

SEX DETERMINATION OF THE BALD EAGLE  
BY RADIOIMMUNOASSAY OF 17 $\beta$ -ESTRADIOL  
AND TESTOSTERONE

Submitted in Partial Fulfillment of the  
Requirements for Graduation with Honors  
to the Department of Biology at  
Carroll College, Helena, Montana

Anthony Lee Baumgartner  
John Patrick Lacey, Jr.  
March 22, 1983



CORETTE LIBRARY CARROLL COLLEGE  
3 5962 00083 074

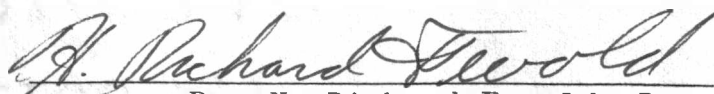
Th-

B327

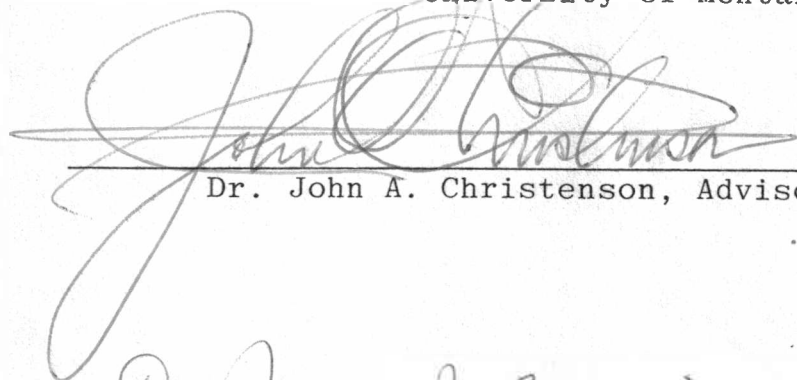
1983

THE BOSTON PUBLIC LIBRARY  
700 STATE STREET  
BOSTON, MASSACHUSETTS 02116

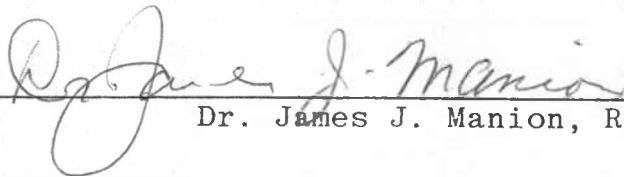
This thesis for honors recognition has been approved  
for the Department of Biology.



Dr. H. Richard Fevold, Director  
Chemistry Department  
University of Montana



Dr. John A. Christenson, Advisor



Dr. James J. Manion, Reader

March 22, 1983

TABLE OF CONTENTS

LIST OF TABLES.....iii  
LIST OF ILLUSTRATIONS..... iv  
ACKNOWLEDGEMENTS..... v  
ABSTRACT..... vi  
INTRODUCTION..... 1  
LITERATURE REVIEW..... 3  
MATERIALS AND METHODS..... 8  
    Estradiol radioimmunoassay..... 8  
    Testosterone radioimmunoassay..... 19  
RESULTS..... 26  
DISCUSSION..... 31  
    Estradiol radioimmunoassay..... 31  
    Testosterone radioimmunoassay..... 32  
LITERATURE CITED..... 35

LIST OF TABLES

<u>Table</u>		<u>Page</u>
1.	Volumes of components used in $17\beta$ -estradiol radioimmunoassay.....	17
2.	Duplicate culture tube contents used in the testosterone radioimmunoassay.....	24
3.	Summary of the bald eagle whole blood samples analyzed by radioimmunoassay of testosterone and $17\beta$ -estradiol.....	27
4.	The age and sex distribution of $B/B_0 \times 100$ for bald eagle samples assayed for testosterone content.....	29
5.	Extraction volumes and computer extrapolation of significant blood testosterone levels found by radioimmunoassay.....	29
6.	Validation of radioimmunoassay using turkey whole blood, and turkey whole blood plus 5.0 ng unlabeled testosterone.....	30
7.	Accuracy, precision, and reliability of $17\beta$ -estradiol radioimmunoassay.....	30

LIST OF ILLUSTRATIONS

<u>Figure</u>		<u>Page</u>
1.	Flow sheet for $17\beta$ -estradiol extraction and radioimmunoassay of bald eagle blood samples.....	18
2.	Flow sheet for the testosterone extraction and radioimmunoassay of bald eagle whole blood samples.....	25
3.	Standard curves for testosterone obtained during four bald eagle radioimmunoassays.....	28
4.	Standard curves for $17\beta$ -estradiol obtained during four bald eagle radioimmunoassays.....	28

## ACKNOWLEDGEMENTS

We owe many thanks to Dr. H. Richard Fevold of the University of Montana for his patience and guidance, as well as for the use of materials and equipment. We also thank Dr. John Christenson and Dr. James Manion for serving as readers.

Special thanks to our parents, who have provided the monetary and spiritual support we've needed during our years at Carroll.

Finally, we thank Cheryl McNurlin for completing the arduous task of typing this thesis.

## ABSTRACT

In order to develop a sex-determined difference in sex steroid levels in bald eagles (Haliaeetus leucocephalus), whole blood samples, obtained by McClelland and coworkers (1982) in Glacier National Park, Montana, were analyzed for  $17\beta$ -estradiol and testosterone levels. Samples from 14 bald eagles, 7 males and 7 females, were analyzed for  $17\beta$ -estradiol by radioimmunoassay (RIA). No significant levels of this steroid were found.

Fourteen bald eagle whole blood samples, taken from 7 males and 7 females, were analyzed for testosterone levels by RIA. Significant levels of this hormone were found in four samples, two taken from males. The testosterone levels found in the two male samples were markedly higher than the levels found in the female samples. Also, testosterone levels in male samples assayed seemed to increase with the age of the eagle. The data obtained from the eagle samples containing testosterone seems to indicate that sex determination is possible by RIA.

## INTRODUCTION

A substantial population of bald eagles (Haliaeetus leucocephalus) is found in Glacier National Park, Montana during the fall of the year. Wintering or migrating bald eagles are attracted to the area by the spawning of salmon in McDonald Creek, in the southwest corner of the Park (McClelland, et al., 1982). Past studies on sex determination of the bald eagle, a monomorphic species, have yielded little success. No reliable field technique exists for sex determination of the bald eagle, therefore only predictions of sex can be made based on beak size, wing span, and weight of the birds.

This study deals with sex determination of bald eagles by analyzing the amounts of the gonadal hormones (estradiol-17 $\beta$ , and testosterone) in the blood, by radioimmunoassay. If an individual eagle had more male hormones (testosterone), than female hormones (estradiol), it was taken to be a male, and vice versa. The sex determined by the radioimmunoassay is then compared to the probable sex of the birds determined in the field. If the two methods have a good percentage of consistency, then the field method will be considered a valid determination of sex.

The determination of sex in the bald eagle is of importance for the study of population dynamics. The synchrony of arrival or departure of the bald eagle in Glacier may be affected by a shift in the sex ratio (McClelland, et al., 1982). Also, since the bald eagle is a monogamous species, any fluctuation in the sex ratio could be detrimental to the population.

## LITERATURE REVIEW

Field sexing techniques of the bald eagle have been limited to external anatomical measurements. Weight and wingspan length are known to vary between sexes. The average weight of adult females is 5.84 kg, while males average 4.10 kg (Grzimek, 1972). The wingspan of female birds is also greater than that of males. Average female wingspan is 211 cm compared to 188-197 cm for the male.

Beak length is another factor considered when field sexing the bald eagle (McClelland, 1983). Since female eagles are usually larger than males, beak length tends to be longer in female birds.

Cloacal sexing techniques have been described for golden eagles (Aquila chrysaetos) and other raptors (Hamerstrom and Skinner, 1971). During the breeding season, which lasts from December to April in the bald eagle (Grzimek, 1972), some raptors can be stimulated to prolapse the vent, thus making accurate sexing possible. Hammerstrom and Skinner stress that the technique has worked only during the breeding season.

There is also a genetic basis for the sexing of birds. The genetic study of birds is complicated by

nuzerous chromosomes which are relatively small in size. Nevertheless, Au and coworkers discussed the sexing of bald eagles through the identification of the W chromosome and sex chromatin by differential staining (1974, 1975). The W chromosome and sex chromatin are present only in female birds. The presence of the W chromosome apparently causes an increased gonadal growth resulting in ovaries (Bloom, 1974). Its absence in male birds results in slower rates of gonadal growth, producing testis.

Arora and Dharamarajan (1969) reported successful genetic sex determination in Gallus domesticus, Columbia livia, and Passer domesticus. Through differential chromatin staining techniques, they found that the sex chromatin body was associated with the nuclei of female birds only. Conversely, the incidence of drumsticks and sessile bodies within the nucleus was predominant in male cells. Several different tissues were analyzed.

Steroid hormone components from the plasma of several avian species, including bald eagles, barn owls (Tyto alba), and black-crowned night herons (Nycticorax nycticorax), were separated by centrifugal chromatography on silica gel columns (Dieter, 1973). Sex determination was accomplished by staining of steroid hormone bands with iodine, charring with sulfuric acid, and observing fluorescence under ultraviolet light. A quantitative difference in testosterone and estradiol levels was

described between sexes. Thus, we attempted to analyze these steroid levels by radioimmunoassay (RIA).

The RIA of estradiol-17 $\beta$  has been fully developed. One of the earliest estrogen RIA procedures published was the solid phase method (Abraham, 1969). Several sensitive RIA's for free plasma estrogens have been described (Niswender and Midgley, 1970; Wu and Lundy, 1971; Nett, et al., 1973). These methods require the purification of the extracted steroid by column chromatography. However, the recent development of highly specific antisera has allowed the assay of estradiol without prior chromatography (England, 1974; Dorenman, 1974 - cited in Haning, et al., 1979).

The RIA of estradiol has been applied to many studies in birds. Levels of estrone and estradiol-17 $\beta$  in the peripheral plasma of laying hens has been reported by Peterson and Common (1972). These investigators studied the levels of estradiol-17 $\beta$  (17 $\beta$ -E<sub>2</sub>) in the blood before and after ovulation. At 26 to 22 hr before ovulation, the 17 $\beta$ -E<sub>2</sub> level was 144 pg/ml; the level then decreased to 66 pg/ml 10 to 6 hr before ovulation. 6 to 2 hr prior to ovulation, the level increases to 180 pg/ml.

Levels of estradiol in chickens at different stages of sexual maturity were also determined by RIA (Senior, 1974). These levels range from 94 pg/ml 7 weeks before the first egg, to 355 pg/ml 2 to 3 weeks before the first ovulation. After laying, 138 pg/ml was present

in the blood.

In ring doves, the levels of  $17\beta$ -E<sub>2</sub> (analyzed by RIA) were found to vary according to their sexual stage and behavior (Korenbrodt, et al., 1974). Levels were markedly different while the birds were mating (85 pg/ml), nesting (67 pg/ml), brooding (not detectable), or isolated (40 pg/ml or less).

Following the development of RIA techniques for estrogens, researchers soon began generating antibodies to testosterone, and several testosterone radioimmunoassay systems were described (Furuyama et al., 1970; Coyotupa et al., 1972; Ismail et al., 1972; Bartke et al., 1973 - cited by Auletta et al., 1979). The RIA technique that was followed for this bald eagle testosterone assay was outlined by Auletta and coworkers (1979).

Since the testosterone assay techniques described above are rapid, specific, and reliable, the RIA can be widely applied to the study of sex steroid levels and sex determination. Sangalang and coworkers (1978) applied the RIA of 11-ketotestosterone to successfully determine the sex of brook trout, rainbow trout, and cod.

Several investigators have applied the RIA technique to avian steroid research. Schrocksnadel and Bator (1971) reported plasma testosterone levels in cocks and hens. They found that male White Leghorn chickens possessed more than twice as much plasma testosterone

than females of the same strain. Also, the plasma testosterone levels in male chickens varied considerably with age. Two-year-old males were shown to possess up to twice as much plasma testosterone as 5-month-old males. This age-determined range was 118 ng/100 ml to 236 ng/100 ml. Furr and Thomas (1970) reported the plasma testosterone level for the male domestic fowl to vary from 84 to 783 ng/100 ml.

Lisano and Kennamer (1977) found that plasma testosterone levels in western wild turkeys (Meleagris gallopavo) increased from 32-63 ng/100 ml at 6 weeks of age, to 87-108 ng/100 ml at 34 weeks. It was also shown that the seasonal variation in plasma testosterone levels in adult turkeys may be marked, with the highest levels occurring in March, April, and May. A seasonal range of 500 ng/100 ml of plasma was described.

The plasma testosterone levels in mature pigeons were reported as 26-39 ng/100 ml of blood plasma in male birds by Jallageas and Attal (1968, cited in Sturkie, 1976). The mallard (Anas platyrhynchos) was shown to possess plasma testosterone levels of 65-270 ng/100 ml by the same researchers (1968, cited in Lisano and Kennamer, 1977).

In our study, blood samples had been frozen, cellular lysis prevented any cytological examination of the corpuscles. Therefore, in an effort to analyze the blood samples for sexual differences, we turned to the RIA of blood sex steroid levels.

## MATERIALS AND METHODS

### I. Estradiol radioimmunoassay

#### 1. Blood samples:

Samples of blood were obtained from 19 bald eagles in Glacier National Park, Montana during the fall of 1981. The birds were first captured, blood was taken from the wing vein, and they were released. The non-heparinized blood was stored in small jars, and frozen until the summer of 1982, when this study was done.

To prepare the blood for the extraction procedure, the following steps were utilized:

1. Let the blood thaw slowly, while on ice.
2. Transfer the volume in the jars to centrifuge tubes. The jars were rinsed with .09 saline (0.1 volume of blood) and the saline added to centrifuge tube.
3. Centrifuge at 2000 rpm for 20 min; remove supernatant and store in separate tubes. Break up residue and re-centrifuge.
4. Pool the supernatants and assay a given volume.

The volume of blood extracted varied from 2 to 4 ml, usually 2 ml.

#### 2. Buffer:

The buffer used in the estradiol RIA is phosphate

buffered saline (PBS). PBS contains 0.01M phosphate, 0.14M sodium chloride, 0.1% gelatin, and 0.1% sodium azide, in deionized water. The addition of the gelatin to the buffer increases the solubility of less polar steroids in the assay buffer and prevents interfering compounds from affecting the assay to a certain extent. The PBS with the gelatin added is termed PBS-ga.

PBS was also made without the gelatin, which is termed PBS-a. PBS-a was used to make the charcoal-dextran suspension. PBS-ga is used in all other assay procedures, other than preparation of the charcoal-dextran suspension.

PBS is prepared in the following way:

To a 1.0 l volumetric flask, add:

0.538 g of monobasic sodium phosphate.  
6.35 g of dibasic sodium phosphate.  
8.2 g of sodium chloride.  
1 g of sodium azide.

Next, add deionized water to a total volume of 1.0 l. The pH should be set at 7.0. These directions are for preparation of PBS-a. To prepare PBS-ga, the assay buffer, we simply add 1 g of gelatin to the buffer just described (PBS-a), and then recheck the pH to be 7.0. The assay buffer is stored at 4°C (as well as PBS-a). This buffer may be used as long as no evidence of mold or bacterial growth is present.

### 3. Solvents:

The solvents ether and ethanol, used in the estradi-

ol RIA were obtained from commercial sources. To obtain the purest form of the solvent, we used glass redistillation. Absolute redistilled anhydrous ether was used for the extraction of the eagle blood samples. Redistilled absolute ethanol was used for the preparation of the estradiol standards. Ethanol was also used for the storage of the final 1 ml volume from the extraction procedure.

#### 4. Counting fluid:

A counting fluid was prepared by mixing 50 mg PPO, 4 g POPOP, and 1 l toluene. Counting fluid is then stored at room temperature and is good for approximately 1 yr. Liquid scintillation counting was performed using a Beckman LS-7500 liquid scintillation counter, or a Nuclear-Chicago Unilux III.

#### 5. Tritiated-estradiol

The 2,4,6,7-<sup>3</sup>H 17 $\beta$ -estradiol (specific activity 85 Ci/mM) was obtained from New England Nuclear Company, and was first diluted to 3.27 uCi/ml with absolute redistilled ethanol. This solution was stored in the freezer at -20°C. Aliquots of the 3.27 uCi/ml solution were dried under nitrogen and redissolved in PBS-ga buffer such that two working solutions of tritiated-estradiol were available: one that had a concentration of 1000 cpm\*/100ul, and the other with a concentration of approxi-

\*cpm=counts per minute in liquid scintillation counter.

mately 10,000 - 15,000 cpm/100ul.

The 1000 cpm/100ul was used in the extraction of blood samples. 100 ul of the 1000 cpm/100ul was added to each blood sample prior to the extraction process. This was done to calculate the percentage recovery and the losses due to the procedure. A 65-75% recovery should be obtained.

The 2,4,6,7,<sup>3</sup>H 17 $\beta$ -estradiol solution of 10,000 - 15,000 cpm/100ul was used in the radioimmunoassay.

#### 6. Unlabeled estradiol standards:

Estradiol-17 $\beta$  was obtained from Sigma Chemical Corporation (St. Louis, Missouri), in the purest form available. A stock solution of estradiol 17- $\beta$  at a concentration of 1 ug/ml was prepared in a solution of absolute-redistilled ethanol. The standard solutions of estradiol used in the assay were obtained by the dilution of 1.0 ug/ml concentration solution. The concentration of the estradiol 17- $\beta$  standards used in this radioimmunoassay study ranged from 500 pg/ml to 12.5 pg/ml. A constant volume (0.5 ml) of each standard was delivered into each of the standard tubes. The standards were stored in suitable containers with lids, and also at 4° C, to cut down on evaporation. Six different standard estradiol solutions were used in each RIA to obtain a standard curve for that RIA. The preparation of standards is very important since any mistake will

influence the results.

7. Estradiol antiserum:

The supply of antibody used in the radioimmunoassay of estradiol 17- $\beta$  was first diluted to 1:25 in PBS-ga buffer. One-half ml aliquots of the 1:25 dilution were frozen in cryo tubes. Fifty ml of a 1:25,000 antibody dilution was prepared to be used in the RIA. This was then stored at 4° C. In some of the later assays of estradiol 17-B, a 1:50,000 dilution of the antibody gave good results. The antiserum dilution should bind 50-70% of the 10,000-15,000 cpm's/100ul  $^3\text{H-E}_2$ .

The antiserum used in this RIA has been tested for cross reactivity with other steroids (Abraham, 1969). This antiserum was found to be highly specific for estradiol 17-B, having minimal cross reaction with the other estrogens tested (estrone, estriol, and 2-hydro-yestriol), and having no detectable cross reaction with the other steroid hormones.

8. Dextran-coated charcoal:

A dextran-coated charcoal suspension was used to absorb unbound steroid and contained 0.25% charcoal plus 0.025% dextran T-70, in PBS-a. This solution was stored at 4° C with a stirring bar in the bottle, and was stirred continuously while being used. This charcoal solution (0.5 ml) was added to each assay tube after the incubation at 4° C.

## 9. Glassware:

The extraction tubes used were 40 - 50 ml citric acid tubes. These tubes, along with all other glassware and pipettes, were washed in Alconox and rinsed in tap water and distilled water.

Disposable 12 X 75 mm glass culture tubes were used for the assay tubes.

### A. Extraction

The following steps were followed for the extraction of the eagle blood samples:

1. I added 100 ul  $E_2$ -2,4,6,7- $^3H$  (approx. 1200 cpm's/100ul) to 40-50 ml centrifuge tubes, and to scintillation vials for reference standard; evaporate the alcohol solvent under nitrogen.
2. Next, I added the desired amount of eagle blood or plasma (usually 1-3 ml) and vortexed.
3. Four ml ether/1 ml of blood or plasma were added; vortex for 1 minute to mix, then centrifuge for 15 minutes at 2000 RPM. After centrifugation, aspirate off ether using plasma lifter into a second set of centrifuge tubes.
4. Repeat extraction with ether. (Step 3)
5. Once all the ether is collected in the second set of tubes, dry down the ether under nitrogen. When dry, add 1 ml of ether to wash the walls of the tubes and concentrate the hormone in the bottom of the tubes. Evaporate off this ether.
6. When all ether is evaporated, add exactly 1 ml of ethanol.
7. From final 1 ml volume take 100 ul to count for recovery purposes (More than 100 ul can be taken for recovery count, if desired).

8. Recovery aliquot is dried down: 10 ml counting medium is added and the sample counted.
9. Store the remaining 0.9 ml ethanol at 4 degrees, until used in RIA. This 0.9 ml is the sample to be used in the RIA.

Calculations for percentage recovery are as follows:

- a. counts/minutes = cpm
- b. cpm in sample - background cpm = net cpm
- c. net cpm in recovery sample/net cpm added for recovery  $\times 100 \times 10^a = \% \text{ recovery.}$

<sup>a</sup>From a total volume of 1 ml, 0.1 ml is removed and counted in order to determine recovery.

#### B. Radioimmunoassay

This estradiol-17 $\beta$  radioimmunoassay is based primarily on the methods of 2 different groups of investigators (Wu et al., 1971; and Nett et al., 1973). All the assay tubes are done in duplicate. The procedure for the assay was as follows:

1. Add 500 ul of the standard amounts of estradiol-17 $\beta$  (250 pg/0.5ml to 6.25 pg/0.5ml) to 12 x 75 mm assay tubes. Evaporate the solvent (ethanol) under nitrogen.
2. Add 0.2 ml of the extracted sample (from step A-9) to the unknown assay tubes. This volume may vary according to the assay. Dry off the solvent under nitrogen.
3. After the alcohol has evaporated off, add 100 ul antiserum (from step I-7) to all assay tubes. Incubate at room temperature for 30 minutes.
4. Place tubes on ice and add 100 ul estradiol-17 $\beta$  radioactive tracer (10,000 to 15,000 cpm/100ul). Mix well and incubate on ice at 4° C for 1 hour. Add the same amount to scintillation vials for counting standard (this is the total counts tube described below).

5. Add 500 ul of ice cold dextran-coated charcoal to each assay tube in an ice bath within 5 minutes and mix well; let stand for 15 minutes.\*
6. Centrifuge for 10 minutes at 2500 rpm.
7. Decant the supernatant from all tubes into scintillation vials and add 5 ml of counting fluid.
8. Shake scintillation vial 150 times, and count by liquid scintillation spectrometry for 10 minutes.

\*The total assay volume is 0.7 ml.

Along with the standard and unknown assay tubes, 4 other tubes are run in each assay. These 4 tubes are: the counting blank, total counts, the assay blank, and the zero standard. The preparation of these 4 tubes, along with an overall diagram for the assay procedure, are shown in Figure 1. A flow sheet for the entire extraction and radioimmunoassay of 17- $\beta$  estradiol is shown in Figure 2.

Calculations for % Bound, and  $B/B_0 \times 100$  values.

- a. Net cpm for Total Counts = Total Counts gross cpm - Counting Blank gross cpm.
- b. Net cpm for standard and unknown tubes =  
= gross cpm of standard or unknown tube - Assay Blank gross cpm.
- c.  $\% B = \frac{\text{standard and unknown net cpm}}{\text{total counts net cpm}} (x 100)$
- d.  $B/B_0 \times 100 = \frac{\text{standard or unknown net cpm}}{\text{zero standard net cpm}} (x 100)$

A standard curve is now plotted in a linear manner using logit transformation. On the graph  $B/B_0 \times 100$  values are plotted on the vertical and pg/ml of the

standards on the horizontal. Values for the pg/ml of the extracted sample are read from this graph.

Calculations for picograms estradiol/ml of blood.

a. Read picograms from the standard curve using  $B/B_0 \times 100$  values for each unknown.

b. pg. estradiol-17 $\beta$ /ml of blood =

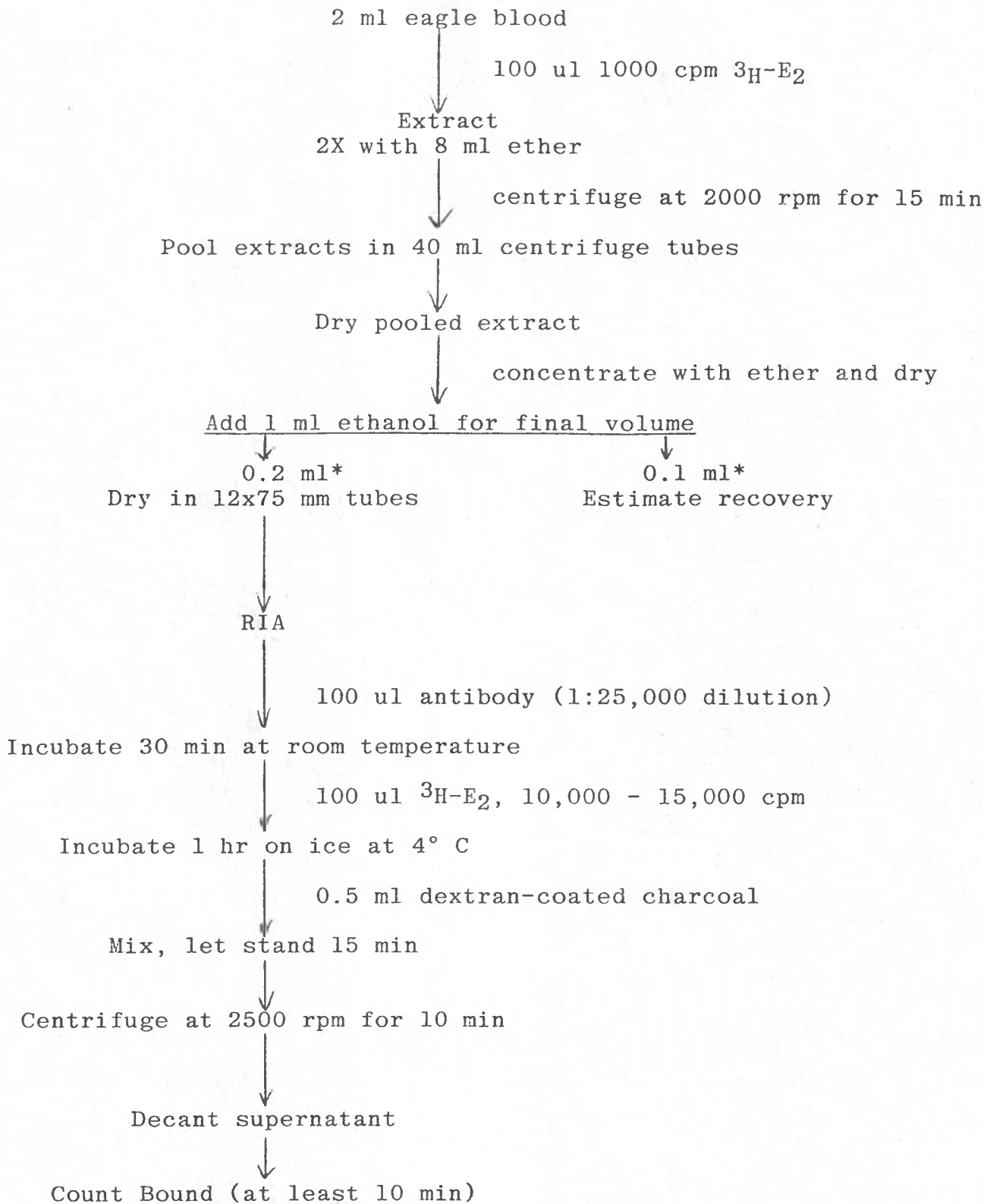
$$\frac{\text{pg from curve}}{=(\text{aliquot of sample used in assay})(\% \text{ recovery}) \text{ ml of blood}}$$

In order to validate the assay, blood and plasma samples from male and female turkeys were assayed. To these samples 1.25 ng of unlabeled 17- $\beta$  estradiol was added, and a percentage recovery was calculated.

SAMPLE	ASSAY BUFFER	STANDARD / UNKNOWN*	ANTIBODY	<sup>3</sup> H-E <sub>2</sub>	CHARCOAL
1. Counting Blank	0.7 ml	--	--	--	--
2. Total Counts	0.6 ml	--	--	--	--
3. Assay Blank	0.1 ml	--	--	0.1 ml	0.5 ml
4. Zero Standard	--	--	0.1 ml	0.1 ml	0.5 ml
5. Standard A 6.25 pg/ 0.5 ml	--	0.5 ml	0.1 ml	0.1 ml	0.5 ml
6. Standard B 12.5 pg/ 0.5 ml	--	0.5 ml	0.1 ml	0.1 ml	0.5 ml
7. Standard C 25 pg/ 0.5 ml	--	0.5 ml	0.1 ml	0.1 ml	0.5 ml
8. Standard D 62.5 pg/ 0.5 ml	--	0.5 ml	0.1 ml	0.1 ml	0.5 ml
9. Standard E 125 pg/ 0.5 ml	--	0.5 ml	0.1 ml	0.1 ml	0.5 ml
10. Standard F 250 pg/ 0.5 ml	--	0.5 ml	0.1 ml	0.1 ml	0.5 ml
-----	-----	-----	-----	-----	-----
UNKNOWNNS	--	0.2 ml	0.1 ml	0.1 ml	0.5 ml

\*Unknown volume may vary depending on assay.

Table I. Volume of components used in <sup>17</sup>β-Estradiol radioimmunoassay.



\*Volume may vary, depending on preference.

Figure 1. Flow sheet for 17- $\beta$  estradiol extraction and radioimmunoassay of bald eagle blood samples.

## II. Testosterone radioimmunoassay

### 1. Blood samples:

Each bald eagle sample had been frozen, and was thawed slowly, using an ice bath. The samples were transferred to centrifuge tubes. The jars containing the samples were rinsed with 0.9% saline solution (one-tenth volume of blood). This saline was added to the centrifuge tube.

The samples were centrifuged at 2000 rpm for 20 min. The supernatant was transferred into a capped storage tube. The residue was broken up with a spatula, and centrifuged again. The two supernatants were pooled, and given volume was assayed.

### 2. Stock buffer:

5.38 gm  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$   
16.35 gm  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$   
9.00 gm NaCl  
1.00 gm Sodium Azide  
1.00 l glass-redistilled (GRD)  $\text{H}_2\text{O}$   
pH adjusted to 7.4

### 3. Buffer "G":

Add 0.1%  $\gamma$ -globulin to stock buffer (100 mg/1.0l).  
Re-check pH - 7.4.  
Good for 2 weeks - store at 4° C.

### 4. Dextran-coated charcoal:

250 mg Charcoal Norit A with fines removed.  
25 mg Dextran T-70.  
200 ml Buffer "G" - stir overnight at 4° C.  
Good for 2 weeks - store at 4° C.

5. 1,2,6,7 <sup>3</sup>H-Testosterone:

The tritiated-testosterone (SA 94 Ci/mMol) was stored, in toluene/ethanol 9:1 v/v at -20° C, under nitrogen. A stock solution of 15.0 ml was kept, under nitrogen, in order to avoid frequent handling of the mother volume.

6. Testosterone antiserum:

The testosterone antiserum was obtained from Dr. Gordon D. Niswender of Colorado State University. The antiserum was diluted in Buffer "G" 1:3000, and stored at -20° C.

7. Redistilled ethyl ether:

Ether was shaken over FeSO<sub>4</sub>/GRD H<sub>2</sub>O (25.0 gm/500 ml) in a separatory funnel. After shaking, the funnel was stored under a hood overnight. The ether was redistilled at a potentiometer setting 36 over CaCl<sub>2</sub>.

8. Redistilled ethanol:

Ethanol was redistilled over CaO.

9. Scintillation cocktail:

4.0 gm PPO  
25.0 mg POPOP  
1.0 l toluene

10. Unlabeled testosterone standards:

10.0 ng/ml, 5.0 ng/ml, 2.0 ng/ml, 1.0 ng/ml, 0.5 ng/ml, 0.2 ng/ml, 0.1 ng/ml Buffer "G." 0.5 ml aliquots

were transferred to cryotubes and frozen.

#### 11. Glassware:

The radioimmunoassay was performed in 12 x 75 mm glass disposable culture tubes. The samples were counted in 10.0 ml plastic counting vials.

#### A. Ether extraction

1. Approximately 1000 cpm  $^3\text{H}$ -testosterone tracer in 0.1 ml buffer "G" was added to 40.0 ml citric acid extraction tubes.
2. Duplicate samples of bald eagle blood were added to the extraction tubes. Duplicate 2.0 ml GRD water blanks were also prepared. These tubes were then shaken gently to ensure solubility of the tracer. (The volume of bald eagle blood extracted varied among samples.)
3. All samples were kept in an ice bath during the extraction process.
4. Four ml redistilled ethyl ether were added for each 1.0 ml of sample. Samples were mixed on a Vortex Jr for 60 sec and centrifuged at 2500 rpm for 15 min at 4° C.
5. The organic phase was aspirated into another citric acid tube and stored on ice. Care was taken to rinse all of the steroid from the aspirator into the tube.
6. The extraction was repeated, as above, and the ether extracts were pooled.
7. Ether extracts were dried under  $\text{N}_2$ . After the ether had evaporated, the testosterone was concentrated by washing the sides of the tubes with 1.0 ml ether. This was also dried.
8. The extraction residue was redissolved in 1.0 ml redistilled ethanol, and 0.4 ml was counted for recovery. Again, these tubes were kept on ice.
9. Two-tenths ml of the final extract was assayed in duplicate.

## B. Testosterone radioimmunoassay

The RIA was performed in 12 x 75 mm glass disposable culture tubes. An ice assay tray was utilized in order to keep the culture tubes cool. All 0.1 ml fractions were pipetted with an SMI automatic pipette.

1. Duplicate aliquots of each standard testosterone solution, 0.01 to 1.0 ng in 0.1 ml buffer "G," were added to 14 culture tubes (standard curve).
2. Duplicate 0.2 ml aliquots of the eagle sample extracts were added to culture tubes. The alcohol was evaporated under N<sub>2</sub> and the residue was taken up in 0.1 ml buffer "G."
3. Duplicate 0.2 ml aliquots of the extract-water blank were also dried and taken up in 0.1 ml buffer "G."
4. Buffer "G" was added to the following additional tubes in duplicate: counting blanks - 1.3 ml; total counts - 1.2 ml; assay blanks - 0.2 ml; zero standard - 0.1 ml.
5. <sup>3</sup>H-testosterone of approximately 10,000 cpm in 0.1 ml buffer "G" was added to all culture tubes but the counting blanks. (The SA of the <sup>3</sup>H-testosterone should be such that the zero standard exhibits approximately 50% binding).
6. One-tenth ml testosterone antiserum, dilution 1:3000, was added to all tubes but the assay blanks, total counts, and counting blanks.
7. The tubes were then vortexed gently for 15 to 20 sec to ensure thorough mixing, and were incubated at 4° C for 2-3 hr.
8. After incubation, 1.0 ml. of dextran-coated charcoal was added to all culture tubes but the total counts and counting blanks. The charcoal addition was done quickly, and the samples were vortexed for 2-3 sec.
9. After 15 min of equilibrium, the tubes were centrifuged at 2500 rpm for 15 min.

10. The supernatant was decanted directly into plastic counting vials and 10.0 ml of counting fluid was added with an automatic dispenser. Each vial was hand-shaken vigorously and counted for 10 min in a Nuclear-Chicago Unilux III liquid scintillation counter.
11. Quantitations of samples are determined using a logit plot with linear regression analysis. The standard curve was obtained by plotting  $B/B_0 \times 100\%$  (ordinate) against the standards (abscissa).

Fourteen bald eagle whole blood samples were assayed. The volume of each sample that was extracted varied. Duplicate 2.0 ml fractions were extracted for most of the samples. However, 2.5 ml whole blood volumes were extracted for sample 629-11601 (band number); 4.0 ml volumes for sample 629-11625; and, 3.5 ml volumes for sample 629-11653.

In order to validate the assay, duplicate 4.0 ml volumes of female turkey blood, and duplicate 4.0 ml volumes of the same blood plus 5.0 ng of unlabeled testosterone were assayed.

Calculations for % bound and  $B/B_0 \times 100$  were made as follows:

- a. Net cpm for Total Counts = Total Counts  
gross cpm - Counting Blank gross cpm.
- b. Net cpm for standards and unknowns = gross cpm  
of tube - gross cpm Assay Blank.
- c. % Bound =  $\frac{\text{standard or unknown net cpm}}{\text{total counts net cpm}} (\times 100)$
- d.  $B/B_0 \times 100 = \frac{\text{standard or unknown net cpm}}{\text{zero standard net cpm}} (\times 100)$

SAMPLE	BUFFER	STD/UK	<sup>3</sup> H-TESTO	ANTISERUM	INCUB.	CHARCOAL
Counting Blank	1.3 ml	--	--	--	2-3 hr	--
Total Counts	1.2 ml	--	0.1 ml	--	2-3 hr	--
Assay Blank	0.2 ml	--	0.1 ml	--	2-3 hr	1.0 ml
Zero Std.	0.1 ml	--	0.1 ml	0.1 ml	2-3 hr	1.0 ml
1.0 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
0.5 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
0.2 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
0.1 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
0.05 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
0.02 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
0.01 ng Std.	--	0.1 ml	0.1 ml	0.1 ml	2-3 hr	1.0 ml
Eagle Samples	0.1 ml	0.2 ml*	0.1 ml	0.1 ml	2-3 hr	1.0 ml
H <sub>2</sub> O Blanks	0.1 ml	0.2 ml*	0.1 ml	0.1 ml	2-3 hr	1.0 ml

\*Alcohol was dried off under N<sub>2</sub>.

Table 2. Duplicate culture tube contents used in the testosterone radioimmunoassay.

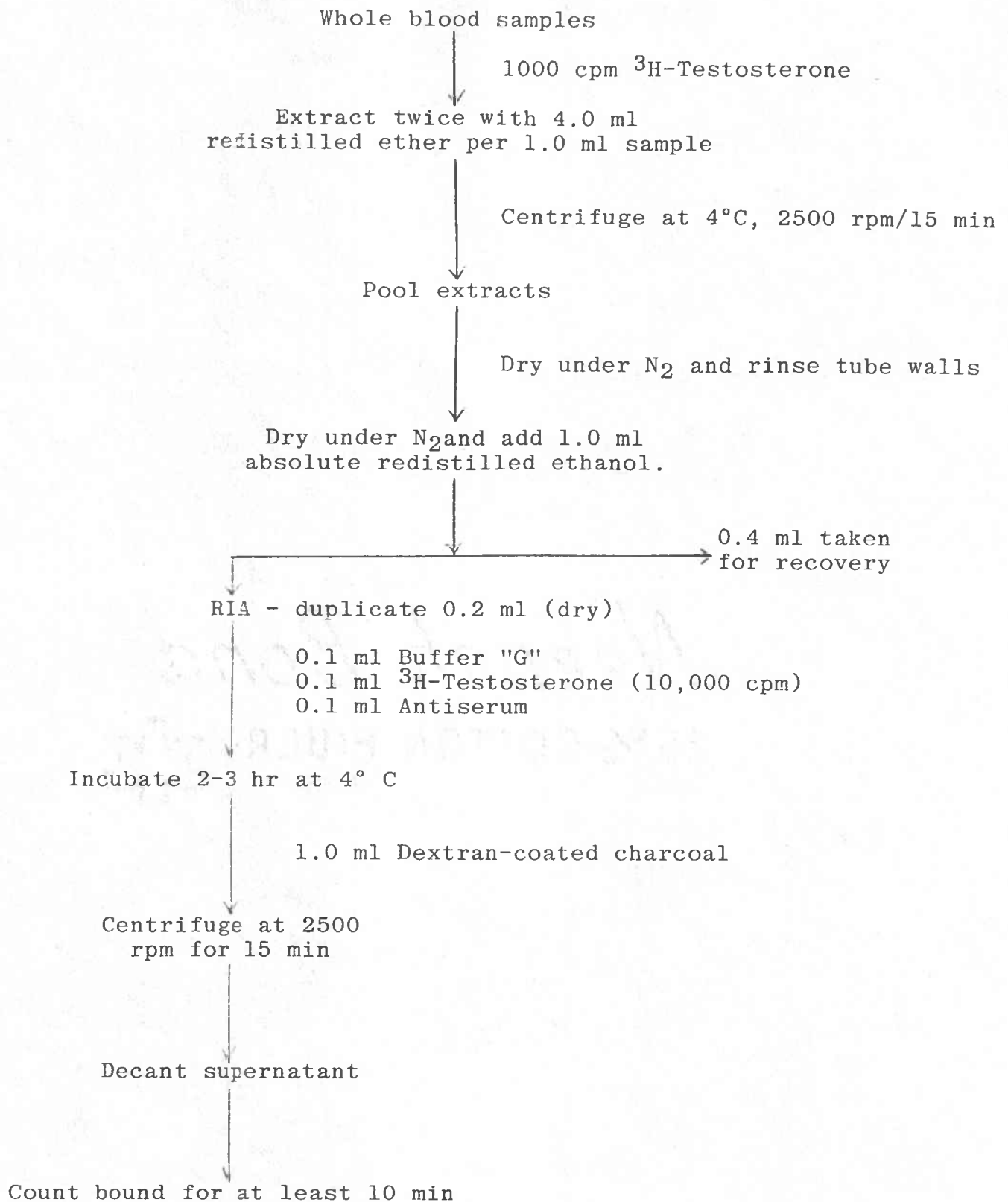


Fig. 2. Flow sheet for the testosterone extraction and radioimmunoassay of bald eagle whole blood samples.

## RESULTS

No detectable levels of 17 $\beta$ -estradiol were found in the 14 bald eagle samples analyzed by RIA. Consistently high B/B<sub>0</sub> x 100 values inhibited accurate extrapolation from the estradiol standard curve. The values obtained for B/B<sub>0</sub> x 100 for the 17 $\beta$ -estradiol RIA are given in Table 3, which also contains a summary of the estradiol RIA.

Significant levels of testosterone were detected in four of the bald eagle samples assayed. Blood testosterone levels in 2 presumptive male samples, taken from a 5-year-old and 2-year-old, contained 0.37 $\pm$ .06 ng/ml and 0.25 $\pm$ .02 ng/ml, respectively.

Blood testosterone levels detected in two presumptive female samples measured .088 $\pm$ .05 ng/ml and .065 $\pm$ .02 ng/ml. Both female samples were taken from HY (hatch year) birds.

No detectable blood testosterone levels were found in the remaining 10 bald eagle samples assayed. A summary of the results of the testosterone RIA is given in Table 3.

Standard curves obtained during the testosterone and estradiol radioimmunoassays are shown in Fig. 3 and 4, respectively.

Band No.	Wt. Kg.	Culmen, mm	Age	Sex*	Testosterone B/B <sub>0</sub> ·100 ng**		Estradiol-17B B/B <sub>0</sub> ·100 pg	
629-08038	4.50	57.2	HY***	F	99.8	ND****	-	-
629-08041	4.00	50.5	HY	M	99.0	ND	-	-
629-08045	4.90	51.7	HY	M	104.5	ND	95.2	ND
629-11601	4.15	49.3	5	M	71.8	0.93	-	-
629-11622	4.70	51.3	HY	M	99.5	ND	-	-
629-11623	3.90	48.1	HY	M	-	-	108.3	ND
629-11624	5.80	53.7	HY	F	99.4	ND	107.5	ND
629-11625	5.85	53.1	HY	F	91.0	0.35	110.8	ND
629-11633	4.55	49.9	2	M	110.8	ND	95.4	ND
629-11635	4.30	50.4	HY	M	-	-	106.9	ND
629-11636	5.40	56.6	HY	F	93.7	0.13	97.5	ND
629-11639	5.40	56.2	HY	F	108.7	ND	97.0	ND
629-11644	4.25	53.4	HY	F	98.7	ND	95.9	ND
629-11645	4.65	49.5	HY	M	104.5	ND	94.1	ND
629-11647	4.10	52.6	2	M	-	-	100.6	ND
629-11648	4.40	55.4	HY	F	-	-	107.2	ND
629-11649	6.15	54.9	2	F	-	-	103.1	ND
629-11653	4.75	50.5	2	M	73.2	0.88	102.6	ND
629-11657	4.05	52.0	2	F	107.3	ND	-	-

- \* Probable sex based on weight and culmen length  
\*\* ng/sample, volume of blood extracted varied  
\*\*\* Hatch year, samples were taken during the fall  
\*\*\*\* Not detectable

Table 3: Summary of the bald eagle whole blood samples analyzed by radioimmunoassay of testosterone and estradiol-17B.

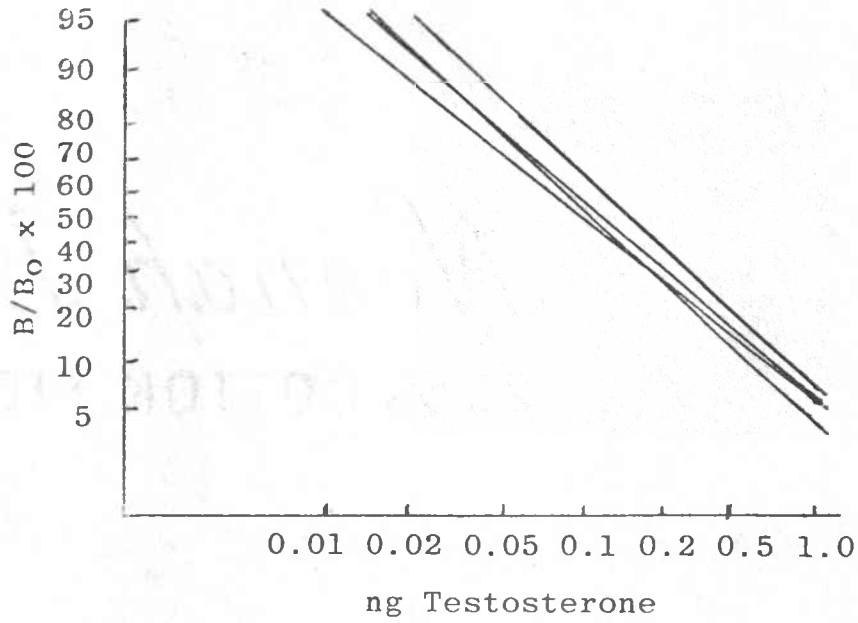


Fig. 3. Standard curves for testosterone obtained during four bald eagle radioimmunoassays.

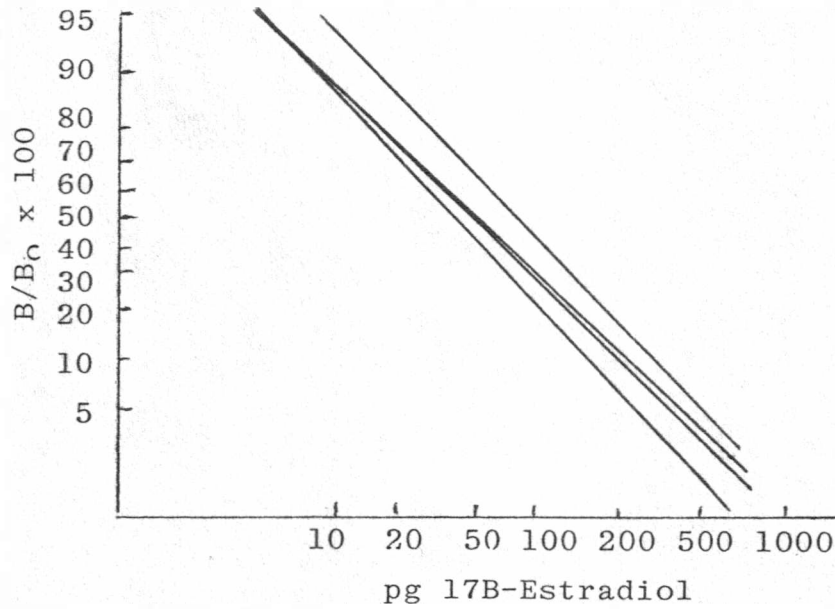


Fig. 4. Standard curves for 17-β estradiol obtained during four bald eagle radioimmunoassays.

B/B<sub>0</sub> x 100

HY		2-year-old		5-year-old
male*	female	male	female	male
99.0	99.8	110.8	107.3	71.8
104.5	99.4	73.2		
99.5	91.0			
104.5	93.7			
	108.7			
	98.7			
Ave. 101.9	98.6	92.0	107.3	71.8

\*Age and sex estimated from morphological measurements.

Table 4. The age and sex distribution of B/B<sub>0</sub> x 100 for bald eagle samples assayed for testosterone content.

Sample	Extract Volume (ml)	Testosterone	
		ng/sample	ng/ml
5-year-old male	2.5	0.93	0.37
2-year-old male	3.5	0.88	0.25
HY female	4.0	0.35	0.09
HY female	2.0	0.13	0.07

Table 5. Extraction volumes and computer extrapolation of significant blood testosterone levels found by radioimmunoassay.

Sample	n*	Ave. B/B·100%	Ave. ng/sample	Net ng	% recovery
female turkey	6	45.0	3.95		
female turkey + 5.0 ng	6	33.1	7.60	3.65	72.3
male turkey	2	98.1	-		
male turkey + 5.0 ng	2	46.0	3.11	3.11	62.1

\*number of samples assayed

Table 6. Validation of radioimmunoassay using turkey whole blood, and turkey whole blood plus 5.0 ng unlabeled testosterone.

Sample	n	ng E <sub>2</sub> * added	ng E <sub>2</sub> recovered	% recovery
H <sub>2</sub> O blank	11	0.00	0.06±.03**	
male turkey plasma	6	1.25	1.23±.25	98±20
female turkey plasma	2	1.25	0.68±.19	54±15
female turkey whole blood	2	1.25	1.08±.07	86±7

\*estradiol-17B

\*\*standard deviation values

Table 7. Accuracy, precision, and reliability of estradiol-17B radioimmunoassay.

## DISCUSSION

### Estradiol-17 $\beta$ RIA

The estradiol-17 $\beta$  RIA was sensitive in the range of 25 pg/ml to 250 pg/ml. When 1.25 ng/ml of standard estradiol-17 $\beta$  was added to plasma or blood, 54 to 98% of it was recovered (see Table 1). Below 25 pg/ml, the RIA did not give consistent results.

The amount of estradiol-17 $\beta$  in the blood may have been less than the minimum detectable level of 25 pg/ml. During the fall, the bald eagle is sexually inactive, and the secretion of estradiol from the ovary is markedly less than that secreted during the breeding season (December to April). During the breeding season ovarian follicles grow, under the influence of FSH and LH, which stimulate the thecal and interstitial cells to secrete estrogen (Murton and Westwood, 1977).

Previous radioimmunoassays, reporting estradiol-17 $\beta$  levels in birds, have usually studied the birds during their sexual cycle when secretion of the hormone is at a maximum (Peterson and Common, 1972; Senior, 1974). However, one RIA of ring doves reported no detectable levels of estradiol in females incubating eggs, while during breeding and nesting there was a marked increase

of estradiol in the blood (Korenbrot et al., 1974).

Although no significant levels of estradiol-17 $\beta$  were detected in any samples of eagle blood, we are confident that further research in this area may produce better results. It is possible that the use of plasma, rather than whole blood, will give better results. Also, no purification of the hormone by column chromatography was used. This step could prove to be beneficial in future assays of bald eagle blood or plasma.

#### Testosterone RIA

The results obtained from the samples containing measurable amounts of testosterone seem to indicate that there is a difference in blood testosterone level between male and female bald eagles. Testosterone levels detected in male birds exceeded levels detected in female birds markedly (see Table 4). Although the number of samples is small, this study seems to indicate that there might be a basis for sexing bald eagles by blood testosterone analysis.

In addition to the higher levels of testosterone detected in the two male birds, blood testosterone levels in the male eagles seem to vary with age. No detectable testosterone levels were found in HY (hatch year) males. These birds were sexually immature, and were expected to possess lower blood testosterone levels than older birds.

Since the levels detected in the older male birds

increased with age (0.25 ng/ml to 0.37 ng/ml from 2 to 5 years, respectively) it appears that the age of the eagle may be a factor affecting testosterone levels. Bald eagles do not develop white heads until about 5 years of age (McClelland, 1983). However, the development of a white head does not appear to be related to the ability of the male to produce testosterone.

Similar age-determined variation was reported in White Leghorn chickens (Schrocksnadel and Bator, 1971), and in eastern wild turkeys (Lisano and Kennamer, 1977). Seasonal androgen levels were shown to vary in the same birds. In seasonal birds, the testis undergo cyclic changes that are much more obvious than in domestic species, and the size of the testis may increase 300 to 500 times (Lake, 1981).

Finally, the results obtained do not consistently validate the field sexing techniques employed (see Table 2). Future eagle plasma testosterone RIA's of samples obtained in various seasons is needed to validate the results reported in this paper.

Taken together, both the estradiol and testosterone RIA's should benefit future research on sex determination of the bald eagle, by observing our results and errors. More blood has been taken from bald eagles in Glacier during the fall of 1982, and plasma was obtained by centrifugation of the blood sample. These samples will be analyzed in the summer of 1983 using the same proce-

dures this study used. It seems evident that the quality and sensitivity of these two RIA techniques will increase in the analysis of more eagle samples.

#### LITERATURE CITED

- Abraham, G.E. 1969. Solid-phase radioimmunoassay of estradiol-17 $\beta$ . *Journal of Clinical Endocrinological Metabolism* 29:866-870.
- Arora, C.L. and Dharamarajan, M. 1969. Sex chromatin and nuclear sexing in birds. *Cytologia* 35:601-608.
- Au, W., and Soukup, S.W. 1974. Identification of the W chromosome in the Bald Eagle. *Mammalian Chromosomes Newsletter* 15:4-5.
- Au, W., Fechheimer, N.S., and Soukup, S. 1975. Identification of the sex chromosome in the Bald Eagle. *Canadian Journal of Genetic Cytology* 17(2):187-191.
- Auletta, F.J., Caldwell, B.V., and Hamilton, G.L. 1979. Androgens: testosterone and dihydrotestosterone. In *Methods of Hormone Radioimmunoassay*. Eds. B.M. Jaffe and H.R. Behrman. Chapter 36, pp. 715-726. Academic Press, New York.
- Bloom, S.E. 1974. Current knowledge about the avian W chromosome. *Bioscience* 24(6):340-343.
- Dieter, Michael P. 1973. Sex determination of eagles, owls, and herons by analyzing the plasma steroid hormones. United States Department of the Interior. Special Scientific Report--Wildlife No. 167. Washington, D.C.
- England, B.G., Niswender, G.D., and Midgley, A.R., Jr. 1974. Radioimmunoassay of estradiol-17 $\beta$  without chromatography. *Journal of Clinical Endocrinological Metabolism* 38:718-720.
- Furr, B.J.A., and Thomas, B.S. 1970. Estimation of testosterone in plasma of domestic fowl. *Journal of Endocrinology* 48:42.
- Grzimek, B. 1972. Grzimek's Animal Life Encyclopedia. Vol. 7, Birds I. Van Nostrand Reinhold, New York.

- Hammerstrom, P., and Skinner, J.L. 1971. Cloacal sexing of raptors. *The Auk* 88:173-174.
- Haning, R., Orczyk, G.P., Caldwell, B.V., and Behrman, H.R. 1979. Plasma estradiol, estrone, estriol, and urinary estriol glucuronide. In *Methods of Hormone Radioimmunoassay*. Eds. B.M. Jaffe and H.R. Behrman. Chapter 34, pp. 675-700. Academic Press, New York.
- Korenbrot, C.C., Schomberg, D.W., and Erickson, J. 1974. Radioimmunoassay of plasma estradiol during the breeding cycle of ring doves. *Endocrinology* 94:1126-1134.
- Lake, P.E. 1981. Male genital organs. In *Form and Function in Birds*. Eds. A.S. King and J. McLelland. Vol. 2, Chapter 1. Academic Press, London.
- Lisano, M.E., and Kennamer, J.E. 1977. Seasonal variations in plasma testosterone level in male eastern wild turkeys. *Journal of Wildlife Management* 41(2):184-188.
- McClelland, B.R., Young, L.S., Shea, D.S., McClelland, P.T., Allen, H.L., and Spettigue, E.D. 1982. The Bald Eagle concentration in Glacier National Park, Montana: origin, growth, and variation in numbers. *The Living Bird, Nineteenth Annual, 1980-1981*. Cornell Laboratory of Ornithology, 133-155.
- McClelland, B.R. 1983. Personal communication.
- Murton, R.K., and Westwood, N.T. 1977. *Avian Breeding Cycles*. Chapter 4, p. 77. University Press, Oxford.
- Nett, T.M., Holtan, D.W., and Estergreen, V.L. 1973. Plasma estrogens in pregnant and postpartum mares. *Journal of Animal Science* 37:962-970.
- Niswender, G.D., and Midgley, A.R., Jr. 1970. Hapten-radioimmunoassay for steroid hormones. In *Immunologic Methods in Steroid Determination*. Eds. F.G. Peron and B.V. Caldwell. Chapter 8, pp. 149-173. Appleton, New York.
- Peterson, A.J. and Common, R.H. 1972. Estrone and estradiol concentrations in peripheral plasma of laying hens as determined by radioimmunoassay. *Canadian Journal of Zoology* 50:395-401.
- Sangalang, G.B., Freeman, H.C. and Flemming, R.B. 1978. A simple technique for determining the sex of fish using 11-ketotestosterone antiserum. *General and Comparative Endocrinology*. 36:187-193.

Schrocksnaefel, H., and Bator, A. 1971. Plasma testosterone levels in cocks and hens. Steroids 18:359-365.

Senior, B.E., and Cunningham, F.J. 1974. Estradiol and LH during the ovulatory cycle of the hen. Journal of Endocrinology 60:206-210.

Sturkie, P.D. 1976. Avian Physiology. Third Edition. Springer-Verlag, New York.

Wu, C.H., and Lundy, L.E. 1971. Radioimmunoassay of plasma estrogens. Steroids 18:91-111.