Non-invasive assessment of endocrine differences in day-old chicks (*Gallus domesticus*) by analysis of the immunoreactive oestrogen excreted in the egg

A. B. Bercovitz, A. Mirsky and F. Frye, Jr

**c/o Research Department, Zoological Society of San Diego, P. O. Box 551, San Diego, CA 92112, U.S.A.**

**Summary.** Oestrone, oestradiol and an uncharacterized polar fraction of oestrogenic substances were monitored by radioimmunoassay in Day-0 domesticated chickens (*Gallus domesticus*) after routine 21.5-day artificial incubation. Paired adrenals and gonads, blood serum and in-ovo excreta (allantoic urates plus cloacal expressions) were collected from 9 female and 10 male chicks. All samples were homogenized, hydrolysed and extracted with ether. Component oestrogens were isolated by celite:ethylene glycol column co-chromatography before assay. All egg excretory oestrogen measurements were higher (*P* < 0.001) in females than in males. Oestradiol was the major excretory oestrogen in both sexes; oestradiol:oestrone ratios (mean ± s.e.m.) in females and males were 18.45 ± 2.42 and 3.03 ± 0.37, respectively. Females had higher (*P* < 0.01) total oestrogens for gonadal and adrenal homogenates and for blood serum samples than did the males; oestradiol was generally the major component.

**Introduction**

Steroidogenic mechanisms of sexual differentiation, particularly avian perinatal endocrine differences, have been well documented for domesticated chickens (*Gallus domesticus*). Oestrone and oestradiol were the first oestrogens isolated from ovarian tissue in 10- to 21-day old embryos (Gallien & Le Foulgoc, 1957, 1958). Several oestrogens have since been identified in chicken embryonic gonads and plasma, i.e. oestrone, oestradiol, oestriol and epoestriol (Ozon, 1965, 1969; Haffen & Cedard, 1968; Weniger, 1969; Weniger & Zeis, 1971; Galli & Wasserman, 1972, 1973; Guichard, Cedard & Haffen, 1973).

Oestradiol produced in the gonad, or total steroid production/24 h/paired gonads, was always greater in females than males (Guichard, Cedard, Mignot, Scheib & Haffen, 1977). Embryonic ovarian oestradiol was 3-fold greater than adrenal oestradiol, which was significantly greater in females than in males (Tanabe, Nakamura, Fujioka & Dori, 1979). More enzyme aromatase activity and greater conversion of androgen precursors to oestrone and oestradiol were noted in 15- to 18-day-old embryonic ovaries (Guichard et al., 1979a; Weniger, Chouraquí & Zeis, 1983). Plasma oestradiol concentrations in 10- and 18-day old embryos were consistently greater in females than in males (Tanabe et al., 1979; Woods, Congoran & Thommes, 1982).

Little has been done to investigate excretory endocrine differences in avian neonates. Excretory steroid analysis, i.e. total oestrogen:androgen ratio evaluation, has been used successfully as a non-invasive sexing method for adult birds of exotic species (Czekala & Lasley, 1977; Bercovitz, Czekala & Lasley, 1978; Stavy, Gilbert & Martin, 1979). Little is known about the excretion of steroidal substances from perinatal birds, except the identity of oestrone and oestradiol from amniotic fluid of 10-day-old embryos and from allantochorionic fluid of 13-day-old embryos (Ozon, 1965, 1969). The purpose of this study was to evaluate immunoreactive oestrogens from excrement.
samples of eggs collected at the time of hatching and to characterize sex-related differences monitored via excretory oestrogen analysis.

Materials and Methods

Sample collection and preparation. Fertile chicken eggs (12-day-old embryos) were purchased commercially and continued through a normal 21.5-day incubation. Only chicks that had hatched or were in the process of hatching were used; they were killed on the day of hatching by decapitation. Blood was obtained by exsanguination, and serum was stored at −20°C until hydrolysis. Paired adrenals, paired gonads and accumulated excrement (allantoic urates and cloacal expressions) were collected and suspended in ethanol to facilitate homogenization with a test-tube tissue grinder. Homogenates were vortex-mixed, incubated overnight at 5°C, desiccated by freeze drying and then reconstituted, before hydrolysis, in 1.0 ml acetate buffer (pH 5.0, with 0.1% gelatin and sodium azide added).

Enzymic hydrolysis was accomplished by the addition of 0.05 ml beta-glucuronidase/arylsulphatase (Helix pomatia preparation; Boehringer Mannheim GmbH, West Germany) to 1.0 ml of the buffered sample and incubation at 37°C for a 24-h period. Blood serum samples (0.3–0.4 ml/chick) were diluted with distilled water to a total volume of 1.0 ml, adjusted to pH 5.0 with acetic acid and were enzymically hydrolysed, like the tissue samples. All hydrolysates were extracted twice with 8 volumes of anhydrous diethyl ether. The aqueous layer was snap-frozen and subsequently discarded; the ether supernates were decanted into a clean tube, dried and resuspended in 1.0 ml iso-octane before co-chromatography with celite analytical filter aid (300G, Fisher Scientific Co., Pittsburgh, PA, U.S.A.).

Chromatography. Celite/ethylene glycol co-chromatography was based on a modification of the method from Anderson, Hooper, Lasley & Yen (1976). Columns were made from 5 ml glass disposable pipettes, packed with 0.9 g celite:ethylene glycol (2:2:1, w/v). Columns were low pressure driven at a rate of 100 drops/min with nitrogen gas and rinsed twice with 3.5 ml iso-octane before samples (1.0 ml iso-octane) were applied and rinsed (0.5 ml iso-octane) into the column bed. Four solvents of increasing polarity were flushed through the loaded columns: (1) 3.5 ml iso-octane; (2) 3.5 ml 15% ethyl acetate in iso-octane; (3) 3.5 ml 40% ethyl acetate in iso-octane; (4) 4.0 ml 100% ethyl acetate. The first effluent was discarded, the second and third were collected for oestrone and oestradiol analysis, respectively. The final effluent, 100% ethyl acetate, was intended to strip the column of all substances more polar than oestradiol and represented an uncharacterized polar oestrogenic fraction. No further definition of the oestrogenicity in this polar immunoreactive fraction was undertaken; it was included in the estimate of total oestrogens. Celite co-chromatographic recovery of purified tritium labelled oestrone and oestradiol displayed a parallel elution profile to peak levels of immunoreactivity. This separation procedure has consistently and routinely demonstrated the ability to distinguish oestrogens from other immunoreactive substances in excrement, tissue homogenates and blood samples.

Methodological losses during extraction and celite chromatography were monitored by co-chromatographic recovery of tritium-labelled oestrone and oestradiol (New England Nuclear, Boston, MA, U.S.A.); specific activities for [2, 4, 6, 7-3H]oestrone and [2, 4, 6, 7-3H]oestradiol were 87 and 104 Ci/mmol, respectively. Approximately 1000 c.p.m. of each isotope was added to each hydrolysate before ether extraction. Specific recovery values (mean ± S.E.M.) after co-chromatography were 74.8 ± 1.3% (n = 64) for oestrone and 67.1 ± 0.9% (n = 73) for oestradiol.

Oestrogen assay. Oestrogens were measured by a standard, single-antibody, charcoal: dextran-prefcipitated radioimmunoassay (Abraham, Buster, Lucas, Corrales & Tell, 1972; De Vane, 1975). An oestriol-trihemisuccinate-HSA antiserum (S310 No. 5; Abraham et al., 1972) was used at a dilution of 1:4000. Cross-reactivity of this antibody was essentially limited to the following C18
Oestrogens in egg excrement of chicks for sex identification

steroids: 140% for oestrone, 140% for oestradiol and 100% for oestriol. Oestriol standards for the assays were serially diluted within a range of 5 to 1000 pg. Assay sensitivity was 3-0 pg/tube; intra-assay variability was 10-0% (n = 26) and interassay variability was 12-0% (n = 13). Water blanks were <6 pg/tube, partly due to an oestrone-like component found in the enzyme preparation used for hydrolysis; sample data were not adjusted for this value. Specific details for this assay system have been previously reported (Anderson et al., 1976; Bercovitz et al., 1978; Erb, Lasley, Czekala, Monfort & Bercovitz, 1982).

Statistical procedures. Comparison between sexes for each individual variable was based on a non-parametric evaluation of centeredness, calculated with a Mann–Whitney test (Ryan, Joiner & Ryan, 1976). No assumption of identical variability was made between data from each sex, although their range of distribution was generally within normal statistical bounds.

Results

Table 1 presents a summary of female and male data for oestrone, oestradiol, an uncharacterized fraction of oestrogenic substances more polar than oestradiol and estimates of total oestrogen derived from the sum of these components. Egg excrement, paired adrenals and gonads and blood serum samples were evaluated for their oestrogen content.

All excretory oestrogenic values were significantly (P < 0.001) greater in females than in males. Oestradiol in excrement from females was 30-fold the median value from males and was clearly the major component of the total oestrogens measured (75-1% in female chicks compared to 46-2% in males). Non-specific polar oestrogen median values were one-fifth of total oestrogens in females and nearly one-third of male total oestrogens. A ratio evaluation (mean ± s.e.m.) of excretory

| Table 1. Summary of oestrogen concentrations in egg excrement and tissues of day-old chicks |
|----------------------------------------|----------|----------|----------|
|                                       | Females  | Males    |
|                                       | Median   | No. of  | Median   | No. of  |
| Egg excrement (pg/sample)             |          | s.d.     |          | s.d.     | Significance* |
| Oestrone                              | 56-00    | 9        | 17-55    | 10        | P < 0.001 |
| Oestradiol                            | 142-00   | 9        | 45-55    | 10        | P < 0.001 |
| Polar oestrogen†                      | 379-00   | 9        | 33-10    | 10        | P < 0.001 |
| Total oestrogen‡                      | 1891-00  | 9        | 98-40    | 10        | P < 0.001 |
| Adrenal homogenate (pg/paired gland)  |          |          |          |          |           |
| Oestrone                              | 8-40     | 9        | 7-20     | 10        | P > 0.62  |
| Oestradiol                            | 9-70     | 9        | 7-70     | 10        | P < 0.04  |
| Polar oestrogen†                      | 12-50    | 9        | 7-00     | 10        | P < 0.006 |
| Total oestrogen‡                      | 32-90    | 9        | 23-00    | 10        | P < 0.01  |
| Gonadal homogenates (pg/paired gland) |          |          |          |          |           |
| Oestrone                              | 24-00    | 9        | 4-35     | 8         | P < 0.002 |
| Oestradiol                            | 25-30    | 9        | 8-30     | 10        | P < 0.001 |
| Polar oestrogen†                      | 13-80    | 8        | 5-50     | 10        | P < 0.003 |
| Total oestrogen‡                      | 69-10    | 8        | 4-35     | 8         | P < 0.002 |
| Blood serum (pg/ml)                   |          |          |          |          |           |
| Oestrone                              | 8-20     | 8        | 8-30     | 9         | P < 0.13  |
| Oestradiol                            | 52-20    | 8        | 23-80    | 9         | P < 0.002 |
| Polar oestrogen†                      | 21-10    | 8        | 20-00    | 9         | P < 0.49  |
| Total oestrogen‡                      | 92-80    | 8        | 54-30    | 9         | P < 0.006 |

- Mann–Whitney test and Minitab statistical software.
- Uncharacterized oestrogens more polar than oestradiol.
- Derived sum of component oestrogens measured.
oestradiol: oestrone also indicated sex \( (P < 0.001) \) in hatching chicks, 18.45 ± 2.42 and 3.03 ± 0.37 for females and males, respectively.

Median oestrogen content of neonatal chick adrenal glands was low for both sexes, <33.0 pg/paired gland. Polar immunoreactive oestrogenic substances were the largest component oestrogen in female adrenal homogenates, 38.0% of total compared with 30.4% for male values. Oestrone was the only adrenal component that did not differ with sex of the chick. All other adrenal evaluations were sexually distinct \( (P < 0.04) \).

Median oestrogen levels for paired gonads were at least 2.3-fold higher in ovaries than in testes with total oestrogen values of 69.1 pg and 18.4 pg, for females and males, respectively. Oestradiol was the predominant gonadal oestrogen in both sexes. Oestrone levels were higher in ovaries than testes. Testes produced a higher proportion of polar oestrogenic substances than ovaries. All gonadal oestrogens were sexually distinct \( (P < 0.003) \).

Blood serum median values for oestradiol and total oestrogens were also sexually distinct \( (P < 0.002) \); oestrone and polar oestrogens did not differ with sex. Median total oestrogens for all sample values were higher in females than in males \( (P < 0.01) \). Oestradiol was the most consistent endocrine evaluator for sex identification in gonadal tissues, blood serum and excrement samples of neonatal chicks \( (P < 0.002) \).

Discussion

Sex-related endocrine differences have been well substantiated in domesticated chickens, during the last half of embryogenesis. The maturing embryonic ovary actively converts dehydroepiandrosterone and testosterone into oestrogens, predominantly oestradiol and oestrone (Weniger et al., 1983). Embryonic oestradiol biosynthesis and secretion have consistently been greater in chick ovaries compared to testes, which produce relatively little oestrogen (Guichard et al., 1977; Teng & Teng, 1977; Woods & Erton, 1978; Guichard, Haffen, Cedral, Mignot & Sheib, 1979b). Adrenal oestradiol was significantly greater in females than in males, with ovarian oestradiol 3-fold greater than adrenal levels (Guichard et al., 1979a; Tanabe et al., 1979). Data from this study were in agreement with these previous reports, except for the lack of sexually distinct oestrone measurements from adrenal homogenates and blood serum samples. Oestrone and oestradiol values were lower than expected. This discrepancy was in part due to sizable amounts of immunoreactive oestrogenic substances, more polar that oestradiol, found in all samples from this study and unaccounted for in previous reports.

Minor oestrogens have been identified in the urine of adult chicken hens: oestriol and epimers of oestriol (16 and 17, α or β epi-oestriol) (Mathur, Anastasiadis & Common, 1966). High-performance liquid chromatography (HPLC), combined with immuno- and cytosol receptor activity, has been used to isolate oestrogenic substances in the plasma, ovary and excrement of adult turkeys: Erb et al. (1982) reported a single immuno- and receptor active peak (more polar than oestradiol) from a turkey ovarian homogenate and 3 distinct polar oestrogenic peaks from turkey hen faeces and plasma samples. These uncharacterized polar oestrogenic substances represented a substantial proportion of the total oestrogen immuno- and receptor activity. Comparative elution profiles (HPLC relative retention values) for these oestrogen-like substances from avian and mammalian sources have not been corroborated to the elution characteristics for any of 16 known steroidal estrogens (Erb et al., 1982). The physical and physiological characteristics of these non-specific polar oestrogenic substances in neonatal chicks remains unknown. Their identity and biosynthetic/metabolic pathways also remain undefined and warrant further investigation.

Quantification of excretory hormonal data to any standardized or comparable unit of measure has not been validated. Little is known about the regulatory factors that influence embryonic steroid excretion. Some obvious and inequitable considerations were apparent in simply stating an
absolute concentration of excretory hormones, expressed in this study as a pg/sample: (1) the effects of incubator environment; (2) duration of hatching; (3) weight, proportion and hormone content of allantoic, colon and urinary components; (4) the variability of creatinine excretion/unit of time from perinatal chicks; (4) the accumulation/resorption of sex steroids in egg excrement. Despite significant differences in excretory oestrogen concentration between sexes, we advise that sexing evaluations be based on data calculated from a dimensionless ratio for egg waste oestradiol/oestrone levels. A similar rationale was used to compare faecal steroid analysis data in sexing a wide variety of adult birds, averting the apparent discrepancies between random single faecal samples collected from a diverse population (Czekala & Lasley, 1977; Stavy et al. 1979; Bercovitz, Collins, Price & Tuttle, 1982).

The basic premise for initiating this study was to verify the presence of steroidal oestrogens in egg excrement and to validate the relationship between oestrogen production from steroidogenic tissues, circulatory transfer and subsequent deposition/accumulation of oestrogens in excrement. These data supported an hypothesis that embryonic ovarian tissue produced more oestrogen than testicular tissue and that this sex distinction could be monitored by egg waste oestrogen analysis. Oestradiol was readily measurable in egg excrement samples from hatching chicks and excretory oestradiol:oestrone ratio evaluation reliably identified the functional sex in domesticated chicks. Female chicks had egg excrement oestradiol:oestrone ratios >11.0 and males had ratios <6.00. Sex identity in domesticated chicks was clearly distinguishable when based on the analysis of egg waste oestrogens. It was also completely non-invasive since samples collected at the time of hatching were removed from the shell remnants.

We thank Ms Jean Hoch and the staff of the Children’s Zoo at San Diego Zoo for their assistance and provision of incubator equipment for this project. This work was supported by the American Federation of Aviculture, the Wildlife Preservation Trust, Int., Inc. and the Zoological Society of San Diego.

References


Guichard, A., Cedard, L., Mignot, Th.-M., Scheib, D. & Haffen, K. (1979a) Radioimmunoassay of steroids produced by chick embryo gonads cultured in the...


Received 21 November 1984