1996

Estrogen Levels in House Wren (Troglodytes aedon) Egg Yolks

V. Brook Waggoner '96
Illinois Wesleyan University

Estrogen Levels In House Wren (*Troglodytes aedon*) Egg Yolks

V. Brook Waggoner, Illinois Wesleyan University

Submitted for Senior Honors

April 19,1996

Abstract

Estrogen, when present in early embryonic development, regulates sexual differentiation in the avian nestling and adult. In this study, I developed a procedure to extract and quantify levels (by radioimmunoassay) of the estrogen,17β-estradiol, in house wren (*Troglodytes aedon)* egg yolk. Levels of 17β-estradiol found in one clutch of eggs increased with the order of laying, indicating female house wrens may be capable of regulating the levels of 17β-estradiol received by the offspring. Since the attraction of mates is often aided by the display of sex differences, maternal control of 17β-estradiol levels in the embryo may influence the future reproductive success of her offspring

Introduction

Steroid hormones present in early embryonic development affect many behavioral, physiological, and morphological characteristics of the avian nestling and adult (Adkins-Regan and Ascenzi 1987, Wingfield et al. 1987, Schwabl 1993, Adkins-Regan et al. 1995). In contrast to maternal resources present in the yolk at the time of embryonic development (e.g. yolk protein, lipid, phosphates), steroid hormones were once considered to be produced exclusively by the embryo (Winkler 1993). However, recent studies (Schwabl 1993, Adkins-Regan et al. 1995) indicate that the close proximity of the steroidogenic ovarian tissue to the developing yolk allows maternal lipophilic hormones to be inherited by the embryo.

Testosterone is a steroid hormone known to have behavioral effects on birds from the time of hatching to adulthood (Winkler 1993). Schwabl (1993) found a positive correlation between the social rank of juvenile canaries (*Serinus canaria*) and the concentration of testosterone in the yolk of the eggs from which they hatched. Thus, maternal control of testosterone levels in the yolk may determine the competitive ability of the offspring. Moreover, it is likely that testosterone enhances the overall growth of the embryonic neuromuscular system (Schwabl 1993). Hence, increased levels of testosterone could help younger, smaller individuals in the nest compete against their older, larger siblings (Schwabl 1993).

Estrogen is a steroid hormone that regulates the sexual differentiation of birds. Maturation of the female is dependent upon ovarian secretion of estrogen (Adkins-Regan et al. 1995, Gahr et al. 1993). Estrogen levels influence the organization of neural circuits in the brain during embryonic development and their activation in adulthood. These neural circuits are primarily involved in adult reproductive behavior such as male

song learning and song production (Gahr et al. 1993, Schlinger 1994, Adkins-Regan et al. 1995). Because attraction of mates is often aided by the display of sex differences, including song repertoire, (Adkins-Regan and Ascenzi 1987; Schlinger 1991, 1992, 1994; Gahr et al. 1993) maternal control of estrogen levels in the embryo may influence the future reproductive success of her offspring.

Although levels of maternally deposited hormones appear to be beneficial to the offspring, constraints may be placed on the female if manufacturing and/or depositing them into the yolk is energetically expensive. If energy costs are great, the quality of the female's eggs could be affected (Winkler 1993). Therefore, despite potential benefits to the offspring, the mother may be forced to deposit low hormone levels to secure her well being and that of all eggs in the clutch.

Although levels of steroid hormones have been quantified in adults, little attention has been paid to levels of the hormones present in the yolks of eggs laid by wild birds. The purpose of this study is to establish an efficient method for the extraction and quantification of 17β-estradiol in house wren (*Troglodytes aedon*) egg yolk. This method can then be used to test the hypothesis that female house wrens control levels of 17β-estradiol in their eggs. If the mother can exert such control, then we expect to find variation of 17β-estradiol within and between clutches produced in the same year.

Methods

Collection of House Wren eggs

Clutches of house wren eggs were collected daily from May-August 1994 from 315 nestboxes located on a 20 ha study area in McLean County, Illinois (40°40' N,

88°53'W) (Harper et al. 1992, 1993). The eggs collected were from early season and late season broods which had modal clutch sizes of seven and six eggs, respectively (Harper et al. 1994). As each egg was collected, a fake, plastic egg was placed in the nest to prevent possible alteration of hormone levels by the female in response to a change in clutch size. At the time of collection, the eggs were frozen. Several months later, the yolks were dissected from the eggs and stored at -20°C until extractions were performed.

Extraction of β-estradiol from egg yolks

At the time of extraction, yolks were thawed and homogenized with 1 ml of distilled water. Tritiated 17 β-estradiol (~10000 DPM/yolk, 85.00Ci/mmol) was added, and the yolks were kept at 4°C overnight. Following the equilibration period, the homogenate was extracted three times with 2 ml of 50:50 diethyl ether:hexane (vol:vol). The three extracts were combined and stored at 4°C until analyzed. For radioimmunoassay (RIA) analysis, 1 ml of the extract was dried and the residue washed with 1 ml of 75% methanol/water to quantitatively recover the steroids.

Radioimmunoassay analysis

Radioimmunoassay (RIA) is a technique that allows for the quantification of a substance by its binding to a specific antibody (Matthews and Van Holde 1996). In this technique, a known amount of radiolabelled compound (radioligand) is added to a small amount of antibody and the amount of radioligand bound is determined. In other tubes, known amounts of the unlabelled (nonradioactive) compound are added to the radioligand and antibody to make a standard curve. The unlabelled compound competes with the radioligand for binding sites on the antibody molecules, causing a decrease in the amount of bound radioligand; this decrease is related to the amount of unlabelled

material present. Unknown amounts of the material can be quantified by comparing the amount of radioactivity bound to that of the standard curve.

Titering of the antiserum: An antiserum for 17β-estradiol was purchased from Arnel Products Co. The binding activity of the antiserum was determined by incubating approximately 5000 DPM of tritiated 17β-estradiol (purchased from NEN-Dupont) and antiserum diluted in varying amounts (a serial dilution: 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600) of RIA buffer (pH 7.2;0,9% NaCl, 0.5 M Phosphate, and 0.1% gelatin) for two hours. After equilibration was reached between the free and antibody-bound 17β-estradiol, 500 µl of dextran-coated charcoal (DCC) (pH 7.2, 1.5% charcoal, 0.8% dextran in RIA buffer) was added to absorb free hormone. After 12 minutes, the assay tubes were then centrifuged (5000 g/min) and 400 µl of the supernatant was added to scintillation cocktail. The amount of radioactivity in this aliquot was quantified using a Packard scintillation counter.

Standard curve: A standard curve was run to examine the effect of unlabelled steroid on the binding of tritiated 17β-estradiol to the antiserum. An aliquot (100 µl) of a 1:25600 dilution of the antiserum was incubated with increasing amounts of 17β-estradiol (i.e. 10, 25, 50, 100, 200, 400, 1000 pg/tube) and approximately 5000 DPM of tritiated 17β-estradiol. After two hours, the tubes were treated with DCC as described above.

Binding specificity: Compounds structurally related to 17β-estradiol were tested for their potential to interact with the antiserum used in this study. One and ten nanograms of dihydrotestosterone, testosterone, progesterone, pregnenolone, or 17α-hydroxy-progesterone were added to assay tubes containing radiolabelled 17β-estradiol and antiserum.

Radioimmunoassay of yolk extracts: Aliquots of the methanolic wash of the yolk extracts were added to assay tubes and dried. Equal volumes (100µl) of radiolabelled steroid and a 1:25600 dilution of antiserum were added to each tube and incubated at room temperature for two hours.

Results and Discussion

In the present study, a procedure was established for extracting and quantifying levels of 17β-estradiol in the yolk of house wren eggs. However, before arriving at the current protocol, extensive preliminary analysis of potential extraction and quantification methods was performed.

Preliminary procedures.---Egg yolk contains large amounts of lipid which will interfere with an RIA. Several extraction procedures were tested to separate 17β-estradiol from lipid found in the yolk. In the first attempt, a triphasic extraction procedure was tested (Borst and Tsukimura 1991). In this method, the yolk was homogenized in saline (2% NaCl) and added to a similar volume of acetonitrile and then extracted with hexane:ether (1:1). It was anticipated that the acetonitrile would prevent most lipids from entering the diethyl ether:hexane extract. Data obtained from the triphasic extraction indicate that the acetonitrile did remove some lipids from the extract. However, a large amount of lipid still remained in each sample, and the recovery of 17β-estradiol was, on average, 80.70 % ($\pm$ 7.97 (SD), N = 6)

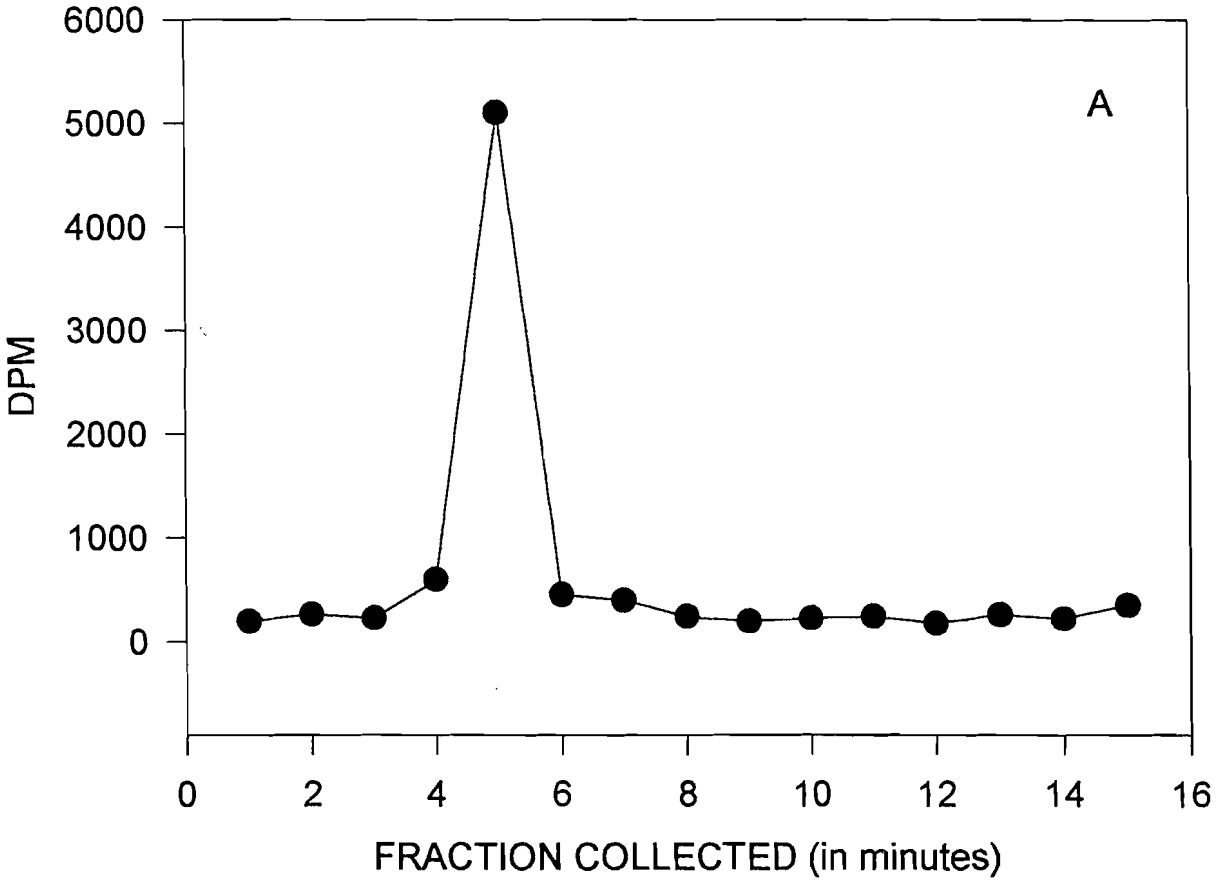
In a second procedure, 17β-estradiol was removed from the yolk by the triphasic extraction and then further purified using a Prepsep silicon mini column. A 10% methanol:methylene chloride solution (vol/vol) was used to elute the 17β-estradiol from the column. Mini column chromatography did not cause a substantial loss of labelled

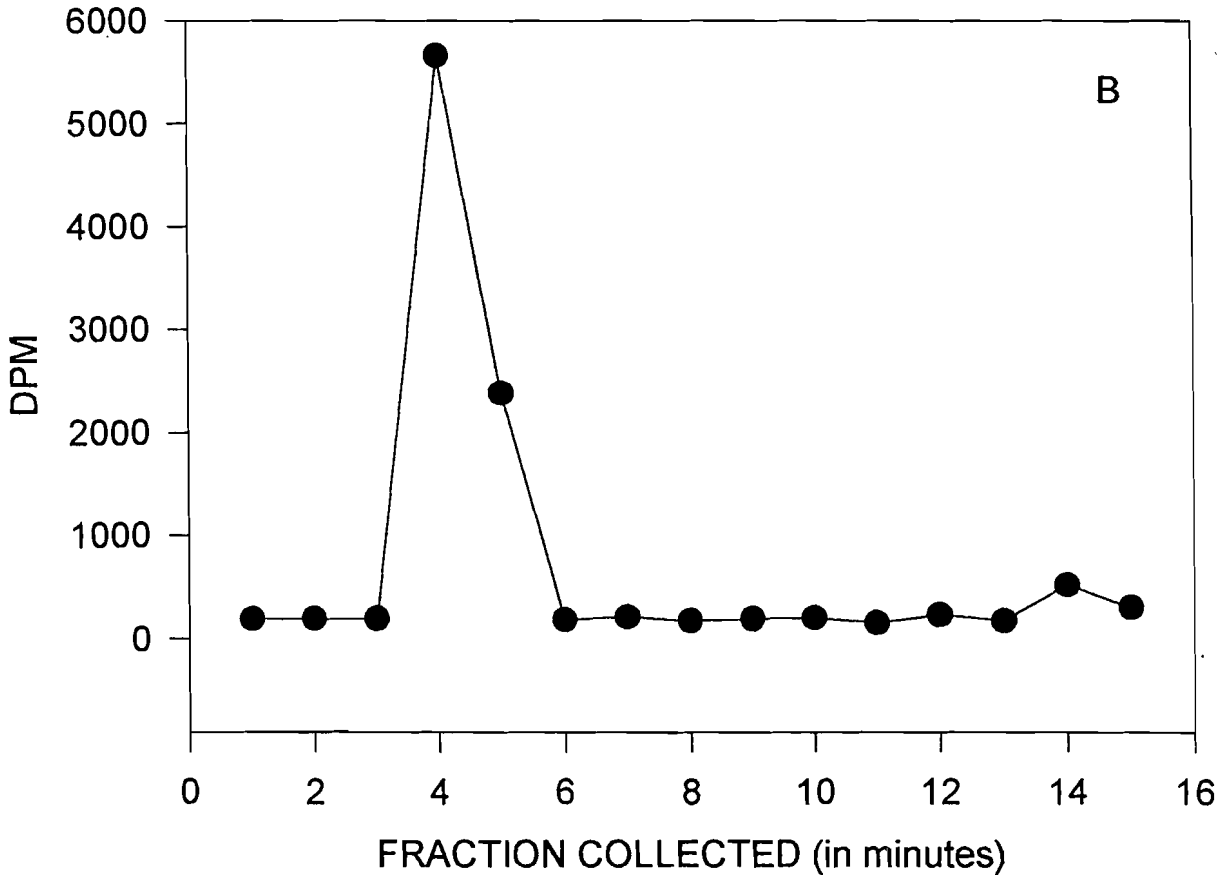
17β-estradiol (<10%). However, separation of the extract from the lipid was not improved with the use of the column.

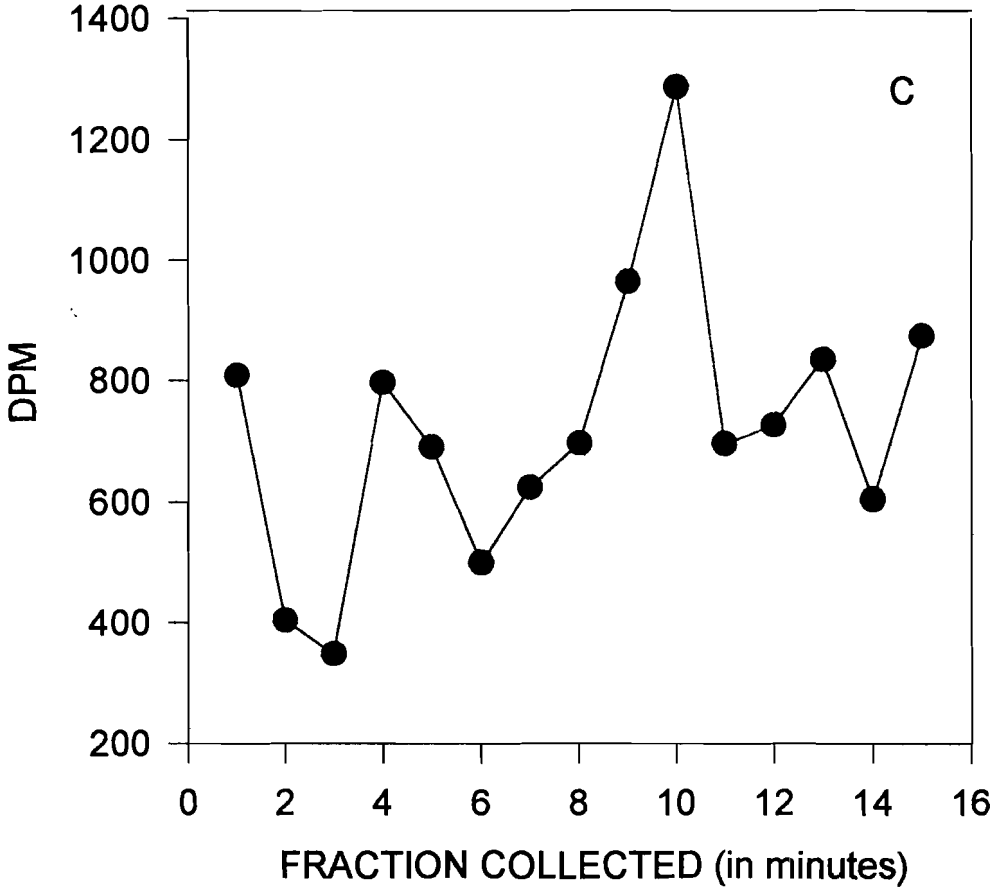
High pressure liquid chromatography (HPLC) was also investigated as a means of purifying 17β-estradiol from other compounds with similar chemical characteristics (i.e. testosterone, dihydrotestosterone, and progesterone). In this procedure, 17β-estradiol was injected into a reverse phase (C18) HPLC (rpHPLC) column with a 60-90% methanol/water gradient. The chromatograph obtained from the standard was used to identify the elution behavior of 17β-estradiol. Extracts of yolk samples were then spiked with tritiated 17β-estradiol and injected into the column. Fractions from the column were collected at one minute intervals for a period of fifteen minutes. Levels of tritiated 17β-estradiol present in each fraction were counted on a Packard scintillation counter. The radiolabelled material was used to identify the time at which 17β-estradiol was eluted from the column. However, rpHPLC did not prove to be an effective method for separating 17β-estradiol from other structurally similar compounds. In particular, testosterone and 17β-estradiol were not separated by rpHPLC (Figure 1A & 1B). Furthermore, when yolk extracts were separated by rpHPLC, 17β-estradiol eluted from the column at various times, yielding low recovery of the 17β-estradiol (Figure 1C & 1D).

The final procedure involved the recovery of the steroid by washing with 75% methanol. Yolk was homogenized in distilled water and extracted with 50:50 diethyl ether:hexane. By adding tritiated 17β-estradiol to the yolk samples, it was shown that the procedure, on average, recovered a high percentage of the steroid 89.07% ($\pm$ 8.41, (SD), N=3). An aliquot of this extract was dried and the lipid residue washed with 75% methanol. This wash quantitatively removed the steroid while leaving most of the lipid

Figure 1: Amount of radioligand present in fractions collected from a reverse phase HPLC column after injecting: A- ^{3}H-testosterone; B- ^{3}H-17β-estradiol; C- a yolk extract containing ^{3}H-testosterone; D- a yolk extract containing ^{3}H-17 β–estradiol


6000
5000
4000
3000
2000
1000
0
DPM
A
0
2
4
6
8
10
12
14
16
FRACTION COLLECTED (in minutes)


6000
5000
4000
3000
2000
1000
0
DPM
B
0
2
4
6
8
10
12
14
16
FRACTION COLLECTED (in minutes)


C
DPM
1400
1200
1000
800
600
400
200
0
2
4
6
8
10
12
14
16
FRACTION COLLECTED (in minutes)

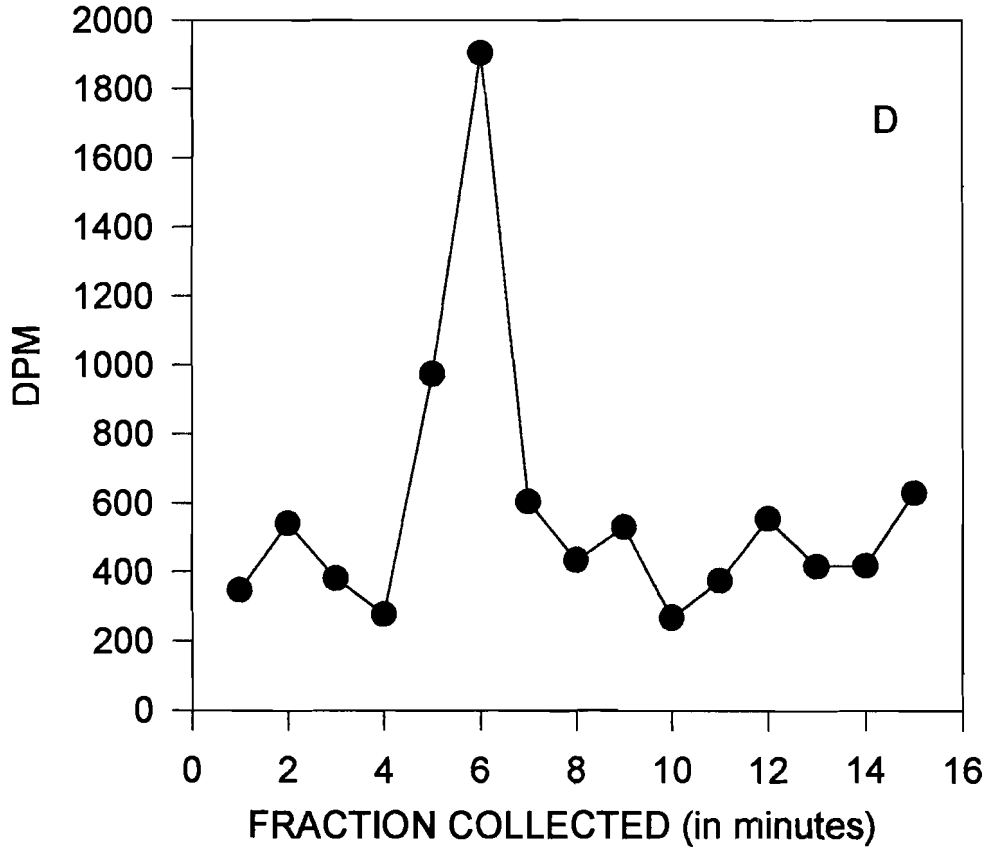
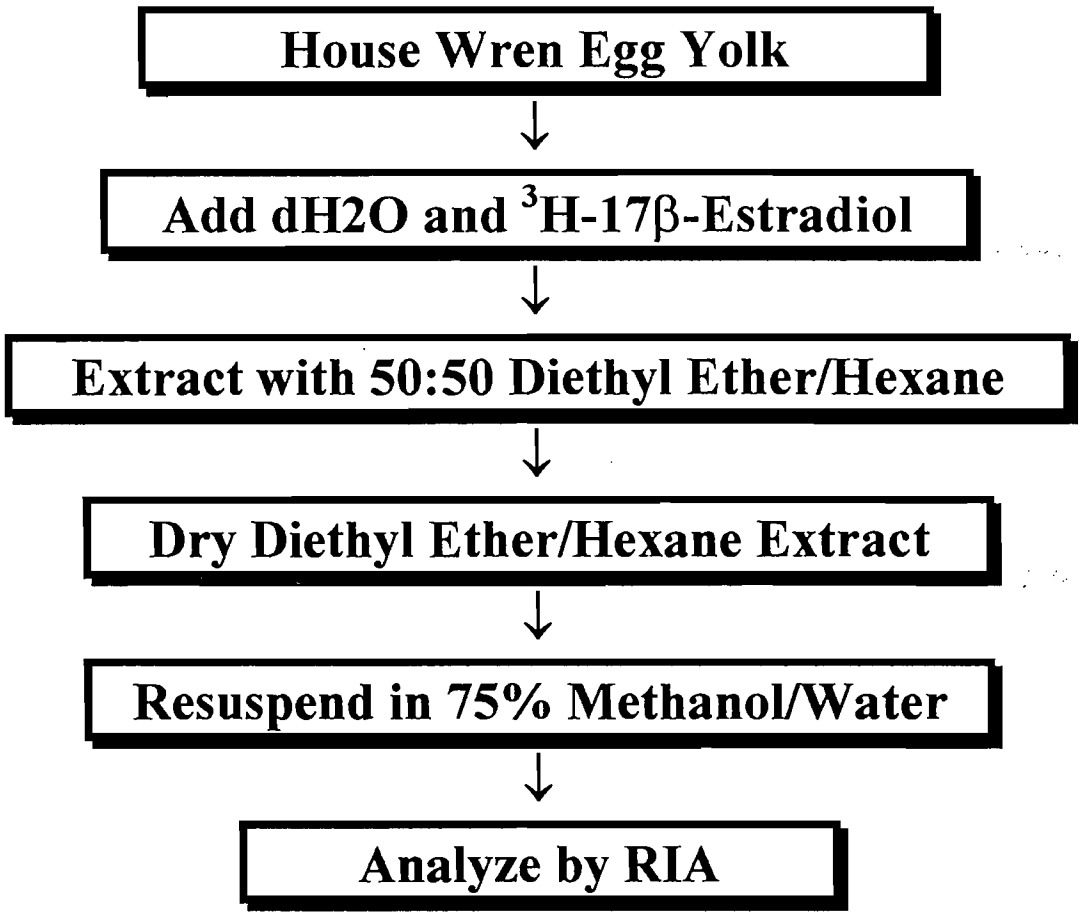

D
DPM
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
FRACTION COLLECTED (in minutes)

Figure 2: An outline of the method used to extract and purify yolk samples.

Extraction Procedure



intact. Subsequent analysis showed that this extract was sufficiently clear for analysis by RIA (Figure 2).

RIA analysis.---Before the antiserum could be used for the RIA, it needed to be characterized. The first step in this characterization was construction of a titer curve (Figure 3), which shows the binding activity of the antiserum for the tritiated steroid. From this curve it was determined that a 1:25600 dilution of the 17β-estradiol antiserum bound approximately 50% of the radioligand present. Because most assays give an optimal response at this point, this dilution was used in subsequent assays.

The effect of increasing amounts of unlabelled 17β-estradiol on the amount of radioligand bound to the antiserum was graphically plotted in order to obtain a standard curve (Figure 4). The lowest amount of 17β-estradiol detectable by the RIA was 25 pg / tube. A standard curve was performed with each assay in order to determine the amount of 17β-estradiol in the yolk samples. The amount of radioligand bound in the presence of the sample was compared to the standard curve to determine the amount of steroid present.

The specificity of the antiserum used in this study was also determined. If other compounds in the yolk bind to the antiserum, then fewer binding sites would be available for 17β-estradiol. As a result, quantification of the 17β-estradiol in the yolk would be inaccurate. Table 1 shows the cross reactions between the antiserum and other compounds which share similar chemical characteristics. As indicated in this table, the amount of cross-reaction is minimal.

Validation of the assay.--- The RIA for 17β-estradiol in yolk samples was validated by comparing various amounts of yolk extract analyzed with or without unlabelled 17β-estradiol. Eight aliquots of each yolk sample were analyzed, all of which contained equal amounts of labelled 17β-estradiol and antiserum. Two 50 µl aliquots of

Figure 3: A titer curve for 17β-estradiol. The point at which 50% of the radioligand was bound to the antiserum is indicated by the arrow.

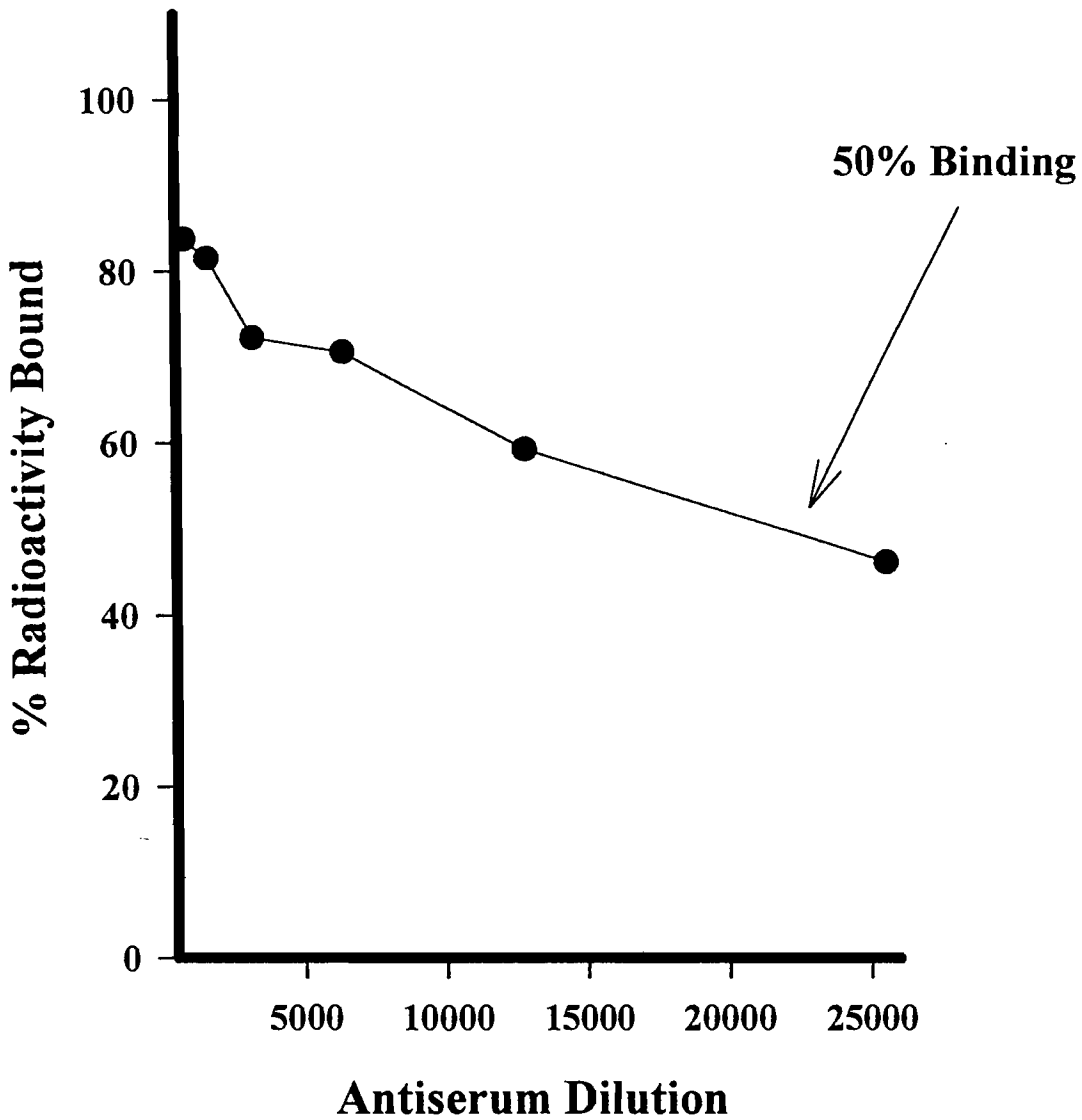

100
80
60
40
20
0
% Radioactivity Bound
50% Binding
5000
10000
15000
20000
25000
Antiserum Dilution

Figure 4: A standard curve for 17β-estradiol. Values given are means ± SE.

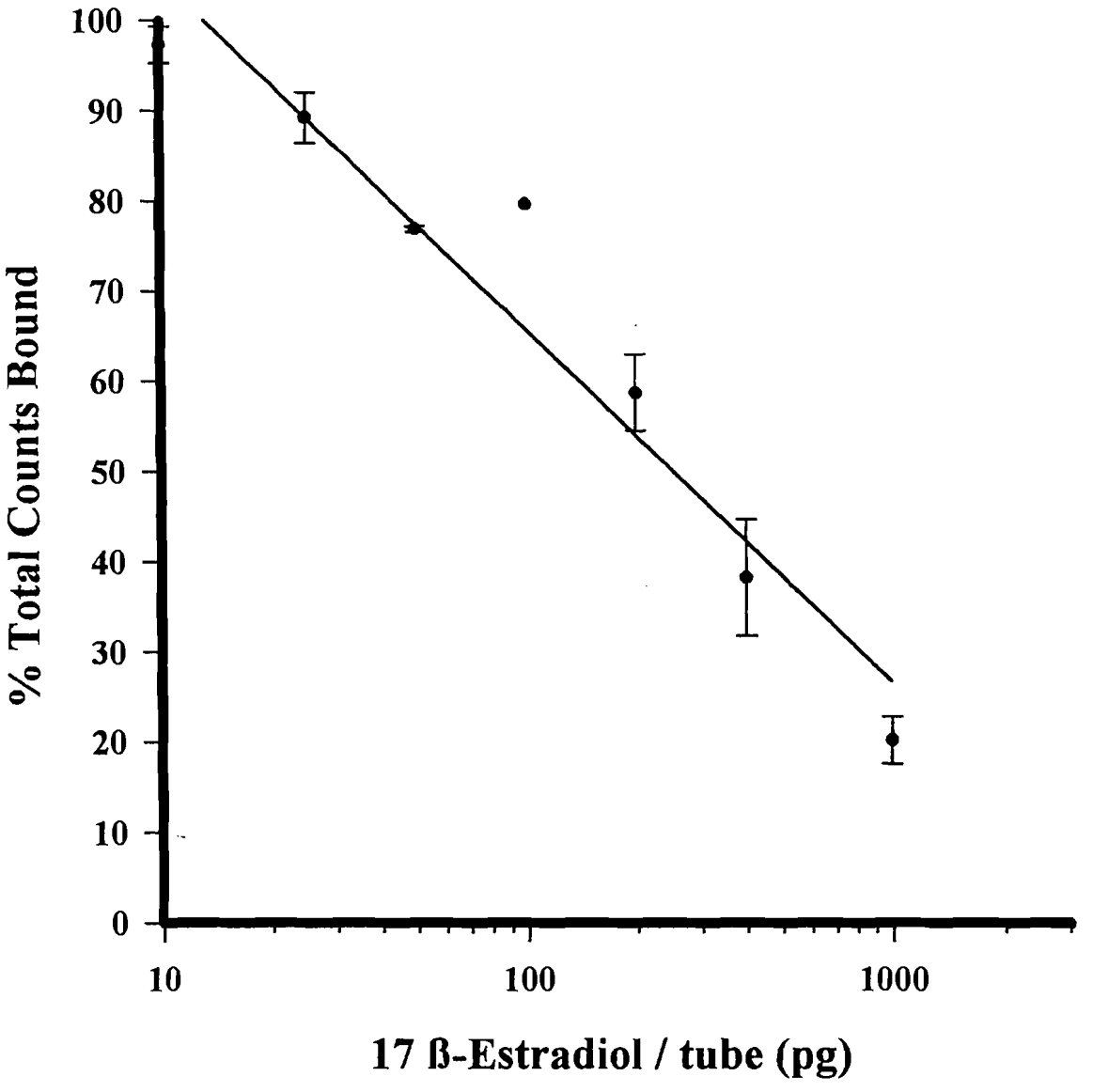

100
90
80
70
60
50
40
30
20
10
0
% Total Counts Bound
10
100
1000
17 ß-Estradiol / tube (pg)

Table 1: Steroid specificity of the17ß-estradiol antiserum

Steroid	ED50*	% Cross reaction
17ß-Estradiol	124.5	100.0
Testosterone	6263.6	2.0
Dihydrotestosterone	10066.4	1.2
Progesterone	10229.7	1.2
Pregnenolone	31272.6	0.4
α Progesterone	19845.3	0.6

*ED50 = amount of steroid (in picograms) needed to inhibit binding by 50%.

extract were incubated with unlabelled estradiol, and two aliquots were incubated without the unlabelled material. Likewise, two 150µl aliquots were incubated with 100 pg of unlabelled 17β-estradiol and two aliquots were incubated without unlabelled 17β-estradiol.

Differences in the concentration of 17β-estradiol detected in the tubes were examined to determine the validity of the assay. For example, the estradiol concentration detected in those tubes with added unlabelled 17β-estradiol should have been 100 pg greater than tubes not receiving added estradiol. The observed difference between these sets was, on average,104.2 pg (± 14.9 (SE), N=9). Likewise, a ratio of the concentration of 17β-estradiol in a 50µl aliquot was compared to that of a 150µl aliquot of the sample. It was anticipated that three times as much steroid would be detected in the 150µl aliquot tubes. The observed ratio of 3.65:1 (± 0.8 (SE), n=5) is close to this value, confirming the validation of this assay.

Hormone levels in eggs.---The RIA analysis of one clutch of house wren egg yolks are shown in Table 2. The data demonstrate an increase in the levels of 17β-estradiol in eggs laid later in the clutch sequence. These preliminary data differ from those of an earlier study which did not detect differences in 17β-estradiol of the yolk of canary eggs (Schwabl 1993). Because only one clutch of eggs was analyzed, no conclusive statement can be made about the variation of 17β-estradiol levels among the eggs. However, if the preliminary data are indicative of levels found in other clutches, then it appears that the female house wrens have some control over the amount of 17β-estradiol that her offspring receives. As previously mentioned, this control could be particularly important in determining the reproductive success of her offspring. The male offspring would likely be affected more than female offspring, since much of their

Table 2: Levels 17ß-estradiol (E2) in a clutch of house wren eggs

Clutch	Order	Egg #	E2 (ng/yolk)
EA8-1	1	154	nd
	2	61	nd
	3	8	3.0
	4	86	4.6
	5	73	3.6
	6	71	10.9
	7	156	9.8

nd = not done

reproductive success is dependent upon estrogen-mediated behavior such as song repertoire.

A possible explanation for the inconsistency of the present data with that of Schwabl's (1993) may be a result of the differences between wild and domestic birds. For example, because domestic birds have resided in a more "controlled" environment for many generations, pressure in the competition for mates may not be as great as it is in wild birds. Thus, differences in maternally-deposited hormone levels may be more advantageous to wild birds.

A second possible explanation for the differences in results lies in the fact that the activation of adult male sexual behavior by testosterone requires conversion of testosterone to estradiol by the enzyme aromatase (Silver and Ball 1987). Since the concentration and efficiency of aromatase can vary from species to species (Silver and Ball 1987), it is possible that canaries have a higher concentration of aromatase in the brain than house wrens. Therefore, smaller levels of estradiol could be deposited into the yolk to achieve similar responses. This speculation is further supported by the fact that higher levels of testosterone were found in house wren eggs than in the canary eggs (Fryzel, personal communication), thus suggesting that the efficiency of the aromatase may be higher in canaries because less substrate (i.e. testosterone) is required.

Future work on this study will include the analysis of hormone levels in remaining clutches.

Acknowledgments

A special thanks to Dr. Given Harper for his encouragement and assistance; Dr. David Borst for his countless hours of work and incredible patience: the ISU endocrinology lab for the use of their equipment; Kim Fryzel for her consistent hard work and cooperation.

Literature Cited

Adkins-Regan, E., and M. Ascenzi. 1987. Social and sexual behavior of male and female zebra finches treated with oestradiol during the nestling period. Anim. Behav. 35: 1100-1112.

Adkins-Regan, E., M.A. Ottinger, and J. Park. 1995. Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. Jour Exp. Zool. 271:466-470.

Borst, D.W., and B. Tsukimura. 1991. Quantification of methyl farnesoate levels in hemolymph by high performance liquid chromatography. Journal of Chromatography 545: 71-78.

Gahr, M., H.R. Guttinger, and D.E. Kroodsma. 1993. Estrogen receptors in the avian brain: Survey reveals general distribution and forebrain areas unique to songbirds. Jour. Comp. Neur. 327: 112-122.

Harper, R.G., S.A. Juliano, and C.F. Thompson. 1992. Hatching asynchrony in the house wren, Troglodytes aedon: a test of the brood-reduction hypothesis. Behavioral Ecology 3 : 76-83.

—. 1993. Avian hatching asynchrony: Brood classification based on discriminant function analysis of nestling masses. Ecology 74: 1191-1196.

—. 1994. Intrapopulation variation in hatching synchrony in house wrens: Test of the individual-optimization hypothesis. Auk 111: 516-524.

Matthews, C.K., and K.E. van Holde. 1996. Biochemistry, 2 ed. Benjamin/Cummings Publishing Company, Inc. Menlo Park, California.

Schlinger, B. 1994. Estrogens and song: Products of the songbird brain. Bioscience 44: 605-612.

Schlinger, B., and A. Arnold.1991. Brain is the major site of estrogen synthesis in a male songbird. Proc. Natl. Acad. Sci. 88: 4191-4194.

—. 1992. Circulating estrogens in a male songbird originate in the brain. Proc. Natl. Acad. Sci. 89: 7650-7653.

Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. Proc. Natl. Acad. Sci. 90: 11446-11450.

Silver, R. and G.F. Ball. 1989. Brain, hormone and behavior interactions in avian reproduction: status and prospectus. The Condor. 91: 966-978.

Wingfield, J.C. and G. F. Ball. 1987. Changes in plasma levels of luteinizing hormone and sex steroid hormones in relation to multiple-broodedness and nest site density in male starlings. Physiol. Zool. 60(2): 191-199.

Winkler, D. 1993. Testosterone in egg yolks: An ornithologist's perspective. Proc. Natl. Acad. Sci. 90: 11439-11441.