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## Radioimmunoassay Analysis of Testosterone Levels in Clutches of House Wrens (*Troglodytes aedon*) Egg Yolks

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Radioimmunoassay Analysis of Testosterone Levels In Clutches of  
House Wren (*Troglodytes aedon*) Egg Yolks

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Thesis Paper for Research Honors in Biology  
Illinois Wesleyan University  
Spring, 1996

## ABSTRACT

Recent evidence indicates that variation in intensity of aggressiveness and competitive ability among nestlings of a clutch may be attributed, at least partly, to differences in maternally deposited yolk testosterone that affects embryonic development (Schwabl 1993). We developed a radioimmunoassay (RIA) for measuring yolk testosterone levels in house wren (*Troglodytes aedon*) eggs. This procedure was used in initial studies to determine whether yolk testosterone varies within a clutch of house wren eggs. The analysis revealed the presence of higher levels of testosterone in yolks of eggs laid later in the clutch sequence. Because later laid eggs tend to hatch later (Winkler 1993), variation in the amount of testosterone deposited by the female may ensure a greater chance of survival for younger, less developed offspring.

## INTRODUCTION

Individual birds within a species exhibit variation in intensity of aggressiveness, competitive ability, and territorial behavior. Such variation serves as a basis on which natural selection can act, whereby the more aggressive, competitive individuals usually attain greater fitness than the less competitive ones. This is especially true among nestlings of a brood; the older, larger nestlings that can gape higher and attain a more favorable position in the nest will out-compete their younger, smaller siblings in obtaining food from the parent (Ryden and Bengtsson 1980, McGillivray and Levenson 1986). Because of their superior competitive ability, larger nestlings are more likely to survive than their smaller siblings (Harper et. al. 1992). These differences in competitive ability are often attributed to genetic variation among nestlings. However, recent evidence indicates that such variation may be attributed, at least partly, to differences in maternally-derived compounds that affect embryonic development (Schwabl 1993).

Avian embryos inherit numerous maternal resources that are used during their development, including steroid hormones, which pass from the mother to the yolk during egg formation (Schwabl 1993). Variations in the concentrations of these hormones (e.g. testosterone) may affect the expression of certain behavioral, physiological, and morphological traits (e.g. Baptista et. al. 1987, Wingfield et. al. 1987, Wingfield et. al. 1990). For example, recent studies have shown a positive correlation between the social rank of juvenile canaries (*Serinus canaria*) and the concentration of testosterone in the yolks of eggs from which each bird hatched (Schwabl 1993). Thus, variation in the level of yolk testosterone may affect the competitive ability of offspring.

If females deposit varying amounts of testosterone in yolks, then eggs of a clutch should contain different levels of testosterone. To test this hypothesis, we developed a

radioimmunoassay (RIA) procedure for measuring testosterone levels in yolk samples from house wren (*Troglodytes aedon*) eggs. We have used this procedure in initial studies to determine whether yolk testosterone varies within a clutch of house wren eggs. This is the first documentation of yolk testosterone levels among the eggs of a clutch of wild birds.

## METHODS

Eggs were collected from nest boxes located on the East Bay (20 ha) study area in McLean County, Illinois from May-August 1994 (40°40'N, 88°53'W). The 315 virtually identical nest boxes were located in upland deciduous forest surrounded by agricultural land (Finke et. al. 1987, Drilling and Thompson 1988, Harper et. al. 1992).

*Definitions.*---House wrens in this population are double brooded, with a modal clutch size of seven in the first ("early season") brood and six in the second ("late season") brood (Finke et. al. 1987, Harper et. al. 1993, 1994). Those nests having egg-1 dates (the date on which the first egg was laid) before the median egg-1 date of the year were designated as early-season broods, while late-season broods had egg-1 dates on or after the median egg-1 date of the year (Harper et. al. 1994).

*General procedures.*---All nest boxes were checked daily between 0700-1200. House wrens lay one egg per day until their clutch is complete, and each egg was collected on the morning it was laid. The last laid egg in a clutch was collected either prior to or early during (within 3-4 hours) the onset of full female incubation. Each collected egg was replaced with an artificial egg to avoid changes in clutch size which in turn might alter the allocation of yolk hormones by the female. Females readily accepted and incubated the artificial replacements (Harper, personal observation). A clutch was considered complete when the same number of eggs was recorded on

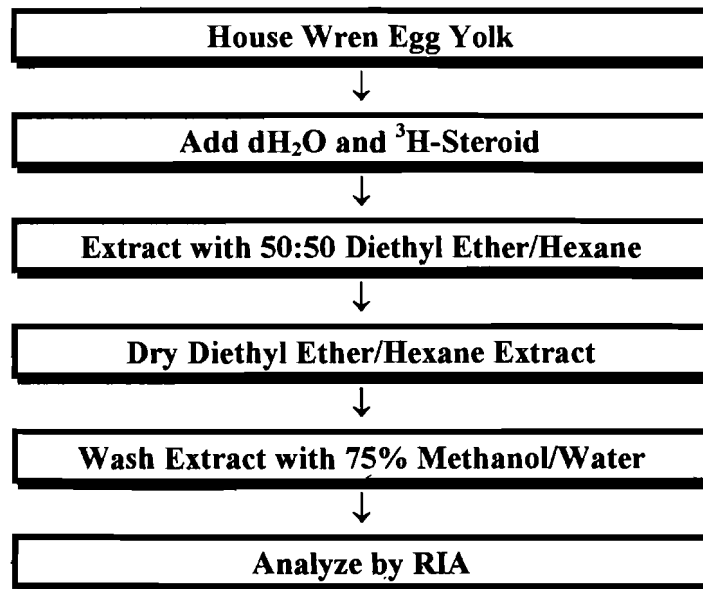
two consecutive visits to the nest (Harper et. al. 1994). The artificial eggs were then removed. At the time of collection, eggs were transported in cartons to the laboratory where they were frozen (-20°C). Several months later, the yolks were dissected from the eggs and stored at -20°C.

*Extraction of Yolks.*---At the time of extraction, the yolk sample was thawed and distilled water (1 ml) added. The sample was homogenized by mixing with glass beads for approximately 5 minutes with a vortex mixer. Approximately 10,000 disintegrations per minute (DPM) of tritiated testosterone (NEN-Dupont, 101.00 Ci/mmol) was added to the homogenate for calculation of steroid recovery and the mixture was allowed to equilibrate overnight. Following equilibration, the homogenate was extracted three times with 2 ml of 50:50 hexane/diethyl ether (vol/vol). The extracts were combined and a 1 ml aliquot was dried and washed with 1.5 ml of 75% methanol/water, a procedure which quantitatively dissolves testosterone (Figure 1).

*Titering of Antiserum.*---Prior to the analysis of the yolk extract, an antiserum for testosterone (Wien Laboratories, Inc.) was titered to determine the optimal dilution for steroid binding. Because most assays give an optimal response when 50% of the radioligand is bound (Borst 1995), the testosterone antiserum was analyzed to determine the dilution giving this level of binding. The commercial antiserum of unknown concentration was diluted in 5 ml of radioimmunoassay (RIA) buffer. This 1:5 dilution was serially diluted in RIA buffer (gel-PBS: 0.05M phosphate buffer, pH 7.2; 0.9% NaCl, and 1% gelatin) to give dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320. Duplicate tubes were used to test each dilution of the antiserum. These tubes contained 50 µl of RIA buffer, 50 µl (approximately 5,000 DPM) of tritiated testosterone, and an aliquot (100 µl) of the indicated antiserum dilution, giving final antiserum dilutions of 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640. The assay tubes were then incubated for

**Figure 1.** Flow diagram of the procedure used to extract and analyze yolk testosterone in house wren eggs.

## Extraction Procedure





two hours at room temperature. Following incubation, each tube was put on ice for 5 minutes, treated with 500  $\mu$ l of dextran-coated charcoal (DCC: 1 g neutralized charcoal, 0.1 g dextran sulfate, and 400 ml RIA buffer) to remove unbound radiolabeled testosterone, and placed on ice for an additional 5 minutes. The tubes were centrifuged at 5,000g x 5 minutes at 4°C. An aliquot of the supernatant (400  $\mu$ l) was placed in a scintillation vial with scintillation cocktail.

To quantify the amount of radiolabeled testosterone present in each sample, a liquid scintillation counter (LSC) was used. When beta particles are emitted by a radioisotope, their kinetic energy is transferred to the solvent molecules of the scintillation cocktail (Kessler 1988). The solvent molecules then transfer their energy to scintillator molecules, causing the molecules to become excited. The scintillation molecules then return to their stable energy state by emitting photons. The photons can be detected using a liquid scintillation counter. The intensity of the detected light is directly proportional to the energy of the beta particle emitted by the radioisotope (Kessler 1988). For these studies, a Packard LSC was used.

*Constructing Standard Curves.*---Standard curves were constructed for testosterone and dihydrotestosterone (DHT) to illustrate the effect of unlabeled testosterone and a structurally similar hormone, DHT, on the binding of tritiated testosterone to the testosterone antiserum. The commercially purchased testosterone standard (10ng/ml) was diluted with methanol to a concentration of 1ng/ml. Increasing amounts (2.5 pg, 5 pg, 10 pg, 25 pg, 50 pg, 100 pg, and 200 pg per tube) of the diluted standard were added to assay tubes and dried with a Speed-vac. Similarly, unlabeled dihydrotestosterone (Sigma Chemical Co.) was diluted in methanol to a concentration of 1 ng/ml. Increasing amounts (5 pg, 10 pg, 20 pg, 40 pg, and 100 pg) of the DHT standard were added to assay tubes and dried. Approximately 5,000 DPM of tritiated testosterone (100  $\mu$ l) was added to each tube, followed by an aliquot (100  $\mu$ l) of the antiserum

(diluted 1:160). The samples were then incubated for two hours at room temperature to allow the competitive binding reaction between radiolabeled testosterone and unlabeled testosterone to go to completion. Following incubation, the assay tubes were placed on ice, 500  $\mu$ l DCC was added to each sample, and the tubes were placed on ice for an additional twelve minutes (Wingfield 1996). The samples were centrifuged at 5,000g x 5 minutes at 4°C. An aliquot of the supernatant (400  $\mu$ l) was placed in a scintillation vial and scintillation cocktail was added. The amount of radioactivity present within each vial was measured using a Packard scintillation counter and the data were analyzed using the SECURIA program accompanying the counter. The computer generated results were plotted using SigmaPlot to construct a curve.

*Binding Specificity of the Antiserum.*---The testosterone antiserum was tested, as above, for its ability to bind to testosterone and DHT. Other steroids (e.g. 17 $\beta$ -estradiol, progesterone, pregnenolone, and 17 $\alpha$ -hydroxyprogesterone) were also tested for their interaction with the antibody by adding 1 ng and 10 ng of each steroid to separate assay tubes. After drying, 100  $\mu$ l of tritiated testosterone (approximately 5,000 DPM) and 100  $\mu$ l of antiserum (1:160) were added to each tube. The tubes were then processed as above.

*Analysis of Yolks To Quantify Testosterone Content.*---To determine the level of testosterone present in yolk samples, 1 ml of each yolk extract was dried and then washed with 75% methanol. Aliquots of the wash were added to eight assay tubes: 4 tubes received 50 $\mu$ l, and 4 tubes received 150  $\mu$ l. Non-radioactive testosterone (25 pg) was added to two of the tubes containing each volume of the methanolic wash. After drying, samples were analyzed by radioimmunoassay as above by adding 100  $\mu$ l of tritiated testosterone (~6,500 DPM) and 100  $\mu$ l of the antiserum

(1:160) to each tube. A standard curve was run with these samples. The amount of radiolabeled antibody-bound testosterone within each vial was quantified using a Packard scintillation counter.

## RESULTS AND DISCUSSION

During the development of this RIA method, several procedural adjustments and modifications were made. The ability to accurately perform a direct assay of yolk samples requires that the extracts be as free of lipid residue as possible. If there is a high concentration of lipids in the extract, the lipid material will form micelles or droplets, trapping the testosterone and interfering with antibody binding (Borst 1994). Producing clean yolk extracts proved especially difficult because lipids are abundant in avian yolk (O'Connor 1984) and may vary in concentration between individual eggs. Several extraction methods were attempted to address this problem.

*Extraction procedures.*---Initial studies compared the extraction procedure used by Schwabl (1993) with a modified procedure described by Borst and Tskimura (1994). Unlike Schwabl's biphasic method of extraction (using distilled water and a 50:50 mixture of hexane/diethyl ether) the modified method was triphasic (using equal volumes of 2% saline, acetonitrile, followed by a mixture of 50:50 diethyl ether/hexane). To perform the triphasic extraction, the yolk sample was thawed and homogenized in 1 ml of 2% saline. Tritiated testosterone (~100,000 DPM) was added to the homogenate for calculation of extraction and purification recoveries. Acetonitrile (1 ml) was added to the homogenate and the mixture was extracted twice with 3 ml of hexane/diethyl ether, 50:50 (vol/vol). The upper phase was decanted from the triphasic solution, combined, and dried using a Speed-vac. The dried extract was then redissolved in 1 ml of 90% ethanol and stored at -20°C.

Preliminary results of the two extraction methods showed that the modified triphasic extraction method recovered approximately 7% more of the tritiated material than the biphasic extraction method. However, during subsequent extractions using the modified triphasic method, it was found that the acetonitrile middle layer was gradually dissolved in the upper hexane/diethyl ether layer. The presence of acetonitrile in the yolk extract made it difficult to analyze the samples. In an attempt to rid the extracts of lipids and the contaminating acetonitrile, the resuspended dry yolk extract was washed with 2 ml of hexane, and then the hexane phase was backwashed with 90% ethanol. The ethanol fraction was dried using a Speed-vac.

*Reverse Phase Mini-Column Purification.*---Because large amounts of lipid residue remained in the extract after the triphasic extraction, the samples were further purified using a reverse phase C18 Prep-Sep mini-column. The dried yolk extract was redissolved in 55% methanol and the reverse phase mini-column was prewashed twice with 2 ml of 55% methanol. After each addition to the column, the column was centrifuged at 1,000g x 3 minutes. Each elution was collected and analyzed for radioactive testosterone. The percent of tritiated testosterone recovered from the column matrix after each wash is reported in Table 1. These results indicate that on average, 62% (n = 2) of the tritiated testosterone in the yolk extract sample was eluted with the first 75% methanol/water column wash. This low percent recovery of the radiolabeled material may reflect the trapping of the steroid on the column matrix.

*Normal Phase Mini-Column Purification.*---A second attempt was made to purify the yolk extract using a normal phase silica gel mini-column. A yolk sample was extracted using the triphasic procedure and the dried yolk extract was resuspended in 2.5 ml of methylene chloride. The silica column was prewashed two times with 2 ml of methylene chloride before applying the

**Table 1.** Percent of radiolabeled testosterone recovered from yolk samples extracted using reverse phase (C18) mini-columns.

Column Wash	Tube #	DPM	% Recovery
#45 Yolk in 55% MeOH/Water	1	239.5	0.23
55% MeOH/Water	2	4701.7	4.56
75% MeOH/Water	3	62456.7	60.5
75% MeOH/Water	4	8085.9	7.84
75% MeOH/Water	5	1084.3	1.05
#131 Yolk in 55% MeOH/Water	1	386	0.42
55% MeOH/Water	2	1129.4	1.24
75% MeOH/Water	3	57625.4	63.4
75% MeOH/Water	4	6243.2	6.87
75% MeOH/Water	5	447.2	0.49

yolk sample. The majority of the tritiated steroid eluted with the first 2 ml of methylene chloride/methanol (9:1). Again, the recovery of the radiolabeled steroid was low ( $63\% \pm 3.3$  (SE),  $n = 3$ ). Table 2 reports the percent of radiolabeled testosterone recovered after each column wash.

*High Performance Liquid Chromatography Analysis.*---High-performance liquid chromatography (HPLC) was used to further purify the elution fractions from the normal phase mini-columns. One ml fractions of the injected yolk sample were collected each minute. High pressure liquid chromatography allows separation of different compounds within the elution fractions because each compound has a different partition constant between the solvent and the column packing material (McMaster 1994). Therefore, the compounds within the sample come off (elute) from this column at different times.

A 40% methanol/water, 90% methanol/water solvent gradient was used on an Alltech reverse phase (C18) column. Solvent flow was 1ml/minute for a 16 minute cycle. An aliquot (200  $\mu$ l) of material eluted with 90% methylene chloride/methanol was dried and resuspended in 1 ml of 75% methanol/water. A fraction (200  $\mu$ l) of the supernatant was injected onto the HPLC column matrix. Tritiated testosterone and dihydrotestosterone standards were also separated on the column to determine their elution times. An aliquot (200  $\mu$ l) of each 1 ml fraction collected was placed in a scintillation vial with scintillation cocktail and the amount of radiolabeled testosterone quantified with a LSC. A graphical representation of the elution of radiolabeled hormone standards is shown in Figure 2. The standards produce a sharp peak. In contrast, when radiolabeled testosterone was mixed with yolk extract, it eluted at various times and was not found in a single fraction (Figure 3). Thus, this method was not successful in purifying the yolk extracts.

**Table 2.** Percent of radiolabeled testosterone recovered from yolk samples extracted using normal phase (Si) mini-columns.

<b>Column Wash</b>	<b>Yolk #</b>	<b>% Recovery</b>
90% Methylene Chloride/MeOH	3	69.7
90% Methylene Chloride/MeOH	107	61.1
90% Methylene Chloride/MeOH	132	59

**Figure 2.**

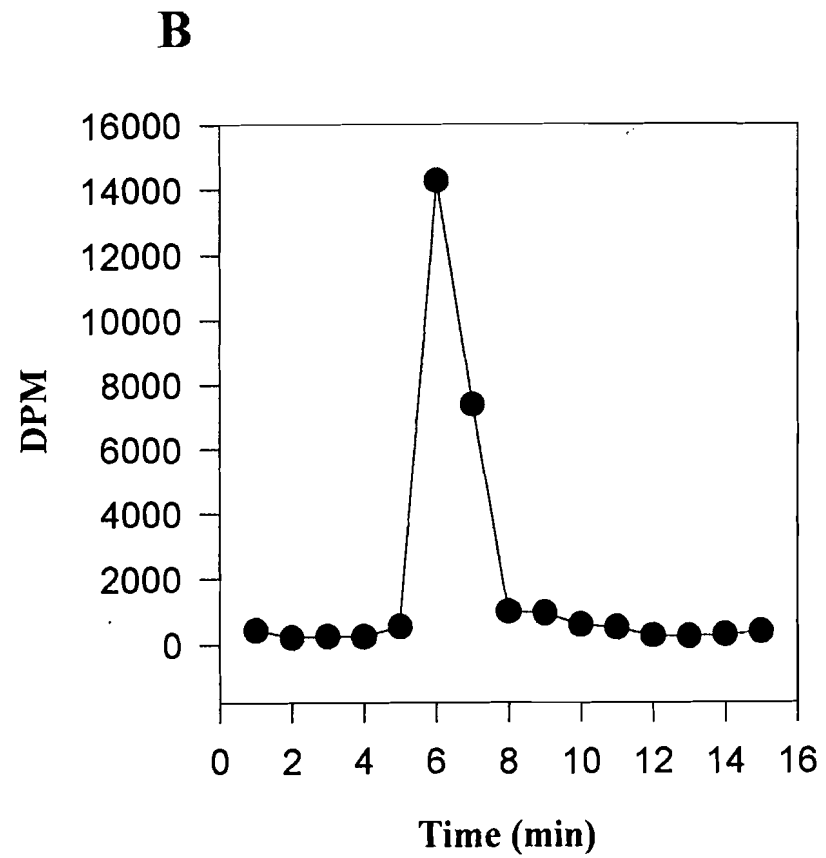
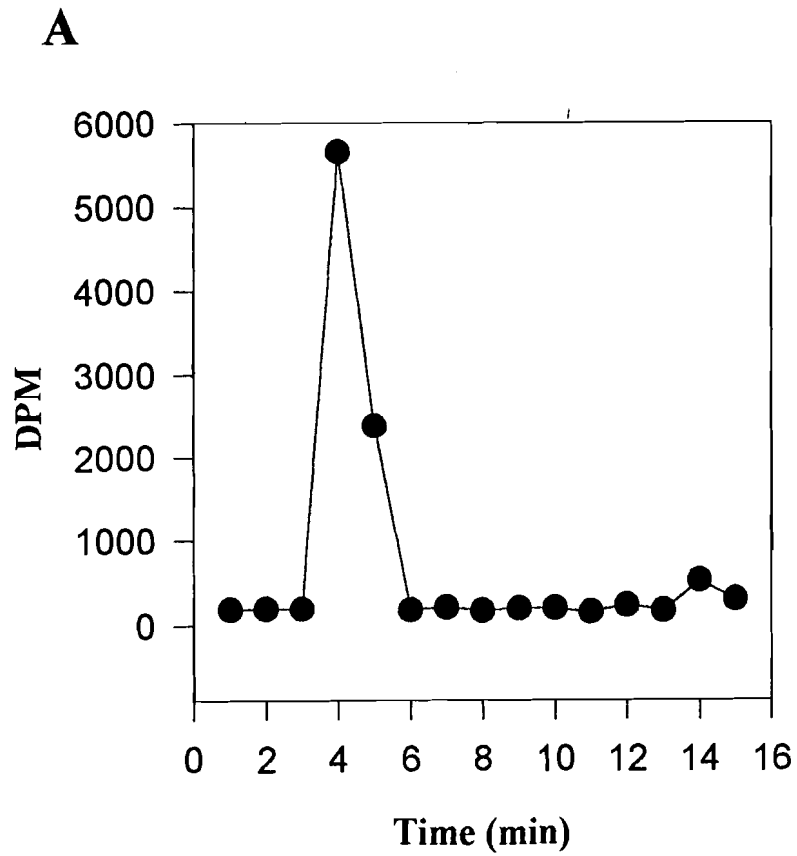
**A. HPLC Elution of Tritiated Testosterone**

The elution time of testosterone on a reverse phase HPLC column using a solvent gradient of 40%, 90% MeOH/water.

**B. HPLC Elution of Tritiated Dihydrotestosterone**

The elution time of dihydrotestosterone on a reverse phase HPLC column using a solvent gradient of 40%, 90% MeOH/water.

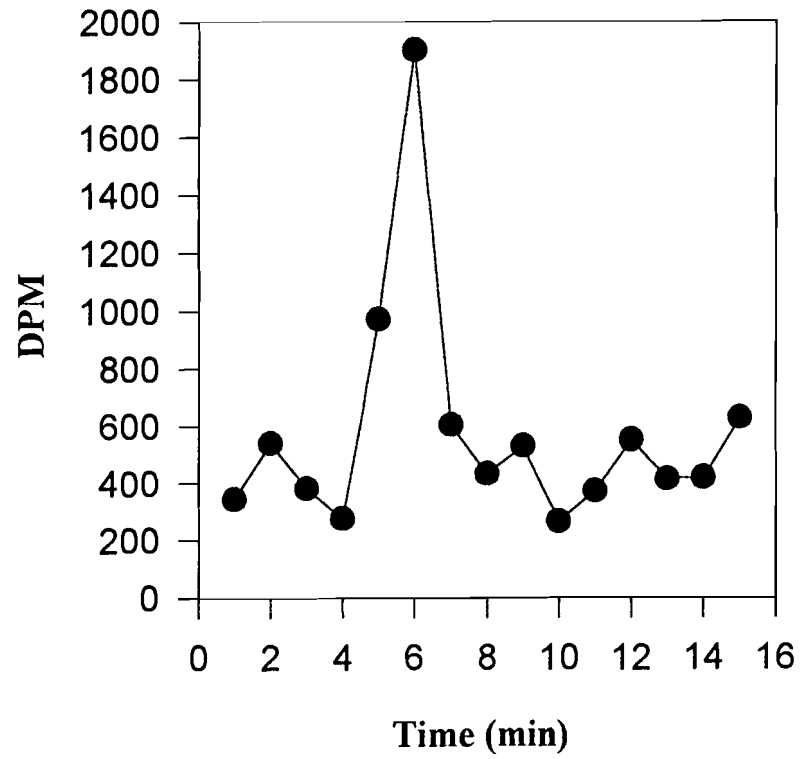
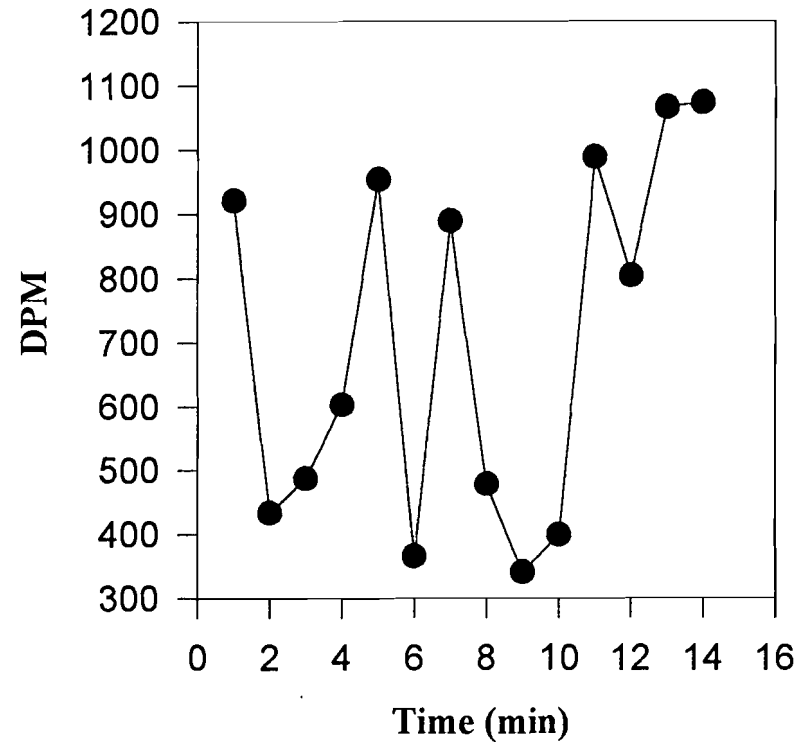




**Figure 3.**

**A. HPLC Elution of Tritiated Testosterone Mixed with Yolk Extract**

**B. HPLC Elution of Tritiated Dihydrotestosterone Mixed with Yolk Extract**

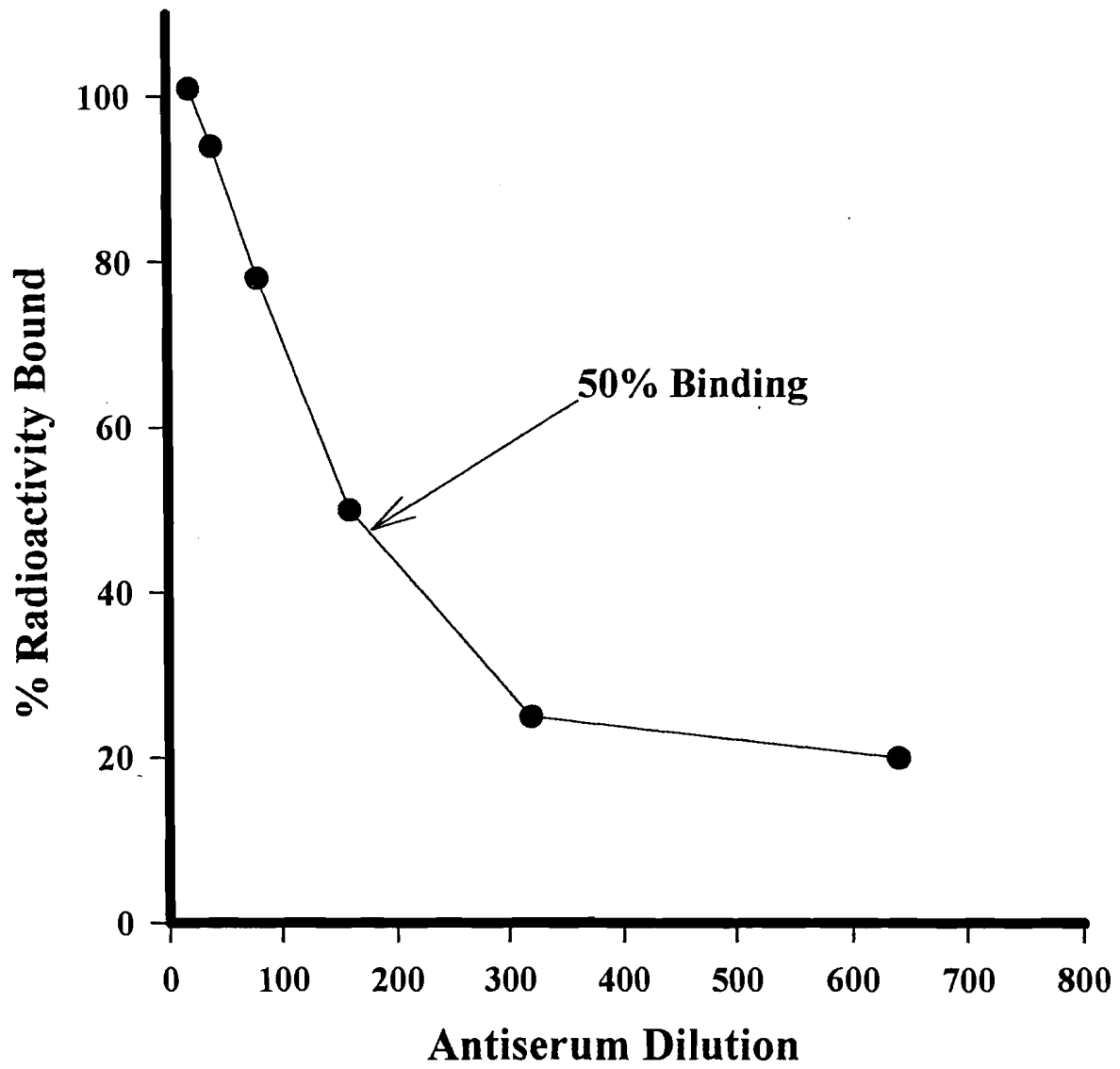
**A****B**

*Preparation of Samples for Radioimmunoassay Analysis.*---During sample preparation for HPLC analysis, it was found that the lipid of the extract was not dissolved in 75% methanol/water. Instead, the lipid material remained deposited at the bottom of the assay tube, despite vigorous mixing with a vortex machine. However, labeled steroids were quantitatively removed in the wash. When this washing procedure was combined with the biphasic extraction (3 x 2 ml) it was found to give nearly quantitative recoveries of labeled testosterone (107%, n = 2). Because this procedure yielded a solution essentially free of lipids, it was used for the RIA.

*Antiserum Characterization.*---Three tests were performed to characterize the testosterone antiserum for use in radioimmunoassay (see above). First, various dilutions of the antiserum were incubated with a known amount of radiolabeled testosterone to determine the percent of total binding activity at each dilution. A 1:160 dilution of the testosterone antiserum gave optimal binding (50%) of the radioligand (Figure 4). This dilution was used in subsequent radioimmunoassays. Results from the second test, which examined the effect of unlabeled steroid on the binding of tritiated steroid to the testosterone antiserum, were used to construct standard curves for testosterone and dihydrotestosterone (Figure 5). Third, the antiserum was tested for its ability to specifically bind to testosterone. In addition, other steroids (Table 3) were tested for their potential interaction with the antiserum. Little antiserum cross-reactivity was detected when  $17\beta$ -estradiol, progesterone, pregnenolone, or  $17\alpha$ -hydroxyprogesterone were incubated with testosterone. However, the presence of a small amount (6.6 pg) of the structurally similar compound, DHT, was found to inhibit testosterone antibody binding by 50%.

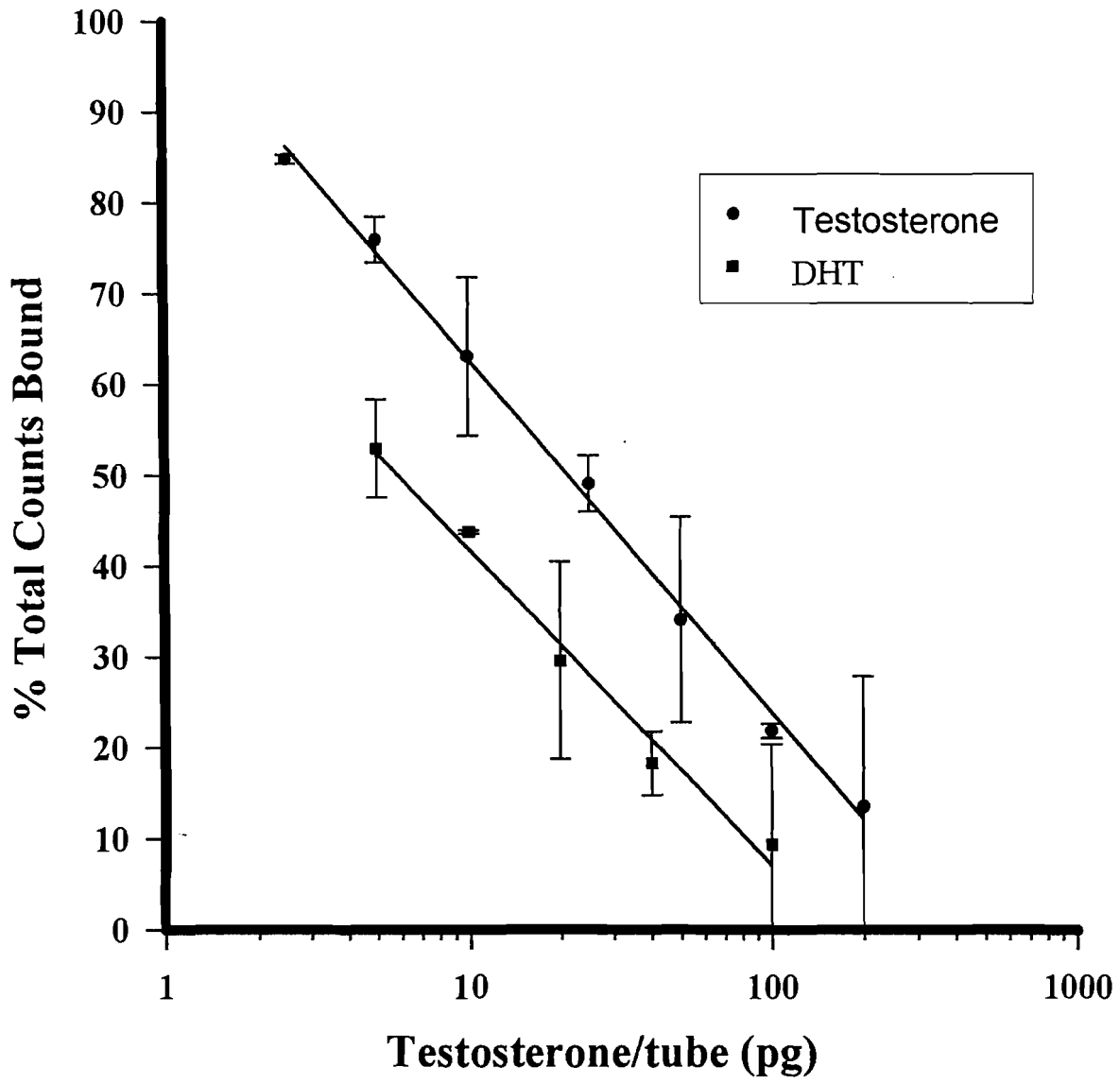
**Figure 4. Titer Curve for Testosterone Antiserum**

The binding activity of testosterone antiserum at various dilutions when incubated with a known amount of radiolabeled testosterone. A 1:160 dilution of the testosterone antiserum bound approximately 50% of the radioligand present.



**Figure 5. Standard Curves for Testosterone (T) and Dihydrotestosterone (DHT)**

The standard curves illustrates the effect of unlabeled testosterone and DHT on the binding of tritiated testosterone to the antiserum. A standard curve for DHT was constructed in addition to a standard curve for testosterone to illustrate the cross-reactivity of DHT with the testosterone antiserum.





**Table 3.** Steroid specificity of the testosterone antiserum. Comparison of the amount of antibody-bound radiolabeled material in tubes containing other steroid hormones to tubes containing unlabeled testosterone demonstrated that there is little cross-reactivity between the antiserum and other structurally similar steroid hormones.

<b>Steroid</b>	<b>ED50*</b>	<b>% Cross reaction</b>
Testosterone	22.9	100.0
Dihydrotestosterone	6.6	347.4
17 $\beta$ -Estradiol	1143.0	2.0
Progesterone	984.0	2.3
Pregnenolone	1497.8	1.5
$\alpha$ Progesterone	1371.7	1.7

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

*Validation of the Radioimmunoassay.*---Two tests were performed to validate this RIA. In the first, 25 pg of testosterone was added to duplicate sets of assay tubes containing aliquots of the prepared yolk extract. The concentration of testosterone detected in the tubes containing 25 pg of testosterone, therefore, should be 25 pg greater than those tubes containing only yolk extract. Results from this study revealed a mean difference between those samples containing added testosterone and those having no added testosterone of 25.0 pg ( $\pm 4.3$  (SE),  $n = 18$ ). A second test involved measuring testosterone levels in 50 and 150  $\mu$ l aliquots of the yolk extract. Those samples containing 150  $\mu$ l of yolk extract should contain three times the testosterone detected in samples containing 50  $\mu$ l of yolk extract. The mean ratio between those samples containing 150  $\mu$ l of yolk extract and those samples containing 50  $\mu$ l of yolk extract was close to this value (Ratio =  $2.42 \pm 0.3$  (SE),  $n = 9$ ).

*Testosterone Levels Within a Clutch.*---RIA analysis of yolk samples from one clutch revealed the presence of higher levels of testosterone in yolks of eggs laid later in the clutch sequence (Table 4). These preliminary data show a trend in yolk testosterone levels among eggs of a clutch. This trend is similar to the one reported in an earlier study in which higher levels of yolk testosterone were detected in canary (*Serinus canaria*) eggs laid later in the clutch.

*Ecological Implications.*---Variations in the concentration of testosterone between eggs of a clutch may affect the aggressiveness and competitive ability of the offspring. Schwabl (1993) found a positive correlation between the social rank of juvenile canaries and the level of yolk testosterone in the eggs from which each bird hatched. Schwabl (1993) suggests two mechanisms by which high levels of yolk testosterone result in a high ranking offspring. The first mechanism suggested is the ability of testosterone to enhance the growth of the neuromuscular

**Table 4.** Levels of testosterone (T) in a clutch of House Wren eggs.

<b>Clutch</b>	<b>Order</b>	<b>Egg #</b>	<b>T (ng/yolk)</b>
EA8-1	1	154	2.1
	2	61	1.5
	3	8	4.1
	4	86	1.0
	5	73	11.0
	6	71	26.6
	7	156	25.0

system. Secondly, Schwabl (1993) suggests that exposure to maternal testosterone during development may modify brain functioning responsible for such traits as aggression. If maternally deposited testosterone does indeed effect growth and brain functioning, then maternal manipulation of yolk testosterone levels may influence sibling competition. Because later laid eggs tend to hatch later (Winkler 1993), increasing testosterone concentrations deposited in eggs laid later in a clutch may reduce the competitive advantage held by older, larger siblings and ensure a greater chance of survival for younger, smaller siblings.

*Future Work.*---Future work will include the analysis of yolk testosterone levels in remaining clutches. Various yolk steroid hormone levels, including  $17\beta$ -estradiol, dihydrotestosterone, and progesterone, will be also be determined. Further studies will focus on the role that these maternal hormones play in the expression of certain behavioral, physiological, and morphological traits in the offspring.

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