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<u>Hormone Analysis of Japanese Quail</u>
<u>(*Coturnix japonica*)</u>

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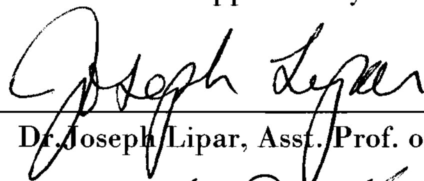
Presented to the faculty of Lycoming College in fulfillment of the requirements for Departmental Honors in Biology

April 2005

Approved by:



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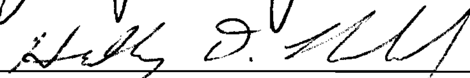

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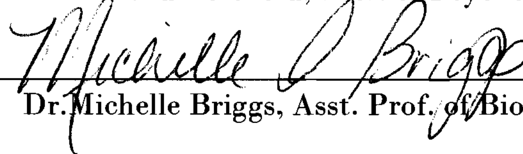

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Abstract

The number of studies applying endocrine techniques to the investigation of developmental and behavioral adaptive strategies of vertebrate species is increasing rapidly. Recent investigations have found that hormones of maternal origin, such as testosterone, are present in the yolks of avian species. It is probable that yolk steroids play a role in avian development and are not simply by-products of the physiological processes of the female (Lipar *et al*., 1999). This honors project consists of two parts that utilize principles and lab techniques of endocrinology to investigate testosterone levels in yolk tissue. Both dealt with the development of quail eggs. I will introduce each part of the project separately for clarification.

The first project involved studying variation in testosterone concentration in yolk layers. The yolk is the source of nourishment for the developing avian embryo. It is thought that testosterone concentration in the yolk might differ among layers so that the bird can be exposed to a specific amount of testosterone at a specific time of development. If this is true, the time of development and the concentration of testosterone can be correlated to determine the developmental function of testosterone at that time. Samples from yolk layers were taken using Japanese Quail (*Coturnix japonica*) eggs.

The second experiment included examining variation in testosterone levels among eggs in clutches of synchronously-hatching birds. Synchronously-hatching birds initiate incubation of all their eggs at the same time; all young are consequently hatched at about the same time. This is in contrast to asynchronously-hatching birds, which initiate incubation of their clutch before all eggs have been laid. This causes some eggs to hatch

before others. It is known that in asynchronously-hatching species, the female bird deposits increasing amounts of testosterone to her young in sequential order. If it is found that synchronously-hatching birds do not increase the amounts of testosterone in subsequently laid eggs, one can postulate that the elevated testosterone levels found in the last eggs of species with hatching asynchrony is an adaptation to enhance the survival of the last and smallest member of the clutch. However, if testosterone levels also increase in synchronous species, then more research into this area will be required to understand the evolution of this trait. Samples of homogenized yolks were taken from the synchronously hatching Japanese quail (*Coturnix japonica*).

Unfortunately, before any of the experiments using the samples could proceed initial testing of the extraction and analysis methods had to be done. The testing proved that a better extraction process was necessary to remove yolk materials other than the hormones. Further work will have to be done to perfect the extraction method. Once this is complete, the samples obtained for the two projects could be analyzed using an enzyme immunoassay (EIA).

Experiment 1- Analysis of Integrity of Yolk Layers During Development

Background

In the eggs of birds, yolk is a source of food for the developing embryo. The yolk also serves as a source of maternal testosterone (Schwabl, 1993). Transfer of other maternal hormones to the embryo, such as corticosterone, 17ß-estradiol, and 5α-dihydrotestosterone, occurs via the yolk as well (Lipar *et al.*, 1999a). Yolk is deposited in concentric spheres during egg formation. The yolk sac is connected to the developing

embryo through blood vessels that transport yolk components into the developing body for use as food. The mechanism by which this occurs may suggest that ingestion of the yolk starts at the outer layers and then proceeds inward (Romanoff, 1960; Lipar *et al.*, 1999b).

Hormones, such as testosterone, may enter the yolk sphere in two different ways. The steroids may be deposited directly by the female during oogenesis. It is highly likely that most testosterone present in the yolk at the beginning of development comes from this maternal source. Later in development, small amounts of steroids from the developing embryo, beginning at the time of gonad production, may enter the yolk through diffusion (Hackl *et al.*, 2003).

As researchers studied hormone concentrations in the yolk layers of freshly-laid eggs in birds and reptiles, they have found something interesting. The concentration of progesterone is significantly higher in the exterior layers of yolk, while the concentrations of 17β-estradiol and testosterone are significantly higher in the intermediate and interior layers. This variation has implications for the timing of embryonic exposure to steroid hormones. These findings are consistent in the painted turtle (*Chrysemis picta,* Bowden *et al.*, 2001), the red-eared slider (*Trachemys sarpta,* Bowden *et al.*, 2001), the red-winged blackbird (*Agelaius phoeniceus,* Lipar *et al.*, 1999b), the dark-eyed junco (*Junco hyemalis,* Lipar *et al.*, 2001) and, most importantly for this study, the Japanese quail (*Coturnix japonica,* Hackl *et al.*, 2003). The steroid concentrations in the yolk parallel steroid concentrations in the ovary of the laying female during egg production.

The development of the avian embryo has been well studied and documented. Steroid hormones in avian yolks have been shown to have developmental effects on embryos (Schwabl, 1996). The varied hormone concentrations in the yolk layers suggest that developing embryos are exposed to different steroid levels as development proceeds (Lipar *et al.*, 1999b). It is believed that the concentration of testosterone in the yolk differs among layers so that the bird can attain a specific amount of testosterone at a specific time of development. If this is true, the time of development and the concentration of testosterone can be correlated to determine the developmental function of testosterone at that time.

The goal of the first experiment was to analyze changes in the testosterone concentration in yolk layers during the initial developmental stages of Japanese quail eggs to establish that yolk layers are or are not maintained during development. It is my belief that mixing may occur during incubation, leading to a consistent concentration of testosterone throughout the egg. It is important to note that the yolk samples were collected from eggs that had only been incubated for 1-4 days to ensure that any hormones in the yolk were not produced by the developing embryo. This should be the case because gonad development in the embryo is not initiated until the fifth day of development (Romanoff, 1960). The separated layers of yolk were analyzed for testosterone concentration using an ELISA method.

Methods

Treatment of Eggs

Dr. Mary Anne Ottinger, from the University of Maryland Department of Animal and Avian Sciences, supplied our lab with over 400 Japanese quail eggs. These eggs

were used for my project as well as for those of two other students working with Dr. Lipar.

The first step of Experiment 1 was to incubate the eggs. The eggs were incubated at 37.5°C. Forty eggs were incubated on Day 0 and an additional 10 eggs were placed directly in a freezer without first being put in an incubator. On Day 1, another 10 eggs were removed from the incubator and placed in the freezer. This continued until Day 4, at which point a total of 50 eggs were placed in the freezer. The eggs were frozen to maintain the yolk layering at that time of development. The eggs were only incubated for a few days to ensure that the testosterone in the yolk had been provided by the mother and not by the embryo's gonads.

Yolk Preparation

In the Fall Semester of 2004, I prepared the yolks for hormone analysis using a procedure outlined by Lipar *et al.* (1999b). I separated the yolk from the albumin, taking into consideration that the albumin thaws quicker than the yolk. After the albumin thawed, it was completely removed from the yolk. The physical appearances of the yolks were analyzed and differences between the stages of development were noted. The frozen yolks were separated into samples of similar mass using a razor blade. The samples were taken from the interior, middle and exterior portions of the yolk. Individual sample masses were weighed and homogenized in 1.5-ml Eppendorf microcentrifuge tubes with 500μl of water using a Vortex mixer. Homogenization was aided by the addition of glass beads to the tubes. These samples were frozen in preparation for hormone analysis using the ELISA system.

Extraction

Before the samples could be put into the EIA kit, the testosterone had to be extracted from the lipids and protein of the yolk. This procedure had to be done for all of the samples used for experiment 1 and 2 as well as for the tests that had to be done to validate the assay kits (see next section). After the frozen samples thawed, they were vortexed thoroughly to ensure all solid material was broken up. Glass beads were added to assist with the vortexing process. The samples were transferred from 1.5ml Eppendorf microcentrifuge tubes into 12 ml conical glass tubes using an Eppendorf P1000 Pipetman. Each sample was rinsed with 500 μl distilled water and the rinse was added to the respective conical tube. Three ml of extraction solvent, which consisted of petroleum ether:diethyl ether (30:70), was added to each sample using an Eppendorf repeat pipette. Immediately following the addition of the extraction solvent, each sample was vortexed for approximately 10 seconds to mix the solutions. The samples sat for 20 minutes to allow phase separation to occur. During this time a snap freeze bath was prepared. The snap freeze bath consisted of dry ice in methanol. Each sample underwent a snap freeze by placing the conical tube in the bath for a few seconds until the bottom layer was solidified. The resulting supernatant was poured off into a 13x100 glass test tube. The entire extraction was performed a second time to increase the efficiency of the extraction. The combined extracts from both the first and second extractions were evaporated under nitrogen gas. After the evaporation, 1ml of 90% ethanol was added to each tube. The tubes were vortexed and the samples were transferred to 1.5ml Eppendorf tubes. The samples were placed overnight at -20°C to allow the precipitation of unwanted lipids and proteins. The samples were then centrifuged at 2000rpm for 5 minutes. This allowed the

formation of a pellet of unwanted material (such as lipids and proteins). The supernatant was transferred to a 13x100 test tube and dried under nitrogen gas once again. The samples were resuspended in 50µl of PBSG, which is a steroid assay buffer. The ingredients for PBSG can be found in Appendix 1.

Validation of Assay Kits and Extraction

To make sure the assay procedure and extraction were valid for this experiment, a set of 5 tests were done using the ACTIVE Testosterone Enzyme Immunoassay (EIA) Kit. For the first test, 50µl of a 10ng/ml solution of testosterone was placed into each of eight wells on a 96-well microplate. A 10ng/ml solution was used because that concentration falls in the middle of the standard curve supplied in the kits and should therefore be easily detected. This test was performed to measure the accuracy and consistency of the values determined by the kit.

The second test used another eight wells of the microplate. The extracts of 50mg yolk samples were placed in each of the wells. These samples came from one homogenized yolk. Although the concentration of testosterone is unknown, the amount in each sample should be the same. This test was performed to measure the consistency of the assay readings

For the third test, eight wells were filled with the extracts of 50mg yolk samples from a homogenized yolk plus an additional 50µl of pure testosterone (10ng/ml). The total values measured by the assay kit should be equal to the sum of the concentrations from tests 1 and 2. If this does not occur, then the yolk may be interfering with the ability of the antibodies to recognize the testosterone, yielding values that are too low.

In the fourth test, 50μl of pure testosterone was added to 50mg yolk samples of homogenized egg yolks prior to the extractions. The entire sample was placed into the wells. This test should indicate the efficiency of the extraction process. It also indicated if a significant amount of testosterone is lost during the extraction process.

For the last test, different amounts of yolk were used. The amounts ranged from 10 to 80 mg of yolk. While these should all have different absorbencies after the EIA test, the amount of testosterone per milligram of yolk should not change with variations in sample mass. If it does, then this might indicate that excess yolk lipids and proteins may be interfering with the EIA process. It will allow me to determine the proper amount of yolk to use in each sample.

Analysis of Testosterone Levels in Yolk Samples

The ACTIVE Testosterone Enzyme Immunoassay (EIA) Kit provides materials for the quantitative measurement of testosterone. The kit follows the basic principle of enzyme immunoassay, where there is a competition between an unlabeled antigen and an enzyme-labeled antigen bound to the antibody binding sites. The amount of enzyme-labeled antigen bound to the antibody is inversely proportional to the concentration of the unlabeled analyte present. The absorbance measured by the plate reader is inversely proportional to the amount of testosterone present in the sample. The concentration of the unknowns can be calculated using a standard curve of absorbance versus testosterone concentration.

The EIA kit was used to analyze the samples for both experiments and all validation tests. The complete procedure can be found in Appendix 2. An automatic

microplate washer was used to wash and aspirate the plates. This increases the kit's precision and accuracy.

Experiment 2- Analysis of Variation in Yolk Testosterone Concentrations Within Clutches of a Synchronously-Hatching Species

Background

A clutch is considered to exhibit hatching asynchrony when its eggs hatch over a span greater than 24 hours. Hatching asynchrony is common in songbirds and produces broods where nestlings vary in age, size, and development. Late hatching members of a clutch are at a competitive disadvantage and often die (Ellis *et al.*, 2001). Hatching asynchrony often occurs in avian species because the mother initiates incubation before the last egg of the clutch has been laid. Since parents begin to feed their young as soon as they hatch, earlier hatched young start to grow before their younger siblings have even been hatched. This often results in size differences among individuals in a clutch depending on hatching order (Lipar *et al.*, 1999b).

When divisible resources are provided by the mother, siblings must compete for food. Access to food brought by parents is largely dependent on the size-related abilities of the young. Variation in offspring size may lead to brood reduction in times of short food supply. Therefore the youngest siblings in a clutch are often at a significant disadvantage (Royle *et al.*, 2001).

There are many hypotheses that have tried to explain the reasons for the existence of asynchronous hatching. Although there has been much interest in asynchronous hatching, the study of this subject is still in its infancy. Many of these hypotheses are based on the idea that the survival of the earlier offspring is promoted. The hypotheses concerning hatching asynchrony fall into four broad categories. Hatching asynchrony

might: (i) arise because of selection on the timing of events during the nesting period; (ii) facilitate an adaptive reduction in brood size; (iii) increase the energetic efficiency of raising the brood, or (iv) result from environmental or phylogenetic constraints (Magrath, 1990).

The late-hatching nestlings in asynchronously-hatching clutches must overcome the age- and size- related disadvantages that are presented to them. Hormonal favoritism may be a common mechanism for avian mothers to influence sibling competitiveness. One way for them to do this is to add additional maternal androgens, such as testosterone, to their eggs (Ellis *et al.*, 2001). Therefore offspring competitive ability may be both directly (hormones) and indirectly (incubation onset) influenced by the mother (Royle *et al.*, 2001).

Mothers often compensate for the competitive advantage given to the earlier born birds by depositing increasing amounts of testosterone in sequential order of the clutch. According to Royle *et al.* (2001), maternally derived traits, such as increased testosterone deposited in egg yolks, may have profound effects on offspring fitness. Offspring with elevated levels of testosterone may have increased aggression and growth, better enabling them to compete with their older and larger siblings. In conjunction with the onset of incubation, high testosterone levels may provide an adaptive mechanism for parental favoritism in response to environmental variability (Royle *et al.*, 2001).

Several experiments have studied the variation of testosterone concentration in clutch order. In 1993, Schwabl discovered that yolk testosterone levels in canary eggs increased with order of laying in a clutch. The social rank of sibling canaries was correlated with the amount of testosterone they were exposed to within the egg. Schwabl

(1993) noted that testosterone enhances overall growth of the neuromuscular system. He hypothesized that the higher testosterone level in the later hatching eggs compensates for the disadvantage of having to compete with older siblings (Schwabl *et al.*, 1993). The effects of incubation onset and testosterone work in opposition. Therefore canary mothers can favor either early-egg (by early hatching) or late-egg (by testosterone in tandem with synchronous hatching) offspring (Schwabl *et al.*, 1996). Brain functions that allow an individual to achieve a higher rank, such as aggression, may be modified in a specific manner by exposure to maternal testosterone in the egg (Schwabl, 1993).

This discovery paved the way for more studies that examined testosterone levels within other asynchronously hatching species. It has been found in red-winged blackbirds, which hatch asynchronously, that the testosterone concentration increases continuously throughout the clutch. Therefore the last egg contains the highest concentration of testosterone (Lipar *et al.*, 1999a). Eising *et al.* (2001) found that black-headed gulls (*Larus ridibundus*) also increased testosterone levels with laying order. Their study also found that increased testosterone causes eggs to hatch sooner, thereby reducing hatching asynchrony. Last-hatched members of the clutch may be at least partly compensated for their handicap by more rapid embryonic development and enhanced post-hatching growth via higher levels of maternal androgens in their eggs (Eising *et al.*, 2001).

It has been shown that not all species exhibit increased testosterone levels in the last egg of the clutch. Siblicidal birds often lay three-egg clutches. The last of the three is usually the smallest and is killed and eaten by the first two siblings. Schwabl *et al.* (1997) conducted a study of cattle egrets (*Bubulcus ibis*), which are siblicidal. They

found that the first two eggs contain much greater amounts of androgens than the last egg. This favors the growth and survival of senior siblings. These results suggest that hormone deposition is a mechanism used by mothers for parental favoritism. If this species differs from other asynchronously-hatching species in testosterone concentration within a clutch, it is a possibility that synchronously-hatching birds also have their own mechanism for testosterone deposition that is different from that of asynchronously-hatching birds.

The goal of the second experiment was to investigate differences in hormone deposition between asynchronously and synchronously hatching species. Asynchronously-hatching birds increase the testosterone concentration as laying order increases. If synchronous birds do not increase their concentration of testosterone, then we might conclude that the presence of such a trait in a synchronously hatching species is directly related to the presence of hatching asynchrony. It is my belief that the synchronous clutches will have equal amounts of testosterone in all the eggs of a clutch. This provides an evolutionary approach to understand the purposes behind an increased concentration of testosterone in the last member of an asynchronous clutch. The last member needs the most testosterone in order to increase its chance for survival. The project entails analyzing testosterone concentrations from all members of the clutches of the Japanese quail, a synchronously-hatching species, to compare to the known pattern found in asynchronous species. This is a novel area of research and is important because it will address a new question in the study of yolk hormones.

Methods

Treatment of Eggs

Dr. Mary Anne Ottinger, from the University of Maryland Department of Animal and Avian Sciences, supplied the lab with eggs once again in March of 2005. These eggs were freshly-laid, unincubated quail eggs. The eggs were marked by order and clutch number. I used five eggs each from four clutches, for a total of 20 eggs.

Yolk Preparation

After removing the albumin, the yolk was mixed and samples were taken from the homogenized yolk. Samples were collected in preweighed Eppendorf tubes and 500µl of deionized water was added to each tube. Samples were placed in the freezer until the extraction process took place.

Extraction & Analysis of Testosterone Levels in Yolk Samples

Using the prepared yolk samples, I extracted and analyzed the samples following the procedures used during the first experiment (see previous experiment).

Results for Validation Testing

Physical Observations for Experiment 1

As I performed the yolk sampling, I noticed that the yolk began to change shape during the first four days of incubation. As the days progressed, the yolk became more pancake shaped than spherical. The albumin decreased in size as well. I could see layers in freshly-laid eggs, but these disappeared after the second day of incubation. However, there was a very light yellow color to the innermost core in all of the eggs.

Validation Tests #1 - #5

All absorbance values were compared to the standard curve found in Figure 1 in order to calculate the correct concentration of testosterone in the samples. This standard curve was created from standards supplied with the EIA kit. In the first validation test (10ng/ml of testosterone), the eight samples' absorbance values yielded concentrations ranging from 19.89 to 22.94 ng/ml. The next test samples (50mg of yolk) yielded concentrations from 48.06 to 58.50 ng/ml. However, two of the samples were 72.43 and 24.03 ng/ml, which were unexpectedly out of range. The third test (10ng/ml testosterone added after extraction) had six concentrations ranging from 33.79 to 55.97 ng/ml. The fourth validation test (10ng/ml of testosterone added prior to extraction) yielded seven concentrations ranging from 42.67 to 48.77 ng/ml as well as 59.65 ng/ml and 112.11ng/ml. The fifth validation test (varying masses of yolk) yielded varying concentrations of testosterone in the six samples of yolk. Table 1 shows the concentrations of testosterone for their respective samples. These results are also shown in a graphical representation in Figure 2.

Figure 1 This is the standard curve that was used for comparison of the absorbencies and the concentration of testosterone.

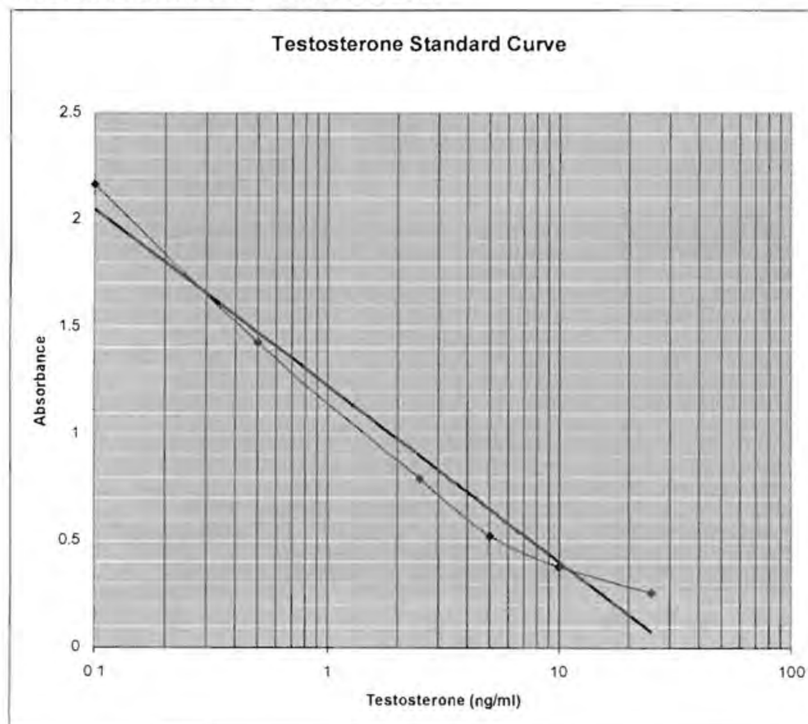
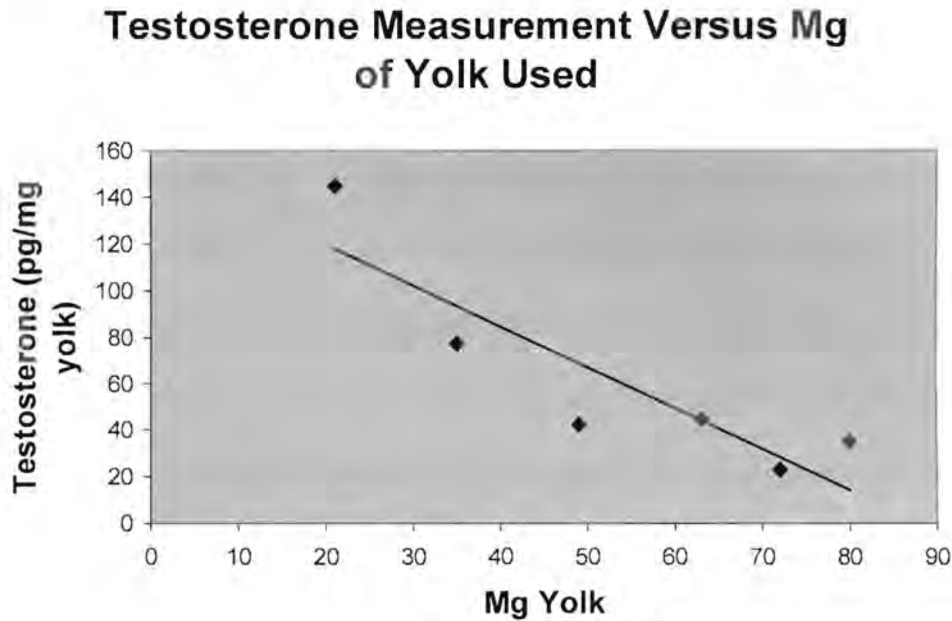


Table 1. Validation Test Results for Test 5. This table represents the mg of yolk used for each sample and the corresponding concentrations.

Mg of yolk	ng/ml
21	144.86
35	77.30
49	42.23
80	34.92
63	44.65
72	22.99

Figure 2. This figure represents validation test 5 results.



Discussion

Since the instruments and extraction process were new and because the EIA kits had not been validated for use with yolk tissue, the validation tests were of the utmost importance to this experiment. None of the samples could be analyzed until it was determined that the extraction process was satisfactory. If the extraction process proved to be less than satisfactory, a new process would have to be developed.

Our first validation study gave us absorbance values that were about twice as much as the 10ng/ml testosterone solution that was used. It was expected that the values would be around 10ng/ml. This could have been the result of miscalculations in the making of the solution. This is the most logical explanation. However, this result did show that the readings are consistent. All of the concentrations determined from absorbance levels were relatively close to one another.

The second validation study once again indicated the consistency of the kit, although the 72.43 and 24.03 ng/ml samples were aberrant. This could be due to some type of error in adding solutions, etc. Taking into account the other samples, which ranged from 48.06 to 58.50ng/ml, it is apparent that the consistency of the readings is acceptable. It was expected that the samples would be relatively close in concentration.

The third validation study is quite important because it showed that the extraction process was unsuccessful at removing enough lipids and proteins. As stated previously, the third test should have yielded concentrations equal to the sum of the first two tests' concentrations. However, this did not occur. The range of the concentrations was 33.79 to 55.97ng/ml. These values are much lower than what I expected. This indicates a problem with the extraction phase of the experiment. Lipids and proteins in the yolk are interfering with the binding of the testosterone to the antibody. To fix this problem, a new extraction technique must be developed. If the experimental samples were run using the current technique, the results would be inaccurate and, in essence, useless.

The fourth validation test once again proved the extraction process was ineffective. The range of concentrations was 42.67 to 112.11 ng/ml with most between 42.67 and 48.77 ng/ml. This indicates that not enough of the lipids and proteins are being removed by the extraction process. In essence, something is hindering the binding of the testosterone to the antibodies; this is most likely the lipids and proteins in the yolk.

The problems with the antibody interference were not surprising, because the EIA kits are designed for use with human blood and not with yolk. These kits could work with the correct extraction process. Once the correct process is found, the samples can be accurately measured.

The fifth validation test yielded varying concentrations of testosterone in the samples. This should not have occurred. As evident by the graph, it appears that the more yolk placed in the kit, the less testosterone concentration is being read by the machine. We expected to see a line with a slope of zero due to the fact that no matter how much yolk was used, the same concentration should be read. Again, this points to a problem with antibody interference in the kits.

From the physical manifestation of the yolk spheres in Experiment 1, the hypothesis of non-existing yolk layers seems probable. No layering appeared in the actual yolks during the later stages of development (that I looked at). Although this is not a scientifically accurate method, it is the best result from the study at this point.

This project is close to completion, contingent on a) a new extraction process that efficiently removes unwanted lipids and proteins and b) the determination of the correct amount of yolk to use per sample. The validation process was essential and proved that using the samples would be a waste of resources if the process didn't work efficiently.

Although the project could not be completed at this time, this project typifies the time and effort that goes into the scientific method. It is clear that troubleshooting and preparation take more time than the actual experiment. . Endocrine research using avian samples is a novel area of research. The techniques and methods used in this experiment are relatively new. Because of that fact, techniques are used on a trial and error basis at this point in time.

Works Cited

Bowden, R.M., Ewert, M.A., Lipar, J.L., Nelson, C.E. 2001. Concentrations of steroid hormones in layers and biopsies of chelonian egg yolks. General and Comparative Endocrinology 121: 95-103.

Eising, C.M., Eikenaar, C., Schwabl, H., Groothuis, T.C.C. 2001. Maternal androgens in black-headed gull (Larus ridibundus) eggs: consequences for chick development. The Royal Society 268: 839-846.

Ellis, L.A., Borst, D.W., Thompson, C.F. 2001. Hatching asynchrony and maternal androgens in egg yolks of house wrens. Journal of Avian Biology 32: 26-30.

Hackl, R., Bromundt, V., Daisley, J., Kotrschal, Möstl, E. Distribuation and origin of steroid hormones in the yolk of Japanese quail eggs (*Coturnix coturnix japonica*). Journal of Comparative Physiology B 173: 327-331.

Lipar, J.L., Ketterson, E.D., Nolan, V. 1999a. Intraclutch variation in testosterone content of red-winged blackbird eggs. The Auk 116: 231-235.

Lipar, J.L., Ketterson, E.D., Nolan, V., Casto, J.M. 1999b. Egg yolk layers vary in the concentration of steroid hormones in two avian species. General and Comparative Endocrinology 115: 220-227.

Lovern, L.B., Wade, J. 2001. Maternal plasma and egg yolk testosterone concentrations during embryonic development in green anoles (*Anolis carolinesis*). General and Comparative Endocrinology 124: 226-235.

Magrath, R.D. 1990. Hatching asynchrony in altricial birds. Biological Reviews 65: 587-622.

Romanoff, A.L. The Avian Embryo. New York: Macmillan & Co, 1960.

Royle, N.J., Surai, P.F., Hartley, I.R. 2001. Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects. Behavioral Ecology 12: 381-385.

Schwabl, H. 1993. Yolk is the source of maternal testosterone for developing birds. Proceedings of the National Academy of Sciences 90: 11446-11450.

Schwabl, H. 1996. Maternal testosterone in the avian egg enhances postnatal growth. Comparative Biochemisty and Physiology 114A: 271-276.

Schwabl, H., Mock, D.W., Gieg, J.A. 1997. A hormonal mechanism for parental favouritism. Nature: 386-231

Appendix 1

Steroid Assay Buffer- PBSG

1 L Distilled Water
1.00g NaN_3
1.00g Gelatin
9.00g NaCl
16.35g $Na_2HPO_4:7H_2O$
5.38g $NaH_2PO_4:H_2O$

Mix in jug and place on a heated, magnetic stirring plate. Heat over low heat overnight while stirring until gelatin is dissolved. A temperature of 50°C is adequate. It must be refrigerated until ready to use.

Appendix 2

Testosterone Enzyme Immunoassay Procedure

Allow all specimens and reagents to reach room temperature and mix thoroughly by gentle inversion before use.

1. Mark the microtitration strips to be used.
2. Pipet 50µL of the standards, controls and unknowns into the appropriate wells.
3. Prepare the enzyme conjugate solution by diluting the enzyme conjugate concentrate in the enzyme conjugate diluent.
4. Add 100µL of the enzyme conjugate solution to each well using a semi-automatic dispenser.
5. Add 100µL of the testosterone antiserum to each sell using a semi-automatic dispenser.
6. Cover and incubate the wells, shaking at a fast speed (500-700rpm) on an orbital microplate shaker, at room temperature for 1 hour.
7. Aspirate and wash each well 5 times with the wash solution using an automatic microplate washer. Blot dry by inverting the plate on an absorbent material.
8. Add 100µL of the TMB Chromogen solution to each well using a semi-automatic dispenser.
9. Incubate the wells, shaking at a fast speed (500-700rpm) on an orbital microplate shaker, at room temperature for 30 minutes. Avoid exposure to direct sunlight.
10. Add 100µL of the stopping solution to each well using a semi-automatic dispenser.
11. Read the absorbance of the solution in the wells within 30 minutes, using a microplate reader set to 450nm.

Appendix 3

This graph contains data from the validity tests including the well number for each sample, the treatment each received and the mg of yolk used per sample.

Well Number	Sample Type	Sample Number	Treatment	Mg of Yolk
A1	Standard Curve	1	Straight Yolk	100
A2	Standard Curve	2	Straight Yolk	54
A3	Standard Curve	3	Straight Yolk	39
A4	Standard Curve	4	Straight Yolk	57
A5	Standard Curve	8	Straight Yolk	55
A6	Standard Curve	10	Straight Yolk	54
A7	Standard Curve	30	Straight Yolk	50
A8		31	Straight Yolk	42
A9		17	T Before Extraction	52
A10		18	T Before Extraction	53
A11		19	T Before Extraction	54
A12		21	T Before Extraction	55
B1	Standard Curve	22	T Before Extraction	53
B2	Standard Curve	23	T Before Extraction	51
B3	Standard Curve	11	T After Extraction	51
B4	Standard Curve	12	T After Extraction	46
B5	Standard Curve	13	T After Extraction	51
B6	Standard Curve	14	T After Extraction	18
B7	Standard Curve	16	T After Extraction	46
B8		25	T After Extraction	53
B9		26	T After Extraction	40
B10		5	Varying Yolk Masses	21
B11		6	Varying Yolk Masses	35
B12		7	Varying Yolk Masses	49
C1	1	28	Varying Yolk Masses	80
C2	2	29	Varying Yolk Masses	63
C3	3	15	Varying Yolk Masses	72

Appendix 4 This table summarizes the calculations made for the validity tests.

Experiment: Quail EIA **Run:** 1 **T Calculations**

Sample	Mean Abs	log(dose)/tube	ng/ml of T		pg T / mg yolk
1	0.0875	1.38069644	24.0268279		24.02682795
2	0.004	1.48194979	30.3354043		56.17667459
3	0.127	1.33279815	21.5178139		55.17388188
4	0.0405	1.43768934	27.3961378		48.06339958
8	0.003	1.4831624	30.4202236		55.30949747
10	0.024	1.45769749	28.6878161		53.12558535
30	0.017	1.46618579	29.2540361		58.50807217
31	0.003	1.4831624	30.4202236		72.42910383
17	0.1215	1.33946753	21.8508095		42.02078743
18	0.0745	1.39646043	24.9149737		47.00938432
19	0.052	1.42374427	26.5304288		49.1304237
21	0.1795	1.26913586	18.5838573		33.78883152
22	0.012	1.47224887	29.6653084		55.97227999
23	0.039	1.43950826	27.5111195		53.94337162
11	0.123	1.33764861	21.7594848		42.6656564
12	0.1345	1.32370354	21.0718922		45.80846129
13	0.0885	1.37948382	23.959835		46.98006869
14	0.15	1.304908	20.1793886		112.1077145
16	0.112	1.35098737	22.4381668		48.77862348
25	0.0655	1.40737397	25.5490036		48.20566717
26	0.09	1.3776649	23.8596957		59.64923934
5	0.003	1.4831624	30.4202236	21	144.8582077
6	0.045	1.43223257	27.0540678	35	77.29733657
7	0.141	1.31582154	20.6929086	49	42.23042562
28	0.0335	1.44617765	27.9368635	80	34.92107939
29	0.031	1.44920918	28.1325554	63	44.6548499
15	0.221	1.21881234	16.5505466	72	22.98687032
Blank	0.001	1.48558763	30.5905744		30.59057441
0.5 ng/ml	0.887	0.41121078	2.57757187		2.577571867
Blank	0.001	1.48558763	30.5905744		30.59057441
0.5 ng/ml	1.358	-0.15993086	0.69194112		0.691941119
5 ng/ml	0.536	0.83683863	6.86813197		6.868131975
10 ng/ml	0.155	1.29884493	19.8996267		19.89962668
10 ng/ml	0.117	1.3449243	22.1270897		22.12708975
10 ng/ml	0.121	1.34007384	21.8813361		21.88133613
10 ng/ml	0.117	1.3449243	22.1270897		22.12708975
10 ng/ml	0.104	1.36068829	22.9450121		22.94501214
10 ng/ml	0.14	1.31703415	20.750767		20.75076698
10 ng/ml	0.132	1.32673507	21.2194964		21.21949642
10 ng/ml	0.123	1.33764861	21.7594848		21.75948477
5 ng/ml	0.446	0.94597398	8.83026989		8.830269889