2.2. Hormonal measurements

All procedures for hormonal measurements were performed as previously described (Kobayashi et al., 2015). Double-antibody radioimmunoassay (RIA) using $^{125}$I-labeled radioligands was performed to analysis the concentrations of testosterone and estradiol (Taya et al., 1985), and corticosterone (Kanesaka et al., 1992) in the eggshell extracts. Anti-sera against testosterone (GDN250) and estradiol (GDN244) were kindly provided by Dr. G.D. Niswender (Fort Collins, CO. USA). Antiserum against corticosterone was goat anti-corticosterone produced by our laboratory (Kanesaka et al., 1992).

### 2.3. Statistical analysis

For statistical analysis GraphPad Prism5 (San Diego, CA, USA) was used. All data are expressed as means ± SD. The concentrations of hormones between the sexes and within and between each time-point were statistically analyzed using one-way analysis of variance (ANOVA) and Tukey post hoc test for significance. Correlations among three hormones were analyzed by Spearman correlation test. $P < .05$ was considered to be statistically significant.

## 3. Results

The initial testosterone concentration was detected in eggshell of freshly laid eggs in the current study. Concomitant with progressing incubation time and embryonic development, testosterone levels declined. A significant decrease was observed in testosterone concentrations between the freshly laid eggshells and after hatching ($P < .001$). On the other hand, no significantly differences were found in the eggshells testosterone concentrations between males and females during incubation and hatching periods (Fig. 1).

Estradiol concentrations were not detected after laying in the eggshell extracts. However, the highest estradiol levels were observed in estradiol levels at 15 th days of incubation and the levels were significantly high in female eggshells compared to male ($P < .01$). In addition, the concentrations decreased at hatching period in female significantly, compared to the levels at 15 th days of incubation ($P < .05$, Fig. 2).

As illustrated in Fig. 3, the initial concentrations of shell corticosterone were low in the fresh laid eggs. However, the shell corticosterone levels increased during the embryonic development and the amounts were much greater in male ($P < .01$) than in female ($P < .05$) after hatching as compared to freshly laid eggshells respectively. Moreover, corticosterone concentrations were

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