

**MATERNAL THYROID HORMONES IN JAPANESE QUAIL EGGS**

by

**Charles Morgan Wilson**

Thesis submitted to the Faculty of the  
Virginia Polytechnic Institute and State University  
in partial fulfillment of the requirements for the degree of  
MASTER OF SCIENCE

in

BIOLOGY

APPROVED:



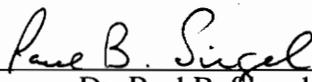
Dr. F. M. Anne McNabb, Chair



Dr. D. Michael Denbow



Dr. Charles L. Rutherford



Dr. Paul B. Siegel

July, 1996

Blacksburg, Virginia

Key words: thyroxine, yolk, maternal, quail, development

c.2

LD  
5655  
V855  
1996  
W557  
c.2

# **MATERNAL THYROID HORMONES IN JAPANESE QUAIL EGGS**

by

**Charles Morgan Wilson**

Dr. F. M. Anne McNabb, Chairman

Department of Biology

(ABSTRACT)

Thyroid hormone content in eggs varies with the thyroid status of the hen and may influence embryonic development prior to the release of appreciable amounts of thyroid hormones by the embryonic thyroid gland. Methods for the measurement of thyroid hormones in egg yolk were verified by demonstrating consistency in the recovery of yolk thyroid hormones following a methanol/chloroform extraction, and in the measurement of thyroid hormones in extracts across a range of dilutions by RIA. Untreated hens produced eggs with yolk T<sub>4</sub> that was low relative to plasma T<sub>4</sub>, but yolk T<sub>3</sub> that was comparable to plasma T<sub>3</sub>. Hens dosed twice daily with L-thyroxine (T<sub>4</sub>; 1x or 3x the daily thyroid secretion rate of T<sub>4</sub> per dose) showed significant increases in T<sub>4</sub> concentrations in their plasma and in the yolk of their eggs. Maternal thyroid hormone deposition in yolk varied with hens' thyroid status. T<sub>4</sub> dosed hens demonstrated "levels" of hyperthyroidism and deposited greater amounts of T<sub>4</sub> into egg yolk compared to controls, yet appeared to regulate T<sub>4</sub> deposition into egg yolk at each level. Pelvic cartilage, a thyroid hormone responsive tissue, showed enhanced growth and differentiation in embryos

from eggs of hens given the highest dose of T<sub>4</sub>. Specifically, alkaline phosphatase activity (a marker of differentiation) and pelvic cartilage weights were significantly greater in embryos from high T<sub>4</sub> eggs than in controls. However, chicks from high T<sub>4</sub> eggs did not have measurable differences in total thyroid hormone content (carcass without the thyroid gland), general body growth (body weight, length, and general morphology), or hatchability when compared to controls.

## ACKNOWLEDGMENTS

I would like to give special thanks to my committee chair, Dr. F. M. Anne McNabb, for her guidance, patience, and faith in my abilities. I would also like to thank the other members of my committee, Dr. Michael Denbow, Dr. Charles Rutherford, and Dr. Paul Siegel, for their suggestions, words of wisdom, guidance, and encouragement.

My family and friends deserve special thanks for their unconditional love, support, and encouragement during this degree.

The following people volunteered their time to assist me with several aspects of the project and deserve special thanks: Allison Wolf, Melissa Goode, Melissa Catena, Heather Solari, Osam Abed, Steve Sklarew, Eric Belling, Megan Harris, Emilio Martinez-Lezama, Cynthia Tate, Matt Stinchcomb, Judy McCord, Amy Wood, David Mullins, Memuna Khan, Kevin Simon, Matt Lovern, and Steve Nunez.

This research was supported by funding from Sigma Xi Grants-In-Aid, the Graduate Research Development Project of Virginia Tech, and the Biology Department of Virginia Tech.

## TABLE OF CONTENTS

	<u>Page</u>
I. ABSTRACT.....	ii
II. ACKNOWLEDGMENTS.....	iv
III. LITERATURE REVIEW.....	1
• Avian Thyroid Function and Its Development.....	2
- General Structure and Function of the Thyroid Gland.....	2
- Avian Thyroid Gland Development.....	3
Hypothalamic-Pituitary-Thyroid (HPT) axis maturation.....	5
Thyroid hormone synthesis and release.....	6
Thyroid hormone deiodination.....	7
• Maternal Thyroid Transfer into Eggs.....	10
- Thyroid Hormone Binding Proteins.....	10
- Avian Oocyte Formation and Maturation.....	12
- Thyroid Hormone Content in Avian Egg Yolk.....	14
- Mechanisms of Thyroid Hormone Transfer into Avian Eggs.....	17
- A Possible Mechanism of Transfer of Thyroid Hormones to the Embryo.....	18
• Thyroid Hormone Effects on Development.....	20
- Avian Embryology, Dynamics of Egg Components, and Yolk Absorption During Development.....	20
- Thyroid Hormone Effects on Vertebrate Development.....	22
Thyroid hormone effects on differentiation in vertebrates.....	24
The effects of thyroid hormones on embryonic skeletal growth and differentiation in birds....	25
IV. INTRODUCTION.....	28
V. MATERIALS AND METHODS.....	31
• Animals.....	31
• Experimental Design.....	31
- Maternal thyroid hormone deposition into egg yolk.....	31
- Manipulation of hen thyroid status and egg thyroid hormone content.....	32

• Sampling Techniques.....	33
• Analytical Methods.....	35
- Yolk thyroid hormone extraction procedure.....	35
- Thyroid hormone radioimmunoassays (RIAs).....	37
- Alkaline phosphatase analysis.....	38
- Statistical analyses.....	39
VI. RESULTS.....	40
• Time Course of Thyroid Hormone Deposition in Egg Yolk.....	40
• The Relationship Between the Thyroid Status of Hens and Their Egg Thyroid Hormone.....	40
• Changes in Weight and Thyroid Hormone Content of Embryos and Egg Compartments During Development.....	42
• Embryonic Development.....	44
VII. DISCUSSION.....	46
VIII. LITERATURE CITED.....	53
VIII. TABLES	
Table 1. Dye Deposition Pattern in Egg Yolk.....	60
Table 2. Comparison of Thyroid Hormones in Chickens and Japanese Quail .....	61
IX. FIGURES	
Figure 1. T4 and T3 RIA Validation.....	62
Figure 2. Hen Plasma and Egg Yolk Thyroid Hormones.....	63
Figure 3. The Relationship Between Individual Egg Yolk T4 and Individual Hen Plasma T4.....	64
Figure 4. Embryonic Plasma Thyroid Hormone Concentrations....	65
Figure 5. Thyroid Hormone Content in Egg Yolk and in Embryos During Incubation.....	66
Figure 6. Embryonic Pelvic Cartilage Weights During Incubation.....	67
Figure 7. Embryonic Pelvic Cartilage Alkaline Phosphatase Activity.....	68
XII. CURRICULUM VITA.....	69

## LITERATURE REVIEW

The material presented in this review represents background information on several aspects of thyroid endocrinology and avian embryonic development. Avian thyroid function, maternal thyroid hormone transfer into eggs, and thyroid hormone effects on avian embryonic development will be addressed. When referring to avian embryonic development, days of incubation for two species of precocial birds, the chicken and the Japanese quail, will be cited. Although these birds have different periods of incubation (21 and 16.5 days, respectively), the pattern in which the thyroid gland develops in both of these precocial avian species is essentially the same (review, McNabb, 1989). Each specific day of incubation that is mentioned will be expressed as a fraction of the total days of incubation (review, McNabb, 1989).

## AVIAN THYROID GLAND FUNCTION AND DEVELOPMENT

### General Structure and Function of the Thyroid Gland

In avian species, the thyroid gland consists of a pair of organs lying on either side of the trachea. These glands are oval in shape and dark red in color. The blood supply to these highly vascularized glands originates from the common carotid arteries and infiltrates the glandular tissue by the cranial and caudal thyroid arteries (review, Wentworth and Ringer, 1986). Thyroid hormones released from the gland enter the blood stream by diffusion into capillaries which surround the thyroid follicles (review, McNabb, 1992).

The functional unit of the thyroid gland is the follicle, which is composed of a single epithelial cell layer surrounding a central, colloid-filled lumen. The colloid is an extracellular fluid or gel composed primarily of thyroglobulin, a thyroid hormone storage protein. The cells that make up the follicle are typically cuboidal in shape under euthyroid conditions and are in direct contact with the endothelial cells of thyroid capillaries at the basal portion of each follicle cell (review, McNabb, 1992).

The general functions of the thyroid gland are to concentrate dietary iodide from the blood stream, synthesize and store thyroid hormones [thyroxine (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>)], and secrete thyroid hormones into the blood stream for subsequent uptake and influence on thyroid hormone-responsive tissues. Current evidence suggests that most effects of thyroid

hormones are mediated by the binding of T<sub>3</sub> to nuclear thyroid hormone receptors (which are considered to be T<sub>3</sub> receptors because they have the highest affinity for T<sub>3</sub>; Oppenheimer et al., 1987). Thyroid hormones regulate basal metabolic rate, cellular respiration, and development (both growth and differentiation). Some actions of thyroid hormones are direct, i.e. they trigger the expression of a gene resulting in mRNA transcription and subsequent translation of a protein (such as in the differentiation of skeletal tissues, muscle, central nervous tissue, lung, and intestinal tissues during development; review, McNabb, 1992). Other functions of thyroid hormones may be indirect, or permissive, where the presence of thyroid hormones enhances the action of another hormone's effect on the expression of a gene or a function of a cell (as in tissue or somatic growth; review, McNabb, 1992).

### **Avian Thyroid Gland Development**

The embryonic thyroid gland appears on day 2/21 in the chicken embryo. During the first phase of thyroid gland development (the precolloid stage), the cells of the gland are arranged in strands or cords, and this arrangement of cells is infiltrated by blood vessels (reviews: Wentworth and Ringer, 1986; McNabb, 1992). The developing gland accumulates radioiodine in concentrations greater than those in the blood by day 7/21, and further concentrates and organically binds radioiodine by day 9/21 in developing chicken embryos (Wollman and Zwilling, 1953). Thommes and Hylka (1978)

report detectable concentrations of T<sub>4</sub> in the plasma of chicken embryos on day 6.5/21 of incubation, while McNichols and McNabb (1988) found detectable concentrations of thyroid hormones in the thyroid gland and the plasma of embryonic quail by day 8/16.5 (the earliest day sampled). In the latter study, the thyroid hormone content of the embryonic thyroid gland increased 300-fold from day 8/16.5 to hatch.

During the second phase of thyroid development, the colloid phase, intracellular spaces begin to enlarge and fill with colloid (review, McNabb, 1992). These spaces or channels appear to migrate and converge, forming the beginnings of thyroid follicles. This process continues as increasing amounts of colloid accumulate (McNabb et al., 1972; review, McNabb, 1992). Thyroid follicles come under the control of the hypothalamic-pituitary axis during this phase of thyroid development (review, Thommes, 1987). Due to an increase in thyroid hormone synthesis and secretion by the gland under the influence of thyroid stimulating hormone, there is approximately a 3-fold increase in plasma concentrations of T<sub>4</sub> from the beginning of the second phase of thyroid development until the beginning of the perinatal period (McNabb, 1988; review, McNabb and King, 1993). Plasma T<sub>4</sub> increases gradually during the latter half of incubation, then increases sharply during the perinatal period. In contrast, plasma T<sub>3</sub> is detectable, but very low (<1 ng/ml), during embryonic life and rises dramatically during the perinatal period, simultaneously with the peak in plasma T<sub>4</sub> (McNabb et al., 1981). After hatch, the concentrations of both

T<sub>4</sub> and T<sub>3</sub> decrease from those of the perinatal peak to low concentrations and then gradually increase to concentrations typical of adults during the third phase of thyroid gland development (reviews: McNabb, 1988; McNabb and King, 1993).

*Hypothalamic-Pituitary-Thyroid (HPT) axis maturation.* As the embryonic thyroid gland develops, the anterior pituitary gland and the hypothalamus are also developing, each independent of the other two. However, just prior to mid-incubation, these three endocrine organs become linked and coordinated to form the hypothalamic-pituitary-thyroid axis (reviews: Thommes, 1987; McNabb, 1992). Thommes and Hylka (1978) report the anterior pituitary of the chicken embryo to be functional prior to axis formation by demonstrating its sensitivity to thyroid releasing hormone from the hypothalamus as early as day 6.5/21 of incubation. Experiments which report similar plasma T<sub>4</sub> concentrations in decapitated chicken embryos (which lack a pituitary and thus thyroid stimulating hormone) and intact embryos demonstrate that the developing thyroid gland is independent of thyroid stimulating hormone from the anterior pituitary during early incubation. However, after the linking of the axis on days 10.5 to 11.5/21, these decapitated embryos showed no increase in plasma T<sub>4</sub> concentration, while intact (unoperated) embryos (now under the influence of thyroid stimulating hormone) demonstrated a 4-fold increase in plasma T<sub>4</sub> during the latter half of incubation (Thommes et al., 1977).

The major increases in the following substances during mid-incubation reflect the activation of the hypothalamic-pituitary-thyroid axis: thyroid iodine content, the rate of radiolabeled iodide uptake by the thyroid gland, amounts of iodotyrosines and T<sub>4</sub> in the thyroid gland, and total T<sub>4</sub> in the plasma (review, Thommes, 1987). The principal hormone produced by the thyroid gland is T<sub>4</sub>, and thyroid stimulating hormone stimulation of the thyroid gland appears to affect primarily T<sub>4</sub> release into the plasma (McNabb et al., 1984). The peak in plasma T<sub>3</sub> during the perinatal period appears to result primarily from extrathyroidal T<sub>3</sub> production (Darras et al., 1992; review, McNabb, 1992).

*Thyroid Hormone Synthesis and Release.* Steps in synthesis of thyroid hormones by the thyroid gland occur within the follicle cells, in the colloid of the follicle lumen, and at the interface between the two (review, McNabb, 1992). Iodide transport from the blood supply to follicle cells occurs against a concentration gradient and is driven by ATPase activity. Once inside the follicle cells, iodide moves through the cell and into the colloid of the follicular lumen down an electrochemical gradient. Within each follicle cell, the thyroid hormone precursor, thyroglobulin, is produced on polysomes of the endoplasmic reticulum, moves through the lumen of the endoplasmic reticulum, and is glycosylated as it is further processed within the Golgi apparatus. Thyroglobulin is then packaged into exocytotic vesicles in the Golgi, sialic acid is added to carbohydrate units on the molecule, and the

vesicles are transferred across the apical membrane of the follicular cell into the colloid (review, McNabb, 1992).

After entering the colloid, the vesicles remain bound to the apical surface of the follicle cells and iodination of tyrosine residues in the thyroglobulin molecule occurs (review, McNabb, 1992). This iodination results in the thyroid hormone precursors, monoiodotyrosine and diiodotyrosine. An enzyme, thyroid peroxidase, then facilitates the coupling of iodinated tyrosine residues, forming primarily thyroxine (3,5,3',5'-tetraiodothyronine, T<sub>4</sub>) and some 3,5, 3'-triiodothyronine (T<sub>3</sub>). Although small amount of T<sub>3</sub> are produced in the thyroid gland, most T<sub>3</sub> is produced by the extrathyroidal deiodination of T<sub>4</sub> (in the liver and kidneys; review, McNabb, 1992).

*Thyroid hormone deiodination.* The idea that T<sub>3</sub> is the most metabolically active thyroid hormone comes primarily from studies showing that thyroid hormone receptors have a higher affinity for T<sub>3</sub> than for T<sub>4</sub> (review, Oppenheimer et al., 1987). Several enzymatic deiodinations are important in determining the plasma patterns of T<sub>3</sub> and thus the availability of T<sub>3</sub> for binding to receptors and causing effects. The prohormone T<sub>4</sub> can be converted to either T<sub>3</sub> (activation) or to reverse-T<sub>3</sub> (rT<sub>3</sub>; an inactive hormone form). The conversion of T<sub>4</sub> to T<sub>3</sub> involves a 5'-deiodination (5'D; a deiodination of the outer-ring), while a conversion of T<sub>4</sub> to rT<sub>3</sub> involves a deactivating 5-deiodination (5D; a deiodination of the inner-ring). These conversions are the result of a specific deiodinase isozymes (reviews: Leonard and Visser, 1986;

Berry and Larsen, 1992). Also important is the degradation of T<sub>3</sub> to inactive 3,3'-diiodotyrosine (T<sub>2</sub>), another example of a 5D reaction. The relationship(s) between the enzymatic conversions to active and inactive thyroid hormone forms is neither simple nor completely understood (review, McNabb, 1992).

The three main types of deiodinations (I-III), are responsible for different conversions of thyroid hormones in different tissues, dependent upon the substrate preference of the enzyme in each tissue (review, Leonard and Visser, 1986). Type I deiodination occurs in the liver, kidney, and thyroid gland, and is capable of both 5D and 5'D activity. The substrate "preference" (i.e. its affinity for substrates) of Type I deiodination is  $rT_3 \gg T_4 > T_3$ , and the physiological role of this enzyme is to supply a source of T<sub>3</sub> for peripheral tissues in the plasma by the conversion thyroidal T<sub>4</sub> to T<sub>3</sub>. The Type II enzymatic pathway occurs in the central nervous system and the pituitary of birds, and in these tissues as well as in brown adipose tissue and the placenta of mammals. This enzyme, which has a preference for T<sub>4</sub> as a substrate over rT<sub>3</sub>, also catalyzes the outer ring deiodination of T<sub>4</sub> to T<sub>3</sub>. The physiological function of this deiodinase enzyme is to regulate the intracellular concentration of T<sub>3</sub> in the pituitary and central nervous system. Finally, the Type III pathway, which occurs in the central nervous system and the placenta in mammals, is a 5D conversion and "prefers" T<sub>3</sub> to T<sub>4</sub> as a substrate. This enzymatic pathway is responsible for the deactivation of T<sub>3</sub>, and its activity parallels the thyroid status of the organism (reviews: Leonard and Visser, 1986; Berry and Larsen, 1992).

In precocial avian embryos, plasma T<sub>3</sub> is very low (although measurements have been made only during the latter half of incubation) because 5D activity predominates. This predominance of the 5D pathway occurs until the perinatal period and is thought to be adaptive to prevent a build-up of T<sub>3</sub>, which is regarded to be toxic to avian embryos (Galton and Hiebert, 1987). During the perinatal period, plasma T<sub>3</sub> peaks due to a shift from 5D conversion of T<sub>4</sub> to rT<sub>3</sub> to a 5'D conversion of T<sub>4</sub> to T<sub>3</sub> (Borges et al., 1980; Borges et al., 1981; Hughes and McNabb, 1986; Galton and Hiebert, 1987; Darras et al., 1992).

## **MATERNAL THYROID HORMONE TRANSFER INTO EGGS**

Little is known about the deposition of maternal thyroid hormones in the developing oocyte. It is possible that maternal thyroid hormones are transferred from the hen's plasma to the yolk of the developing oocyte either by simple diffusion down a concentration gradient, or by transport in association with plasma binding proteins. Only a few recent studies have addressed the possible transfer of thyroid hormones into yolk bound to plasma proteins, and no studies have addressed the transfer of maternal thyroid hormones by simple diffusion. It is the purpose of the following sections to give background information about plasma thyroid hormone binding proteins, the formation and maturation of avian oocytes, and to address the proposed mechanism(s) of thyroid hormone transfer into oocytes from the hens plasma and into the embryo from the yolk.

### **Thyroid Hormone Binding Proteins.**

In euthyroid chickens and quail, >99% of plasma thyroid hormones are associated with plasma binding proteins, which have a high affinity for thyroid hormones (review, Robbins and Edelhoeh, 1986). Binding proteins maintain an extrathyroidal pool of thyroid hormones, providing a buffering system against changes in thyroid hormone release from the thyroid gland and the degradation and excretion of thyroid hormones. Thus, they help to regulate the

supply of thyroid hormones to tissues (review, Robbins and Bartalena, 1986). The free and bound fraction of thyroid hormones in the blood are in equilibrium, so that when free thyroid hormones pass from blood to tissues, the free fraction is maintained by disassociation of thyroid hormones from binding proteins (review, McNabb, 1992). Binding proteins may play a role in the metabolic clearance rates or half lives ( $t_{1/2}$ ) of thyroid hormones; the half-lives of thyroid hormones in birds are relatively short when compared to those in mammals (in chickens and quail:  $t_{1/2}$  of thyroid hormones is 3.25 to 5.5 hr, Singh et al., 1967; in mammals:  $t_{1/2}$  of T<sub>4</sub> is several days, and  $t_{1/2}$  of T<sub>3</sub> is  $\approx$  1 day, review, Van Middlesworth, 1974).

In euthyroid adult Japanese quail, thyroid hormones are presumed to remain relatively constant ( $\approx$  10 to 15 ng T<sub>4</sub>/ml;  $\approx$  1 to 5 ng T<sub>3</sub>/ml) and are considerably lower than the peak in thyroid hormones which occurred during the perinatal period (review, McNabb, 1988). In adult Japanese quail, plasma thyroid hormones are bound to albumin, transthyretin (thyroxine-binding prealbumin), and to  $\alpha$ - and  $\gamma$ -globulins; T<sub>4</sub> binds primarily to albumin and secondarily to transthyretin, while most T<sub>3</sub> binds to  $\alpha$ -globulin and to albumin and  $\gamma$ -globulin to a lesser extent (McNabb and Hughes, 1983). This pattern of binding is similar to the pattern of thyroid hormone binding in chickens, pigeons, and turkeys (Farer, et al., 1962; Davison, et al., 1978). Although the pattern of the binding of thyroid hormones in avian blood is different from

that found in mammals (birds do not have the specific thyroxine binding globulin found in man and other mammals), the amount of free thyroid hormones in avian blood is similar to that found in humans (McNabb and Hughes, 1983).

### **Avian Oocyte Formation and Maturation**

An ovum which is ready to rupture from its follicle has undergone yolk deposition periodically since the hen was two months old; however, up until the point of rapid follicular development, only white yolk was periodically laid down in the oocyte (review, Romanoff and Romanoff, 1949). When sexual maturity is reached in galliform birds, the deposition of yolk in oocytes of the single functioning ovary is accelerated. Ripening follicles mature in an orderly, hierarchical fashion, balanced by both follicle atresia and ovulation (Johnson, in press). Several days prior to ovulation, large amounts of yolk are deposited, yellow yolk (rich in lipid and carotenoid pigments) during the day, and white yolk (composed mostly of water and small amounts of lipids and proteins) during the later part of the night (review, Romanoff and Romanoff, 1949). During this rapid follicular development, materials necessary for the formation of yolk are passed from the hen's blood to the oocyte (review, Romanoff and Romanoff, 1949).

Several studies have addressed yolk deposition and ovarian follicle growth and maturation in birds. Bacon and colleagues addressed rapid

follicular growth in three galliform species (turkeys: Bacon and Chermis, 1968; chickens: Bacon and Skala, 1968; quail: Bacon and Koontz, 1971) by orally dosing laying hens with lipid-soluble dyes. The rapidly developing oocytes quickly accumulated the different colored dyes, and factors such as number, rate, and duration of rapid oocyte development were assessed. The time frame for yolk deposition in the avian oocyte was investigated by Grau (1976). Results of this study indicated that egg yolks have a ring structure as a result of sequential peripheral yolk deposition within each oocyte. A pair of one light and one dark ring, demonstrated using a dye feeding technique followed by staining of the egg yolk with potassium dichromate solutions, indicated yolk deposition during each of several 24 hr periods. Although each individual egg yolk showed a distinct pattern, the number of days of dye deposition was fairly consistent within a species (Japanese quail: 4 to 6 days; chickens: 7 to 11 days; turkeys: 10 to 12 days; cackling geese: 12 days).

The major precursors of the oocyte yolk, vitellogenin and very-low density lipoproteins, are produced in the maternal liver and transferred to the developing oocyte (Barber, et al., 1991). Likewise, other yolk components, such as phosphovitins and lipovitellins, are incorporated into oocyte yolk during formation and are thought to be derived from the vitellogenin precursor in the hen plasma (Griffin, et al., 1984).

Hormones found in the yolk of unincubated avian eggs [(testosterone: Schwabl, 1993) (thyroid hormones: Prati, et al., 1992; Sechman and Bobeck,

1988; Hilfer and Searls, 1980) (insulin and insulin-like growth factor I , Scavo et al., 1989)] are incorporated into the yolk from the maternal plasma. However, the mechanism(s) by which maternal hormones enter the yolk is not clear.

Although water-soluble proteins (levitins) found in the yolk are responsible for the transfer of some vitamins (biotin, riboflavin, thiamin, vitamin A, and cholecalciferol) and immunoglobulins from the hen plasma to yolk (Griffin, et al., 1984), thyroid hormones are not soluble in water. There is evidence for the incorporation of proteins and lipoproteins into oocytes by receptor mediated endocytosis (Barber, et al., 1991; Nimpf, et al., 1989; Vieira, et al., 1995; Vieira and Schneider, 1993). Because thyroid hormones are lipophilic, it is possible that they cross the plasma membrane and enter the yolk of oocytes by diffusion down a concentration gradient from the plasma to the yolk. Thyroid hormones enter tissues either by diffusion in a free form or by active transport across the cell membrane (review, McNabb, 1992). The following sections summarize the reports of thyroid hormones in avian egg yolk and proposed mechanisms of maternal thyroid hormone transfer into avian egg yolk and to the embryo.

### **Thyroid Hormone Content of Avian Eggs.**

Hilfer and Searls (1980) proposed that the majority of the T<sub>4</sub> found in the blood of chicken embryos early in development was derived from maternal thyroid hormones in egg yolk, and appear to be the first to have published thyroid hormone concentrations in avian egg yolk. They reported 1.0 to 2.0 ng

% T<sub>4</sub> ( $\approx$  15 to 20 ng T<sub>4</sub>/g yolk) in the egg yolk by radioimmunoassay (RIA).

However, the methods used to attain these results are of some concern. It appears that egg yolk was added directly to the RIA system (without extraction to isolate thyroid hormones). Lipids can interfere with the accuracy of the RIA by binding to the thyroid hormone primary antibody, depressing the binding of labeled and unlabeled hormone, and inflating values reported by the assay. There also was no mention of validations of the techniques used in this study.

A study by Sechman and Bobeck (1988) also addressed the presence of thyroid hormones in the yolk of chicken eggs. They reported 6 to 10 ng T<sub>4</sub>/g yolk and 1.5 to 3.5 ng T<sub>3</sub>/g yolk. Although a method was used to separate thyroid hormones from the yolk in this study, Sechman and Bobeck also failed to account for potential lipid effects on the primary antibody. Yolk and oocytes were roughly homogenized and aliquoted onto an ion exchange chromatography column. A known amount of a radiolabeled iodothyronine was added, the proteins and inorganic iodide eluted from the column, and a primary antibody and barbital buffer added to the column. The presumed hormone/antibody complex was then eluted from the column and analyzed by RIA. The radiolabeled elution was reported to be "proportional to the concentration of non-radioactive iodothyronine in the specimen," but the authors did not present data to support the adequacy of this claim. Furthermore, the statement would be more appropriate reading "inversely proportional" instead of the implied direct proportion. Despite these questions

about the accuracy of the methods used, the studies of Hilfer and Searls (1980) and Sechman and Bobeck (1988) do suggest that maternal thyroid hormones are stored in egg yolk and could influence avian embryonic development before the production and secretion of thyroid hormones by the developing embryo.

In a more recent study, Prati et al. (1992) addressed the amount of thyroid hormones available to the chicken embryo before and after the presumed time of onset of embryonic thyroid gland secretion. It is clear from the methodology reported that the techniques for extracting thyroid hormones from the yolk, as well as assessing yolk and tissue thyroid hormones by RIA, were well validated. Samples of yolk, albumen, whole embryos, and several embryonic tissues were each homogenized in methanol and purified using a methanol/chloroform extraction procedure, followed by an additional purification of the thyroid hormone extracts using ion exchange chromatography. Data obtained in this study indicate that thyroid hormones were found in chicken egg yolk prior to incubation and were of maternal origin (T<sub>4</sub>: 3.8 ng/g; T<sub>3</sub>: 1.5 ng/g). The yolk thyroid hormone content of these unincubated eggs represented 95% of the total thyroid hormones found in these eggs. Thyroid hormones were present in different portions of the chicken embryo during its early and later development, but it was not possible to distinguish their origin (maternal or embryonic).

## **Mechanisms of Thyroid Hormone Transfer into Avian Eggs.**

Mitchell and colleagues have demonstrated the binding of small amounts of thyroid hormones to lipoproteins and vitellogenin in the blood of chickens. They propose that lipoproteins and vitellogenin play a crucial role in the transfer of thyroid hormones into avian egg yolk (Mitchell, 1984; Mitchell, et al., 1985; Mitchell and Stiles, 1985). Both very-low-density lipoproteins and low-density lipoproteins are synthesized in the liver and deposited in oocytes during follicle development (Griffin, et al., 1984). The lipoproteins found in avian plasma are rich in triglycerides and are very similar to those found in egg yolk. This similarity of plasma and yolk lipoproteins suggests that thyroid hormones (which are lipid soluble) may be transfer from the hen's plasma to the egg yolk with these lipoproteins (Griffin, et al., 1984). Both very-low density lipids and vitellogenin are important components in the yolk of chicken eggs and have been shown to bind to a receptor on ovarian membranes of laying chicken hens (Barber, et al., 1991). In chicken hen plasma, a relatively small (but significant amount) of T<sub>3</sub> is bound to very-low and low-density lipoproteins, a small amount of both thyroid hormones is bound to vitellogenin, and a substantial amount of T<sub>4</sub> (20 to 30%) is bound to a low molecular mass protein, suspected to be transthyretin or  $\delta$ -globulin (Mitchell and Stiles, 1985).

Transthyretin, a non-lipoprotein in the plasma, binds thyroid hormones. Data from a very recent study by Vieira et al. (1995) demonstrate the presence

of a putative transthyretin receptor on the oocyte membrane which has not previously been described. They demonstrated that transthyretin found in egg yolk was derived from the plasma of the laying hen. Neither the granulosa cells of the chicken ovary nor embryonic fibroblasts possess the transthyretin-binding membrane protein, indicating the protein is oocyte membrane specific. It is clear from the data presented in this study that the binding of thyroid hormones to transthyretin is a mechanism for the transfer of thyroid hormones from the hen's plasma to oocyte.

#### **A Possible Mechanism of Transfer of Thyroid Hormones to the Embryo**

The expression of the transthyretin gene occurs very early in chicken embryonic development (extraembryonic membranes, day 2/21; embryonic liver, days 3 to 5/21; the choroid plexus, days 5 to 9/21; yolk sac, day 7/21; Southwell et al., 1991), prior to the presence of high thyroid hormone concentrations in the embryonic circulation and the onset of appreciable embryonic thyroid gland function. Therefore, the potential for transfer of thyroid hormones from the yolk to the embryo, and the subsequent availability of thyroid hormones bound to transthyretin in the embryonic plasma, is apparent. Because the expression of the transthyretin gene occurs prior to the onset of appreciable secretion of thyroid hormones by the thyroid gland, it is possible that thyroid hormones of maternal origin could be transported in the embryonic plasma to the tissues if they are taken up from the

yolk by the embryo. The action of these thyroid hormones on embryonic tissues would then depend upon the movement of thyroid hormones into cells and the binding to thyroid hormone receptors.

## THYROID HORMONE EFFECTS ON DEVELOPMENT

### **Avian Embryology, Dynamics of Egg Components, and Yolk Absorption**

#### **D ring Development**

The incubation period of Japanese quail is 16 to 16.5 days at 39°C.

Although there is some variation in the development of embryos within a set of eggs during the first week of incubation (because of initial egg warm-up time, variation in physical factors involved in incubation, and individual egg variation), variation in embryonic development during the latter half of incubation within a set of eggs is minimal (Padgett and Ivey, 1960).

Early embryonic development (i.e., the first few days of incubation) in avian species is usually described in terms of the number of somites present in the embryo. By day 3 of development, embryonic quail have limb bud primordia, distinctions in the five regions of the brain, and the allantois is present but variable in size and position (Padgett and Ivey, 1960). During this time, the yolk becomes increasingly vascularized (the area vasculosa), and the allantois (a diverticulum of the small intestine) functions as the embryonic respiratory organ and the site of urinary waste deposition by the embryonic kidneys (review, Romanoff, 1960). Also, the yolk stalk (which attaches the yolk sac to the embryonic intestine) has appeared (review, Romanoff, 1960). The endoderm of the yolk sac is continuous with the endoderm of the gut; the yolk

sac is thus as an extension of the intestine. However, yolk does not pass directly into the gut under normal circumstances until very late in incubation, at which time only small amounts enter the intestine (review, Romanoff, 1960).

Throughout most of incubation, the absorption of material from the yolk by the embryo occurs by the endocytosis of yolk by endodermal cells that line the internal surface of the yolk sac (review, Romanoff, 1960). After endocytotic uptake, the yolk inclusions are degraded by intracellular enzymes (lipases and proteases) and the digestive products enter blood vessels of the yolk sac. As the cells of the yolk sac continue to develop, processing of yolk within the endodermal cells is replaced by the transport of enzymes into the yolk for extracellular processing and subsequent absorption of the enzymatic products (review, Romanoff, 1960).

By about 1/3 the way through incubation in Japanese quail (day 6/16.5), digits of the limbs are distinct, feather germs are first recognizable, and the eyes are large, bulging, and heavily pigmented (Padgett and Ivey, 1960). During this time period, the amount of total yolk decreases because of yolk absorption by the embryo (review, Romanoff, 1960). During mid-incubation, many morphological characteristics of the embryo have developed: the appendages (wings and feet) become distinctly avian in characteristics, the pipping tooth becomes prominent, and the body is covered with feather tracts (Padgett and Ivey, 1960). By the latter half of incubation, the quail embryo has acquired the general shape and outer morphology present at hatch. More

subtle distinctions in plumage, length, and body position within the egg, as well as organ development, continue until piping (Padgett and Ivey, 1960). On the day prior to hatch, the rate in which yolk is absorbed by the chicken embryo (and presumably by the quail embryo) suddenly increases; it has been suggested that as the yolk sac is retracted into the abdominal cavity, yolk is forced into the intestine (review, Romanoff, 1960). Yolk within the abdominal cavity is assimilated within the first few days of posthatch life (review, Romanoff, 1960).

### **Thyroid Hormone Effects on Development**

Thyroid hormones can have either direct or indirect (permissive) actions on development during embryonic, neonatal, and juvenile life (review, McNabb, 1992). Vertebrate development can be defined as the combination of the processes of growth and differentiation. Growth is the irreversible increase in size or mass by cellular hypertrophy or hyperplasia, and differentiation (maturation) is the result of irreversible changes in the structure or function of cells, leading to the diversification of cell types and specialization of cell functions (reviews: Legrand, 1986; McNabb and King, 1993).

The development of thyroid function has been divided into three general phases based on the availability of thyroid hormones to the vertebrate embryo or neonate. Timing of these phases during prenatal and postnatal life, varies with the timing of development in different species (review, McNabb and King,

1993). Phase one of this development involves the beginning of thyroid hormone synthesis by the embryonic thyroid gland, phase two involves follicular development of the gland and increases in hormone production and secretion, and phase three involves the gradual change in plasma thyroid hormone concentrations to those found in adults (review, McNabb and King, 1993).

Vertebrates, such as sheep and Japanese quail, which reach a relatively advanced state of development by the time of birth/hatch (precocial development) have a maturing hypothalamic-pituitary-thyroid axis, measurable concentrations of plasma thyroid hormones, and thyroid hormone-responsive receptors by the latter half of incubation/gestation. In vertebrates, such as laboratory rats and Ring doves, which are born/hatch in a much less-developed state and require extensive parental care (altricials), the linking of the hypothalamic-pituitary-thyroid axis and the production and secretion of thyroid hormones by the thyroid gland occur after birth/hatch (reviews: Legrand, 1986; McNabb, 1992; McNabb and King, 1993). Although this seems to be a general picture for thyroid development in vertebrate species, several recent studies on the altricial rat suggest maternal thyroid hormones transferred across the placenta affect fetal development prior to maturation of the fetal hypothalamic-pituitary-thyroid axis (Morreale de Escobar et al., 1985; Morreale de Escobar et al., 1988; Morreale de Escobar et al., 1989).

*Thyroid hormone effects on differentiation in vertebrates.* It is generally accepted that thyroid hormones play an indirect or permissive role in the growth of vertebrates, but have specific, direct effects on the differentiation of vertebrate tissues (reviews: Schwartz, 1983; McNabb and King, 1993). The effects of thyroid hormones on differentiation in thyroid hormone-responsive tissues have been addressed in some species of all vertebrate classes. The tissues of the central nervous system, striated muscle, and the skeletal system are influenced by thyroid hormones during development (reviews: Legrand, 1986; McNabb and King, 1993). Cell proliferation and migration, the creation of synapses, the myelination of neurons, and the specification of neurotransmitters are important aspects of differentiation of the central nervous system (review, Nunez, 1984). Reduction in brain weight, alterations in synaptic patterns, decreased myelination, and alterations in the ratio of neurons to glial cells can result from a thyroid hormone deficiency and are evidence of the need for thyroid hormones for normal development (reviews: Schwartz, 1983; McNabb, 1992; McNabb and King, 1993).

Thyroid hormones affect the growth and development of both striated and cardiac muscle cells. Because striated muscle represents a large proportion of body mass, it can be used as an indicator of thyroid hormone effects on development (reviews: Legrand, 1986; McNabb, 1992). Increases in muscle cell RNA content and protein synthesis, as well as a shift from the neonatal myosin isoform to the adult form of the myosin heavy chain, occur under the influence

of thyroid hormones (review, McNabb and King, 1993). In laboratory rats, the administration of thyroid hormones accelerates the expression of adult myosin and its mRNA in cardiac and striated muscle, while the administration of the goitrogen propylthiouracil prevents the transition to the adult isoform of myosin, decreases muscle protein composition, and decreases skeletal muscle mass (reviews: Legrand, 1986; McNabb, 1992). Premetamorphic tadpoles show similar responses to thyroid hormones administration; T<sub>3</sub> causes an acceleration in tropomyosin synthesis and the transition of muscle myosin isoforms (review, McNabb and King, 1993).

Thyroid hormones have been shown to have specific effects on the development of the skeleton in both birds and mammals [reviews: (birds) King and May, 1984; (mammals) Schwartz, 1983; Legrand, 1986]. The development of the skeleton in embryos and neonatal vertebrates involves cartilage formation and its subsequent ossification. In prenatal altricial vertebrates, only growth hormone and insulin-like growth factors appear to be necessary for skeletal growth; however, thyroid hormones are also essential for the conversion of cartilage to bone (reviews: Schwartz, 1983; Legrand, 1986). During precocial vertebrate development, the growth and differentiation of the skeleton requires both growth hormone and thyroid hormones (review, McNabb and King, 1993).

*The effects of thyroid hormones on embryonic skeletal growth and differentiation in birds.* Normal growth and differentiation of the skeleton of chicken embryos

requires thyroid hormones. Injection of the goitrogen thiourea onto the chorio-allantoic membrane of chicken eggs (day 8/21) resulted in a reduction in the rate of differentiation of chondrocytes by days 10 to 12/21, and inhibited cartilage matrix formation by day 18/21 (Hall, 1973). This goitrogen treatment also resulted in a reduction in weight and length of embryonic tibiae, excessive cartilage erosion and vascularization, and a general loss of cartilage integrity.

A deficiency in thyroid hormones can cause retardation in skeletal growth and maturation, while hyperthyroidism can cause slight increases in growth of skeletal tissue and pronounced acceleration of skeletal maturation, specifically causing premature closure of epiphyseal plates (Burch and Van Wyk, 1987). Studies on the pelvic cartilage of the chicken embryo, conducted by Burch and colleagues, demonstrate the effects of physiological concentrations of T<sub>3</sub> on both growth and maturation of embryonic chick pelvic cartilage using a serum-free, *in vitro* culture system. Triiodothyronine (0.0015 to 15 nM) added to the culture media supporting 9-day chicken embryonic pelvic cartilage caused significant, dose dependent increases in cartilage wet weight, dry weight, length, and total soluble protein when compared to controls. The addition of T<sub>3</sub> to the culture media resulted in small increases in total DNA content, indicating the occurrence of cellular hypertrophy rather than hyperplasia (Burch and Lebovitz, 1982). When compared to pelvic cartilages incubated in media alone, embryonic cartilages treated with T<sub>3</sub> (0.015 to 15 nM) showed histological and biochemical changes indicative of

accelerated differentiation. The chondrocytes of cartilages incubated with T<sub>3</sub> had larger, rounded nuclei and vacuolated cytoplasm when compared with controls, indicative of mature (hypertrophied) chondrocytes. When compared to controls, T<sub>3</sub> treated pelvic cartilages also had significantly higher concentrations of alkaline phosphatase, an enzyme found in high concentrations in hypertrophied cartilage (and very low concentrations in immature cartilage) and used as a marker of cartilage differentiation (Burch and Lebovitz, 1982).

Burch and colleagues, using the same *in vitro* serum free culture system for embryonic chicken pelvic cartilage, hypothesized that T<sub>3</sub> affected embryonic chicken growth plate cartilage either through the enhancement of the effects of the insulin-like growth factor somatomedin, or through the stimulation of maturation by accelerating cartilage differentiation (Burch et al., 1985; Burch and Van Wyk, 1987). Their data indicate that T<sub>3</sub> did not increase somatomedin production in the embryonic cartilage, but did enhance the sensitivity of embryonic cartilage to the effects of somatomedin. An antibody to somatomedin added to the culture system blocked the stimulation of growth resulting from T<sub>3</sub>, but did not affect the acceleration of differentiation by T<sub>3</sub> (increases in alkaline phosphatase activity; Burch and Van Wyk, 1987). The data obtained from these *in vitro* studies of embryonic chicken pelvic cartilage clearly demonstrate that T<sub>3</sub> stimulates the growth and directly affects the differentiation of avian embryonic cartilage.

## INTRODUCTION

Thyroid hormones are essential for the control of development in all vertebrate classes (Gorbman, et al., 1983; McNabb, 1992; McNabb and King, 1993). However, some early mammalian studies suggested that maternal thyroid hormones did not cross the placenta, and therefore were not presumed to play a role in embryonic development (Fisher et al., 1977; Schwartz, 1983). It is now known that maternal thyroid hormones are available to embryos of many vertebrates. Maternal thyroid hormones are transferred into fish egg yolk (Brown, et al., 1987; Kobuke et al., 1987; Tagawa and Hirano, 1987) and across the placenta in rats (Morreale de Escobar et al., 1985). In striped bass, treatment of prespawning females with triiodothyronine (T<sub>3</sub>) increases yolk T<sub>3</sub>, accelerates larval development and swimbladder inflation, and enhances larval survival (Brown et al., 1988). Data obtained from laboratory rats provide evidence that maternal thyroid hormones are important in early mammalian fetal development, prior to appreciable fetal thyroid gland function (Morreale de Escobar et al., 1985; Morreale de Escobar et al., 1988).

Although thyroid hormones are known to affect the development of avian embryos and chicks (review, McNabb and King, 1993), few studies have addressed hormone effects on early embryonic stages or the transfer of maternal thyroid hormones into avian egg yolk. The first reports of thyroid hormones in avian eggs (chickens; Hilfer and Searls, 1980; Sechman and Bobeck,

1987) are difficult to evaluate because they provided no verification that the methods used effectively extract or accurately measure yolk thyroid hormones. However, the data from both studies, as well as those from a more recent, thoroughly verified study by Prati et al. (chickens; 1992), do suggest that maternal thyroid hormones are present in avian egg yolk.

If utilized by the embryo, maternal thyroid hormones in egg yolk could influence development before appreciable synthesis and release of hormones by the gland. During the first 1/3 to 1/2 of incubation, the thyroid gland appears but possesses a very limited capability to synthesize hormones (chickens: Thommes, 1987; Japanese quail: McNichols and McNabb, 1988). The presence of thyroid hormones in blood and tissues during early development may suggest that the embryonic gland is producing and releasing small amounts of thyroid hormones. Thommes and Hylka (1978) report that thyroxine (T<sub>4</sub>) is detectable in embryonic chicken plasma by day 6.5 of the 21 day incubation period. Prati et al. (1992) report that T<sub>4</sub> and T<sub>3</sub> are detectable in chicken embryonic tissues on days 4 and 6. It is clear, however, that in chicken embryos the linking of the hypothalamic-pituitary-thyroid axis does not occur until days 10.5-11.5, marking the beginning of substantial T<sub>4</sub> release from the gland (Thommes et al., 1977; Thommes, 1987). The presence of T<sub>4</sub> in the embryonic blood stream and tissues prior to the linking of the axis may therefore suggest that the embryo is obtaining maternal thyroid hormones

from the yolk, releasing thyroid hormones from its thyroid gland as they are produced, or both.

What remains unclear is the mechanism(s) by which maternal thyroid hormones are deposited in yolk, whether there is control over the amount of thyroid hormone deposition in yolk, and the effect(s) thyroid hormones may have on early avian embryonic development. It is the purpose of this study to investigate the transfer and availability of thyroid hormones in avian egg yolk, and the effect(s) of maternal thyroid hormones on early embryonic development, using Japanese quail as a study model.

The objectives of this study were: 1) to verify consistent recovery of thyroid hormones in extracts of avian egg yolk, and to verify the measurement of thyroid hormones in extracts by radioimmunoassay (RIA), 2) to determine if a laying hen deposits thyroid hormones in egg yolk in proportion to her own thyroid status, and 3) to determine if thyroid hormones in the yolk of fertilized eggs affect thyroid hormone-responsive developmental events before the embryonic thyroid gland is functional.

## MATERIALS AND METHODS

### *Animals*

Adult Japanese quail (*Coturnix japonica*), ages 8 to 12 months, from a random-bred colony, were paired and maintained under a 14L:10D photoperiod in 20" x 10" x 10" wire-mesh cages arranged in racks (4 across x 5 high). A commercial game bird ration (18 to 24% protein; Big Spring Mills, Shawsville, VA) and water were available *ad libitum*. Eggs were incubated at  $39 \pm 1^\circ\text{C}$  and > 90% relative humidity in a forced air incubator (Humidaire Hatchette Incubator; New Madison, OH). During experiments, laying hens were orally dosed with compounds by inserting a 2" piece of tygon tubing (I.D. = 4.7 mm; O.D. = 4.8 mm) down each bird's throat to the crop. This tubing was connected to a tuberculin syringe used to administer each measured dose.

### *Experimental Design*

*Maternal thyroid hormone deposition in yolk.* To determine the number of days thyroid hormones could potentially be deposited in the yolk of an individual egg, adult Japanese quail hens (n = 8) were dosed orally at 0800 hr for 10 days, alternating two lipid soluble dyes (technique modified from Bacon and Chermis, 1968). Either Sudan Black B or Sudan IV Red was administered (200  $\mu\text{l}$  of 0.01 g dye/ml vegetable oil; dyes from Sigma Chemical Co., St.

Louis, MO). Eggs were collected daily and boiled for 20 min. The shells were removed and the eggs were sliced longitudinally so that the resultant colored rings of yolk could be counted and photographed.

*Manipulation of hen thyroid status and egg thyroid hormone content.* To determine if hens deposited thyroid hormones in eggs relative to their own thyroid status and to obtain eggs with high thyroid hormone content, adult laying hens (n = 12 per treatment) were made hyperthyroid by an oral dose of L-thyroxine (T<sub>4</sub>; Sigma Chemical Co., St. Louis, MO) in 200 µl of 0.9% NaCl. In a preliminary experiment to determine a dosing protocol, blood from hens was collected prior to dosing and then every 3 hr for 12 hr after the 0800 hr dose of T<sub>4</sub>. The results of this experiment indicated T<sub>4</sub> needed to be administered twice daily (0800 hr and 1800 hr) to maintain elevated plasma thyroid hormone concentrations. For T<sub>4</sub>, dosage regimes were a multiple (either 1x or 3x) of the normal daily thyroid secretion rate of T<sub>4</sub> (TSR T<sub>4</sub>) for adult Japanese quail (2.78 µg/100 g body weight/day; Singh et al., 1967). Control hens were dosed with 200 µl of a saline vehicle (0.9% NaCl) at 0800 hr and 1800 hr. Blood was collected weekly from saline, 1x TSR T<sub>4</sub>, and 3x TSR T<sub>4</sub> dosed hens (3 to 4 hr after the morning dose in all cases). For the analysis of thyroid hormones in egg yolk, eggs were collected from individual saline and T<sub>4</sub> dosed hens on the day blood was sampled. Each egg was identified by the individual hen that laid it.

Eggs collected from saline and T<sub>4</sub> dosed hens were incubated until hatch. Chicks were visually inspected for gross morphology, weighed, measured (length in mm, crown to rump), and the approximate time of hatch recorded. Other eggs from the hens of each treatment were incubated and opened during development (days 7 and 15 for embryo and egg compartment analysis; days 9 and 14 for embryonic blood collection) to assess possible developmental effects resulting from increased yolk T<sub>4</sub> content.

To determine if hypothyroid hens produce thyroid hormone deficient eggs, and to attempt to obtain eggs with low thyroid hormone content, adult laying hens (n = 8) were given an oral dose of 4 mg of methimazole (a goitrogen that inhibits thyroid hormone synthesis; each dose in 200 µl 0.9% NaCl) at 0800 hr each day for 30 days. Because this treatment did not lower plasma thyroid hormone concentrations, the daily dose of goitrogen was increased to 8 mg/bird/day for 40 days. Soon after this dosage increase, the hens ceased laying. Because the hens showed no decrease in plasma thyroid hormone concentrations and ceased laying after the dosage increase, the experiment was aborted, the hens were sacrificed and weighed, and their thyroid glands removed and weighed.

### ***Sampling Techniques.***

Blood was collected from the brachial vein of laying hens and from the chorioallantoic artery of day 9 and day 14 embryos after removing each

embryo from its shell and separating it from the egg compartments. All samples were collected in heparinized capillary tubes, centrifuged (10 min, 6500 rpm, 25°C), and the plasma stored in heparinized capillary tubes at -20°C.

The eggs from saline and T4 dosed hens were collected each day blood was taken from the hens. Prior to incubation and/or on days 7 and 15 of incubation, the compartments (yolk, albumen, and allantois) and embryo of each egg were separated, weighed, homogenized, and the thyroid hormones extracted from a 0.5 g sample of homogenate.

Additional eggs from saline and T4 dosed hens were collected and incubated for the analysis of embryonic pelvic cartilage wet weight, dry weight, and alkaline phosphatase activity (used as a biochemical marker of differentiation). Embryos from days 6 to 12 of incubation were removed from eggs and immediately decapitated. Embryonic pelvic cartilage was dissected out of the embryos, connective tissue and muscle were removed, and the cartilage was blotted and weighed. To obtain the dry weight, pelvic cartilages were dried to constant weight at 37°C (18 to 24 hr). For alkaline phosphatase analysis, pelvic cartilages were homogenized in 0.9% NaCl (7X v/w) using a mortar and pestle, and the homogenate centrifuged (12,500 g, 4°C, 2 min). The post-mitochondrial supernatant fraction (PMF) was removed and stored at -20°C (method modified from Suvarna et al., 1993).

## ***Analytical Methods***

*Yolk thyroid hormone extraction procedure.* Thyroid hormones were extracted from egg yolk using a modification of a methanol/chloroform extraction procedure used by Denver (1993) to extract thyroid hormones from amphibian larvae. Egg yolk was separated from the shell and albumen, weighed, diced with scissors, and homogenized using a 50 cc glass syringe (18g, 1.5 inch needle; each yolk was drawn into and expelled from the syringe 3 times). A 0.5 g sample of homogenized yolk was transferred to a 17 x 100 mm polypropylene test tube, and 2 ml of methanol containing 1 mM PTU (propylthiouracil; an inhibitor of the deiodination of T<sub>4</sub>) were added to the yolk homogenate. Labeled thyroid hormone (approximately 2000 cpm of either <sup>125</sup>T<sub>3</sub> or <sup>125</sup>T<sub>4</sub>; high specific activity: T<sub>3</sub>, 1200 μCi/μg; T<sub>4</sub>, 1250 μCi/μg New England Nuclear; Boston, MA) was added, the sample vortexed, and counted for 10 min. After extraction for 10 min on a multi-tube shaker (150 oscillations/min), the sample was centrifuged (2400 rpm, 4°C, 10 min) and the supernatant was decanted into a 50 ml graduated conical polypropylene tube. The remaining yolk precipitate was resuspended in 1 ml of methanol (with 1 mM PTU), the thyroid hormones were again extracted by further mixing on a multi-tube shaker, the sample centrifuged, and the resultant supernatant decanted into a second 50 ml conical tube. The two separate supernatants each received 5.0 ml of chloroform (CHCl<sub>3</sub>) and 0.5 ml ammonium hydroxide

(2N NH<sub>4</sub>OH), and were shaken and centrifuged as above; the upper phase (methanol/aqueous phase) from both tubes was removed and placed in a single 17 x 100 mm polypropylene tube. To the CHCl<sub>3</sub> phase remaining in each 50 ml tube, 0.5 ml 2N NH<sub>4</sub>OH was added and the solution was shaken and centrifuged. The upper phase from each tube was removed and added to the methanol/aqueous pool for each sample, which was then dried under a filtered air stream (for 6 to 7 hr) in a fume hood. When dry, the sample was resuspended in 1 ml 2N NH<sub>4</sub>OH, briefly vortexed, shaken, centrifuged (as above), and the supernatant decanted into a clean 17 x 100 mm polypropylene tube. The general extraction procedure was then repeated with 1 ml CHCl<sub>3</sub>, the upper phase removed and dried under forced air, resuspended in 150 µl 75% EtOH, and counted (10 min) to determine extraction efficiency of the trace amount of labeled thyroid hormones added to the original homogenate. Recovery studies demonstrated consistent extraction efficiency for thyroid hormones: 63 ± 1% (mean, ± SE) for <sup>125</sup>I-T<sub>4</sub> and 61 ± 3% for <sup>125</sup>I-T<sub>3</sub> (n = 6 for each hormone). These recoveries are comparable to those found in the literature from similar extraction techniques for yolk or tissue thyroid hormones [45 to 70% recovery: Tagawa and Hirano (1990) fish; Niinuma et al. (1991), amphibian; Denver (1993), amphibian]. Individual recoveries were determined for all samples and used to calculate thyroid hormone concentrations. The reconstituted extracts were stored at -20°C until analysis.

The extraction of thyroid hormones from albumen and embryonic allantois was done following the procedure described above. For the extraction of thyroid hormones from embryos, each sample was prepared by decapitating the embryo, removing the area containing the thyroid gland, and homogenizing the carcass (minus the thyroid gland) in a volume of methanol (with 1 mM PTU) twice the embryo's body weight using a Brinkman tissue homogenizer (model PT 10/35; 110 Volts, 6.3 Amps, 60 Hz; model PTA 10 generator, saw tooth; Westbury, NY). After homogenization, the blades were washed with an equivalent volume of methanol (with 1 mM PTU) which was then added to the sample homogenate. Thyroid hormones in the embryonic sample were then extracted in the manner described above for yolk.

*Thyroid hormone radioimmunoassays (RIAs).* Plasma T<sub>3</sub> and T<sub>4</sub> concentrations were measured with a double antibody RIA by the method of McNabb and Hughes (1983) using hormone standards prepared in hormone-free chicken plasma and previously verified for use on quail plasma. Primary antibodies were purchased from Endocrine Sciences (Calabasas, CA), <sup>125</sup>T<sub>4</sub> and <sup>125</sup>T<sub>3</sub> were from New England Nuclear (Boston, MA; high specific activity: T<sub>3</sub>, 1200 μCi/μg; T<sub>4</sub>, 1250 μCi/μg), and carrier immunoglobulin was from Antibodies Incorporated (Davis, CA). Secondary antibody was kindly provided by Dr. John McMurtry (USDA; Beltsville, MD). Assay volumes were 12.5 μl for T<sub>4</sub> and 25 μl for T<sub>3</sub>. Precision tests showed ± 2 SE was 9.4% of the mean for T<sub>4</sub> (n

=10) and 9.6% of the mean for T<sub>3</sub> (n = 10). Lower limits of assay sensitivity were 1.25 ng/ml for T<sub>4</sub> and 0.125 ng/ml for T<sub>3</sub>.

Concentrations of T<sub>3</sub> and T<sub>4</sub> in extracts of yolk, albumen, allantois, and embryonic tissue were determined using RIAs. Hormone standards for the RIAs on extracts were made with 75% ethanol, the solvent used for the final dissolution of the dried hormone extract. For validation of each RIA, samples from a pooled yolk extract (n = 6) were diluted with 75% ethanol or spiked with hormone standards prepared in 75% ethanol. Precision tests performed on yolk extracts show  $\pm 2$  SE was 13.7% of the mean for T<sub>4</sub> (n = 10) and 14.8% of the mean for T<sub>3</sub> (n = 8). There was good correspondence between the measured and predicted regression lines in both the T<sub>3</sub> and T<sub>4</sub> RIAs on yolk samples, indicating consistent measurement of hormone concentrations over this range of concentrations. A Thiel-Sen test showed no significant difference between the slopes of the measured and predicted (slope of 1.0) lines for both RIAs (T<sub>4</sub>: k = 3, p = 0.431, Fig. 1a; T<sub>3</sub>: k = 9.0, p = 0.376, Fig. 1b).

*Alkaline phosphatase analysis.* An alkaline phosphatase assay was verified for measuring enzymatic activity in embryonic pelvic cartilage PMF by demonstrating linearity of enzyme activity with time, and by proportionality between enzymatic activity and PMF for cartilages from 6, 9, and 12 day embryos. Once the assay had been verified, pelvic cartilage PMF diluted with 0.9% NaCl (10  $\mu$ l PMF to 10  $\mu$ l 0.9% NaCl for days 7 and 8; 1  $\mu$ l PMF to 19  $\mu$ l 0.9%

NaCl for days 8 to 12) was added to 300  $\mu$ l of alkaline phosphatase reagent (Sigma Diagnostic Alkaline Phosphatase Components Kit; Sigma Chemical Co., St. Louis, MO) in a narrow light path cuvette, the solution was mixed by rapid shaking, and absorbance readings were taken at 0 and 30 min. The hydrolysis of phosphate by alkaline phosphatase converts the substrate p-nitrophenyl phosphate into p-nitrophenol and inorganic phosphate (Sigma Diagnostics, 1990) resulting in a color change which was measured using a Beckman DU-640 spectrophotometer (25°C,  $\lambda$  = 405 nm). Precision of this technique on 10 aliquots of a pooled sample of pelvic cartilage PMF showed that  $\pm$  2 SE was 8.3% of the mean. A unit of alkaline phosphatase activity is defined as “that amount of alkaline phosphatase which will produce one  $\mu$ mol of p-nitrophenol per min at 25°C” (Sigma Diagnostics, 1990).

*Statistical Analyses.* Student’s t-tests were used to evaluate the effects of treatments on yolk and plasma thyroid hormones. A Theil-Sen simple linear regression procedure was used to compare the slope of regression lines to a slope of 0 and a slope of 1 (the Theil-Sen test statistic is reported as k). Pelvic cartilage wet and dry weights expressed as a percentage of individual embryonic body weight were normally distributed (Shapiro-Wilks test for normality); the effects of treatments on embryonic pelvic cartilages were compared by ANOVA (general linear models procedure). A value of  $p \leq 0.05$  was used to statistical significance.

## RESULTS

*Time Course of Dye Deposition in Egg Yolk.* Eggs from hens given alternating doses of two lipid-soluble dyes showed alternating rings of dye in egg yolk. The outermost ring corresponded to the color of dye administered the day before the egg was laid. For hens that laid one egg each day, 5 to 6 alternating, colored rings were present in each egg yolk after 5 to 10 days of dosing with the dyes. Examples are shown in Table 1.

*The Relationship Between the Thyroid Status of Hens and Their Egg Thyroid Hormone Content.* The administration of T<sub>4</sub> to hens produced a hyperthyroid condition (increased hen plasma T<sub>4</sub>) and elevated the yolk T<sub>4</sub> content in their eggs. Hens dosed with 1x TSR T<sub>4</sub> or 3x TSR T<sub>4</sub> had plasma T<sub>4</sub> concentrations 8 to 15-fold those of controls by 3 hr after the administration of the initial dose, but by 9 to 12 hr after the initial dose, the plasma T<sub>4</sub> concentration in these hens had dropped 2 to 3-fold. Therefore, hens were dosed with T<sub>4</sub> twice daily (0800 and 1800 hr) to sustain a high plasma T<sub>4</sub> concentration over a 24 hr period.

The plasma T<sub>4</sub> concentrations of hens dosed twice daily with 1x TSR T<sub>4</sub> were significantly elevated over controls (4-fold), but their plasma T<sub>3</sub> concentrations did not differ from controls. Yolk from eggs of 1x TSR T<sub>4</sub> dosed hens had significantly higher T<sub>4</sub> concentrations (6.4-fold) and T<sub>3</sub> concentrations (1.3-fold) compared to yolk from eggs of control hens (Fig. 2). The plasma T<sub>4</sub>

and T<sub>3</sub> concentrations of hens dosed twice daily with 3x TSR T<sub>4</sub> were significantly elevated over those of controls (T<sub>4</sub>: 12-fold; T<sub>3</sub>: 1.8-fold). Yolk from eggs of 3x TSR T<sub>4</sub> dosed hens had significantly higher T<sub>4</sub> concentrations (22-fold) and T<sub>3</sub> concentrations (1.9-fold) than yolk from eggs of control hens (Fig 2).

Because hens made hyperthyroid lay eggs with very high concentrations of T<sub>4</sub> in the yolk, the relationship between hen plasma T<sub>4</sub> concentrations and yolk T<sub>4</sub> concentrations was investigated. Yolk T<sub>4</sub> concentrations did not increase in simple proportion to increases in hen plasma T<sub>4</sub> concentrations [i.e., the slope of the Theil-Sen regression line for yolk versus hen plasma T<sub>4</sub> concentrations was significantly less than a slope of 1.0 for each treatment group ( $p = 0.00001$ ); Fig. 3b, c, and d]. Instead, yolk T<sub>4</sub> concentrations showed a stepwise pattern of increase with increasing T<sub>4</sub> treatment of the hens (Fig. 3a). When the slopes of the Theil-Sen regression lines for yolk versus hen plasma T<sub>4</sub> concentrations for each treatment group were compared to a slope of 0, the slopes for the control and 3x TSR T<sub>4</sub> groups were statistically greater than a slope of 0 ( $p = 0.038$  and  $0.010$ , respectively).

For the control hens, variation in T<sub>4</sub> content among the eggs laid by individual hens (mean of the standard deviations = 1.67,  $n = 4$ ) was similar to the variation in T<sub>4</sub> content of eggs laid by different hens (std dev = 1.74,  $n = 6$ ). The variation in high T<sub>4</sub> eggs increased with the increase in the T<sub>4</sub> dose given to the hens (1x TSR T<sub>4</sub>, mean of the std dev = 9.05,  $n = 4$ ; 3x TSR T<sub>4</sub>, mean of the std

dev = 34.54, n = 4). These variations were similar to the variation in T<sub>4</sub> content in eggs from different hens for each T<sub>4</sub> treatment (1x TSR T<sub>4</sub> treated hens, std dev = 7.45, n = 5; 3x TSR T<sub>4</sub> treated hens, std dev = 40.20, n = 6).

The administration of methimazole at 4 mg/day for one month and 8 mg/day for a second month did not significantly decrease hen plasma thyroid hormone concentrations compared to controls. However, the thyroid glands of the methimazole treated hens were significantly heavier than those from control hens (thyroid gland weights as a percentage of hen body weight: methimazole treatment =  $9.13 \times 10^{-4}\% \pm 1.3 \times 10^{-6}\%$ ; saline treatment =  $1.02 \times 10^{-4}\% \pm 1.6 \times 10^{-5}\%$ ;  $p = 0.0001$ ). Yolk of eggs laid by hens receiving either the 4 mg/day or 8 mg/day methimazole did not differ significantly in the concentration of either thyroid hormone from the yolk of eggs laid by control hens. Egg laying in methimazole dosed hens was sporadic during the 4 mg dose and ceased soon after the start of the 8 mg dose.

***Changes in Weight and Thyroid Hormone Content of Embryos and Egg Compartments During Development.*** The total weight of the embryo, albumen, and allantois on days 0, 7, and 15 did not differ in control and high T<sub>4</sub> eggs from hens which received either T<sub>4</sub> dose. The weight of the embryo and egg compartments changed in the directions expected during development: embryonic body weight increased 9-fold from day 7 to day 15, albumen weight decreased 2.8-fold from day 7 to day 15, and yolk weight increased 1.4-fold

from day 0 to 7, then decreased 3-fold by day 15. This pattern of yolk weight increase followed by a decrease occurs as a result of influx of water into the yolk from the albumen, followed by the uptake of yolk by the embryo during the latter part of embryonic development (Romanoff, 1967). Allantoic weight did not differ in control and high T<sub>4</sub> eggs on day 7 and could not be dissected for measurement on day 15.

The concentration of T<sub>4</sub> and T<sub>3</sub> in the plasma of day 9 embryos was below detection (< 1.25 and 0.125 ng/ml, respectively) in control and high T<sub>4</sub> eggs. Both thyroid hormones were detectable in the plasma of day 14 embryos, but there was no significant difference in the plasma concentration of either hormone in embryos from control and high T<sub>4</sub> eggs (Fig. 4).

Yolk T<sub>4</sub> content of high T<sub>4</sub> eggs decreased dramatically during incubation. Although initially the T<sub>4</sub> content in eggs from the 3x TSR T<sub>4</sub> dosed hens was 26.5-fold and those from the 1x TSR T<sub>4</sub> dosed hens was 7.5-fold greater than controls, by day 15 of incubation, the yolk T<sub>4</sub> content in eggs from 1x TSR T<sub>4</sub> treated hens did not differ from controls. Yolk T<sub>4</sub> content in eggs from 3x TSR T<sub>4</sub> dosed hens was similar to (1.9-fold that of controls), but significantly different from, controls ( $p = 0.002$ ; Fig. 5a).

The T<sub>4</sub> and T<sub>3</sub> contents of embryos (with the thyroid glands removed) from control and high T<sub>4</sub> eggs were very low, but detectable, on day 7 of incubation, and were not significantly different in control and high T<sub>4</sub> eggs. By day 15, the embryonic T<sub>4</sub> and T<sub>3</sub> content had increased significantly from day 0

(T<sub>4</sub>, ≈ 60-fold; T<sub>3</sub>, ≈ 25-fold), but did not differ in control and high T<sub>4</sub> eggs (Fig. 5b).

Before incubation, the T<sub>4</sub> content of albumen from eggs of 3x TSR T<sub>4</sub> treated hens was significantly higher than in albumen from eggs of control and 1x TSR T<sub>4</sub> dosed hens (which did not differ significantly); the T<sub>3</sub> content of albumen did not differ in control and high T<sub>4</sub> eggs. The T<sub>4</sub> content of the albumen was 21.4% of the total T<sub>4</sub> content in unincubated eggs from control hens, but only 4.9% and 4.4% of the total T<sub>4</sub> content in unincubated eggs from 1x TSR T<sub>4</sub> and 3x TSR T<sub>4</sub> dosed hens, respectively. On day 7, the thyroid hormone content of egg albumen remained significantly higher in eggs from 3x TSR T<sub>4</sub> dosed hens than in eggs from control and 1x TSR T<sub>4</sub> dosed hens.

The allantois was sampled from control and high T<sub>4</sub> eggs on day 7 only. The T<sub>4</sub> content in the embryonic allantois of eggs from 1x TSR T<sub>4</sub> dosed hens was significantly higher than that from control and 3x TSR T<sub>4</sub> dosed hens, but did not differ in eggs from control and 3x TSR T<sub>4</sub> dosed hens. There were no significant differences in allantoic T<sub>3</sub> content in control and high T<sub>4</sub> eggs. The T<sub>4</sub> content of the allantois on day 7 was 4.1%, 3.3%, and 0.5% of the total T<sub>4</sub> content in unincubated eggs from control, 1x TSR T<sub>4</sub>, and 3x TSR T<sub>4</sub> dosed hens, respectively.

***Embryonic Development.*** Chicks hatched from control and high T<sub>4</sub> eggs (from hens on either T<sub>4</sub> dosage) showed no significant differences in gross morphology, crown to rump length, or body weight. Hatchability of chicks

from control and high T<sub>4</sub> eggs was similar (0.9% NaCl = 75%, 1x TSR T<sub>4</sub> = 68%; 0.9 % NaCl = 65%, 3x TSR T<sub>4</sub> = 66%; n = 50 eggs per treatment). However, chicks from eggs of control hens began to hatch several hours earlier than those from eggs of 1x TSR T<sub>4</sub> and 3x TSR T<sub>4</sub> dosed hens.

In eggs from hens dosed with 3x TSR T<sub>4</sub>, embryonic pelvic cartilage wet weights, dry weights, and pelvic cartilage to body weight ratios were significantly greater than controls overall when compared by analysis of variance ( $p = 0.038$  and  $0.032$ , for wet and dry weights, respectively;  $p = 0.013$  and  $0.002$  for weight ratios, respectively; Fig. 6a and b). However, the increased yolk T<sub>4</sub> content in eggs from 1x TSR T<sub>4</sub> dosed hens did not significantly affect these variables.

The alkaline phosphatase activity in the pelvic cartilages of embryos from eggs of 3x TSR T<sub>4</sub> dosed hens was significantly higher than controls overall when compared by analysis of variance ( $p = 0.0045$ ; Fig 7b). There appears to be a divergence from controls in embryos from 3x TSR T<sub>4</sub> dosed hens on days 9 to 12 of incubation. The alkaline phosphatase activity of pelvic cartilage of embryos from 1x TSR T<sub>4</sub> dosed hens did not differ from controls.

## DISCUSSION

Two preliminary studies were conducted (1) to determine the frequency of thyroid hormone dosing required to study the effects of alterations in hen thyroid status on yolk thyroid hormone content, and (2) to determine the length of time a hen deposits thyroid hormones into each egg. Dosing hens twice daily (0800 and 1800 hr) with T<sub>4</sub> was needed to maintain sustained high plasma T<sub>4</sub>. Laying hens deposited yolk into each developing ovum for 5 to 6 days prior to oviposition, as indicated by alternating colored rings in hard-boiled egg yolks from hens dosed with two lipid-soluble dyes on alternate days (Table 1). These findings are consistent with those of Bacon and Koontz (1971), who reported yolk deposition for a period of  $6.1 \pm 1.1$  days during rapid follicular development in Japanese quail.

In addition to the present study, several previous studies of chicken egg yolk have suggested that thyroid hormones of maternal origin are transferred into the egg prior to laying [Hilfer and Searls (1980), Sechman and Bobeck (1988), and Prati, et al. (1992)]. The T<sub>4</sub> and T<sub>3</sub> concentrations found in the yolk of Japanese quail eggs were similar to those reported by Prati et al. (1992) and Sechman and Bobeck (1988) for chicken eggs (Table 2). These three studies all separated/extracted thyroid hormones from yolk in some way, whereas Hilfer and Searls (1980) used yolk samples directly in the RIAs. Thus, the high thyroid hormone concentrations reported by Hilfer and Searls (1980) may have been

inflated as a result of lipid interference with thyroid hormone-to-primary antibody binding. Such interference can decrease the bound fraction of radiolabeled thyroid hormones and thereby inflate hormone readings by the assays.

To my knowledge, this is the first report that addresses the relationship between the hen's thyroid status and the deposition of thyroid hormones in her eggs. Overall, the data show that hyperthyroid hens, with high concentrations of T<sub>4</sub> in their plasma, deposit increased amounts of maternal thyroid hormones in the yolk of their eggs. However, when the yolk T<sub>4</sub> concentration of each egg is plotted against the plasma T<sub>4</sub> concentration of the hen during the period just prior to laying the egg, there is evidence of some regulation of thyroid hormone deposition. Thus, within each treatment, hens regulated yolk T<sub>4</sub> concentrations independent of variations in their plasma T<sub>4</sub> concentrations (see Fig. 3).

My attempts to study the effects of hen hypothyroidism on yolk T<sub>4</sub> content suggest that thyroid hormone deficient eggs may not be produced. Laying hens were very resistant to the effects of the goitrogen, methimazole, and there was no significant reduction in the thyroid hormone concentrations in either their plasma or egg yolk. Assuming that the goitrogen treatment decreased thyroid hormone synthesis, it appears that thyroid hormone reserves in the thyroid gland were sufficient to meet the needs of the hen's tissues and supply thyroid hormones for deposition in the eggs. When the methimazole

treatment was doubled (second month), plasma thyroid hormone concentrations remained in the normal range (despite thyroid gland hypertrophy suggesting low thyroid hormone concentrations), but egg laying became sporadic and quickly ceased. These results suggest the possible adaptive significance for the cessation of egg laying, rather than the production of eggs with low maternal thyroid hormone content.

Potential mechanism(s) for the transfer of thyroid hormones from the hen to the yolk have been addressed. Mitchell and colleagues (Mitchell, 1984; Mitchell et al., 1985; Mitchell and Stiles, 1985) demonstrate that lipoproteins in the plasma of chicken hens bind small but significant amounts of plasma thyroid hormones and are responsible for the transport of these hormones into the yolk of developing follicles. This proposed mechanism of transfer seems plausible because low-density lipoproteins are synthesized by the liver and deposited in oocytes during follicle development (Griffin et al., 1984), and the lipoproteins found in hen plasma and egg yolk are very similar (Griffin et al., 1984). Vitellogenin also binds thyroid hormones in hen plasma and binds to receptors on ovarian membranes in chickens (Barber, et al., 1991). A recent study by Vieira et al. (1995) demonstrates that transthyretin (thyroxine binding prealbumin) found in egg yolk is derived from the hen's plasma. Further results from this study indicate the presence of a putative transthyretin receptor on the oocyte membrane. Therefore, it is probable that one or more plasma binding proteins are responsible for the transfer of maternal thyroid hormones

into the egg and could be important in regulating thyroid hormone concentrations in the yolk.

Embryonic uptake of maternal thyroid hormones from yolk prior to the onset of appreciable thyroid gland function in birds was addressed in this study and has been addressed previously in chicken embryos (Prati et al. 1992). Data from the current study indicate that 7-day quail embryos contained small amounts of both T<sub>4</sub> and T<sub>3</sub>. However, 7-day embryos from high T<sub>4</sub> eggs did not appear to take up greater than normal amounts of T<sub>4</sub>. At this stage of development, the embryonic thyroid gland contains only small amounts of thyroid hormones (day 8 of the 16.5 day incubation: 1.7 ng T<sub>4</sub>/gland pair and 1.5 ng T<sub>3</sub>/gland pair; McNichols and McNabb, 1988) compared to the amount of T<sub>4</sub> in the yolk of eggs from 3x TSR T<sub>4</sub> dosed hens ( $\approx$  400 ng/yolk; this study; Fig. 5). Therefore, a difference in the total embryonic T<sub>4</sub> content should have been apparent on day 7 if greater than normal amounts of T<sub>4</sub> were taken up by embryos of high T<sub>4</sub> eggs and retained by the embryos in this hormone form.

Very high T<sub>4</sub> content in Japanese quail eggs (3x TSR T<sub>4</sub> dose to hens) accelerated the differentiation and growth of embryonic pelvic cartilages, a tissue previously shown to be thyroid hormone responsive (Burch and Lebovitz, 1982). Alkaline phosphatase activity measured in pelvic cartilages from 6 to 12 day embryos indicated a significant acceleration of cartilage differentiation in high T<sub>4</sub> eggs versus controls (Fig. 7b). Significantly increased pelvic cartilage wet and dry weights, as percentages of embryonic body weight,

provided evidence that cartilage growth also was accelerated by high yolk T<sub>4</sub> availability (Fig 6a and b). In contrast to these effects on pelvic cartilage, high T<sub>4</sub> content in eggs did not affect hatchability, body length, body weight, or the gross morphological appearance of quail chicks at hatch. This result is not surprising because thyroid hormones appear to be permissive with respect to the stimulation of body growth, and there is no simple relationship between growth and thyroid hormone concentrations (McNabb and King, 1993).

It is generally thought that most thyroid hormone action in birds is triggered by T<sub>3</sub>, as is the case in mammals (McNabb and King, 1993). Thyroxine in the yolk of high T<sub>4</sub> eggs might be deiodinated in the yolk or in embryonic tissues to T<sub>3</sub>, to inactive reverse-T<sub>3</sub>, or to one or both of these hormones, followed by further deiodinations. The conversion of T<sub>4</sub> to T<sub>3</sub> in the yolk was not suggested by any of the data in this study except for a small (but significant) increase in total yolk T<sub>3</sub> in eggs from 3x TSR T<sub>4</sub> dosed hens on day 7 of incubation (see Fig. 5a, lower panel). However, this increase in yolk T<sub>3</sub> is not reciprocal to the dramatic decrease in yolk T<sub>4</sub> from day 0 to 7 of incubation. The possibility of deiodinase activity in egg yolk has not been investigated in egg producing vertebrate species, so that this possibility cannot be ruled out.

Increased deiodination of T<sub>4</sub> to T<sub>3</sub> within pelvic cartilage of embryos from high T<sub>4</sub> eggs would be consistent with the acceleration of cartilage development demonstrated in this study. However, chicken embryos (based on studies of the liver) at comparable ages have little or no T<sub>4</sub> to T<sub>3</sub>

conversions; most T<sub>4</sub> is converted to reverse-T<sub>3</sub> (Borges et al., 1980; Galton and Hiebert, 1987; Darras et al., 1992).

The data from this study demonstrate that very high concentrations of maternal T<sub>4</sub> in egg yolk can accelerate both the growth and differentiation of thyroid hormone-responsive embryonic tissues (e.g. pelvic cartilage). Therefore, these data provide a basis for addressing additional questions about maternal transfer of thyroid hormones into eggs and the possible effects of these maternal hormones on growth and differentiation of the embryo.

The mechanism(s) by which maternal thyroid hormones are deposited in egg yolk remains unclear; plasma binding proteins such as transthyretin are clearly involved in this transfer (Vieira et al., 1995). Thus, the apparent regulation of T<sub>4</sub> in the egg yolk by hens in the current study could reflect modulation of individual circulating thyroid hormone concentrations by different levels of plasma binding proteins within different levels of thyroid hormone status. The simple diffusion of thyroid hormones from the hen's plasma to the developing oocyte in the case of high plasma T<sub>4</sub> concentrations in hens also may be a possibility and cannot be ruled out.

The pelvic cartilage data from this study demonstrate that the development of at least one thyroid hormone-responsive tissue in the embryo is influenced by maternal thyroid hormones from the yolk. However (1) the extent to which maternal thyroid hormones in the yolk are available to the embryo during early development, (2) the possibility and/or extent of

deiodination of T<sub>4</sub> in the yolk and in the early embryo, and (3) the mechanism(s) of transfer of maternal thyroid hormones from the yolk to the embryo (whether by simple diffusion down a concentration gradient, or regulated by a transport protein such as transthyretin) remain unclear.

## LITERATURE CITED

- Bacon, W. L.; and F. L. Chermis. 1968. Ovarian follicular growth and maturation in the domestic turkey. *Poultry Sci.* 47:1303-1314.
- Bacon, W. L.; and M. Koontz. 1971. Ovarian follicular growth and maturation in Coturnix quail. *Poultry Sci.* 50:233-236.
- Bacon, W. L.; and J. H. Skala. 1968. Ovarian follicular growth and maturation in laying hens and the relation to egg quality. *Poultry Sci.* 47:1437-1442.
- Barber, D. L.; Sanders, E. J.; Aebersold, R.; and W. J. Schneider. 1991. The receptor for yolk lipoprotein deposition in the chicken oocyte. *J. Biol. Chem.* 266(28):18761-18770.
- Berry, M. A.; and P. R. Larsen. 1992. The role of selenium in thyroid hormone action. *Endocrine Rev.* 13(2):207-219.
- Borges, M.; Eisenstein, Z.; Burger, A. G.; and S. H. Ingbar. 1981. Immunosequestration: A new technique for studying peripheral iodothyronine metabolism in vitro. *Endocrinology* 108:1665-1671.
- Borges, M.; LaBourene, J.; and S. H. Ingbar. 1980. Changes in hepatic iodothyronine metabolism during ontogeny of the chick embryo. *Endocrinology* 107:1751-1761.
- Brown, C. L.; Doroshov, S. I.; Nunez, J. M.; Hadley, C.; Vaneennaam, J.; Nishioka, R. S.; and H. A. Bern. 1988. Maternal triiodothyronine injections cause increases in swimbladder inflation and survival rates in larval striped bass, *Morone saxatilis*. *J. Experiment. Zool.* 248:168-176.
- Brown, C. L.; Sullivan, C. V.; Bern, H. A.; and W. W. Dickhoff. 1987. Occurrence of thyroid hormones in early developmental stages of teleost fish. *Am. Fisheries Soc. Symp.* 2:144-150.
- Burch, W. M.; Corda, G.; Kopchick, J. J.; and F. C. Leung. 1985. Homologous and heterologous growth hormones fail to stimulate avian cartilage growth in vitro. *J. Clin. Endocrinol. Metab.* 60:747-750.
- Burch, W. M.; and H. E. Lebovitz. 1982. Triiodothyronine stimulation of in vitro growth and maturation of embryonic chick cartilage. *Endocrinology* 111: 462-468.

- Burch, W. M.; and J. J. Van Wyk. 1987. Triiodothyronine stimulates cartilage growth and maturation by different mechanisms. *Am. J. Physiol.* 252: E176-E182.
- Darras, V. M.; Visser, T. J.; Berghmans, L. R.; and E. R. Kühn. 1992. Ontogeny of type I and type II deiodinase activities in embryonic and posthatch chicks: relationship with changes in plasma triiodothyronine and growth hormone levels. *Comp. Biochem. Physiol.* 103:131-36.
- Davison, T. F.; Flack, I. H.; and E. J. Butler. 1978. The binding of thyroxine and tri-iodothyronine to plasma proteins in the chicken at the physiological pH. *Res. Vet. Sci.* 25:280-283.
- Denver, R. J. 1993. Acceleration of anuran amphibian metamorphosis by corticotrophin-releasing hormone-like peptides. *Gen. Comp. Endocrinol.* 91:38-51.
- Farer, L. S.; Robbins, J.; Blumber, B. S.; and J. E. F. Rall. 1962. Thyroxine-serum protein complexes in various animals. *Endocrinology* 70:679-696.
- Fisher, D. A.; Dussault, J. H.; Stack, J.; and I. J. Chopra. 1977. Ontogenesis of hypothalamic-pituitary-thyroid function and metabolism in man, sheep, and rat. *Rec. Progr. Horm. Res.* 33:59-116.
- Galton, V. A.; and A. Hiebert. 1987. The ontogeny of the enzyme systems for the 5'- and 5-deiodination of thyroid hormones in chick embryo liver. *Endocrinology* 120:2604-10.
- Gorbman, A.; Dickhoff, W. W.; Vigna, S. R.; Clark, N. B.; and C. L. Ralph. 1983. The thyroid gland. In *Comparative endocrinology*. pp. 185-276. John Wiley & Sons. New York, NY.
- Grau, C. R. 1976. Ring structure of avian egg yolk. *Poultry Sci.* 55:1418-1422.
- Griffin, H. D.; Perry, M. M.; and A. B. Gilbert. 1984. In *Physiology and biochemistry of the domestic fowl*. Vol. 5. pp. 345-380. New York: Academic Press.
- Hall, B. K. 1973. Thyroxine and the development of the tibia in the embryonic chick. *Anat. Rec.* 176:49.
- Hilfer, S. R.; and R. L. Searls. 1980. Differentiation of the thyroid in the hypophysectomized chick embryo. *Dev. Bio.* 79:107-118.

- Hughes, T. E.; and F. M. A. McNabb. 1986. Avian hepatic T3 production by two pathways of 5<sup>1</sup>-monodeiodination: effects of fasting and patterns during development. *J. Exp. Zool.* 238:393-99.
- Johnson, A. L. In Press. The avian ovarian hierarchy: a balance between follicle differentiation and atresia. *Poult. Avian Rev.*
- King, D. B.; and J. D. May. 1984. Thyroidal influence on body growth. *J. Exp. Zool.* 232:453-460.
- Kobuke, L.; Specker, J. L.; and H. A. Bern. 1987. Thyroxine content of eggs and larvae of coho salmon, *Oncorhynchus kisutch*. *J. Experiment. Zool.* 242: 89-94.
- Legrand, J. 1986. Thyroid hormone effects on growth and development. In *Thyroid hormone metabolism*. G. Hennemann (ed). pp. 503-534. New York: Marcell Dekker, Inc.
- Leonard, J. L.; and T. J. Visser. 1986. Biochemistry of deiodination. In *Thyroid hormone metabolism*. G. Hennemann (ed). pp. 189-229. New York: Marcell Dekker, Inc.
- McNabb, F. M. A. 1988. Peripheral thyroid hormone dynamics in precocial and altricial avian development. *Amer. Zool.* 28:427-40.
- McNabb, F. M. A. 1989. Development and aging of the thyroid in homeotherms. In *Development, maturation, and senescence of neuroendocrine systems*. pp. 333-351. New York: Academic Press.
- McNabb, F. M. A. 1992. *Thyroid hormones*. New Jersey: Printice Hall.
- McNabb, F. M. A.; and T. E. Hughes. 1983. The role of serum binding proteins in determining free thyroid hormone concentrations during development in quail. *Endocrinology* 113:957-963.
- McNabb, F. M. A.; and D. B. King. 1993. Thyroid hormone effects on growth, development, and metabolism. In *The endocrinology of growth, development, and metabolism in vertebrates*. pp. 393-417. New York: Academic Press.

- McNabb, F. M. A.; Stanton, F. W.; Weirich, R. T.; and T. E. Hughes. 1984. Responses to thyrotropin during development in Japanese quail. *Endocrinology* 114:1238-1244.
- McNabb, F. M. A.; Weirich, R. T.; and R. A. McNabb. 1981. Thyroid function in embryonic and perinatal Japanese quail. *Gen. Comp. Endocrinol.* 43:218-226.
- McNabb, R. A.; Stouffer, R. L.; and F. M. A. McNabb. 1972. Thermoregulatory ability and the thyroid gland: their development in embryonic Japanese quail (*Coturnix coturnix japonica*). *Comp. Biochem. Physiol.* 43A:187-193.
- McNichols, M. J.; and F. M. A. McNabb. 1988. Development of thyroid function and its pituitary control in embryonic and hatchling precocial Japanese quail and altricial Ring doves. *Gen. Comp. Endocrinol.* 69:109-118.
- Mitchell, M. A. 1984. Binding of tri-iodothyronine and thyroxine to  $\beta$ -lipoprotein in chicken plasma. *J. Physiol.* 343:86P.
- Mitchell, M. A.; and M. S. Stiles. 1985. Some new observations on the binding of thyroxine and triiodothyronine to plasma proteins and lipoproteins in the domestic fowl. *Gen. Comp. Endocrinol.* 57: 309-319.
- Mitchell, M. A.; Raza, A.; and P. Uglow. 1985. Transfer of maternal thyroid hormones to the embryo in the domestic fowl. *J. Physiol.* 360:74P.
- Morreale de Escobar, G.; Obregon, M. J.; Ruiz de Ona, C.; and F. Escobar del Rey. 1988. Transfer of thyroxine from the mother to the rat fetus near term: effects on brain 3,5,3'-triiodothyronine deficiency. *Endocrinology* 122:1521-1531.
- Morreale de Escobar, G.; Obregon, M. J.; Ruiz de Ona, C.; and F. Escobar del Rey. 1989. Comparison of maternal to fetal transfer of 3,5,3'-triiodothyronine versus thyroxine in rats, as assessed from 3,5,3'-triiodothyronine levels in fetal tissue. *Acta Endocrinol.* 120:20-30.
- Morreale de Escobar, G.; Pastor, R.; Obregon, M. J.; and F. E. Del Rey. 1985. Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function. *Endocrinology* 117: 1890-1900.

- Niinuma, K.; Tagawa, M.; Hirano, T.; and S. Kikuyama. 1991. Changes in tissue concentrations of thyroid hormones in metamorphosing toad larvae. *Zool. Sci.* 8:345-350.
- Nimph, J.; Radosavljevic, M. J.; and W. J. Schneider. 1989. Oocytes from the mutant restricted ovulator hen lack receptor for very low density lipoprotein. *J. Biol. Chem.* 264(3):1393-1398.
- Nunez, J. 1984. Effects of thyroid hormones during brain differentiation. *Mol. Cell. Endocrinol.* 37:125-132.
- Oppenheimer, J. H.; Schwartz, H. L.; Mariash, C. N.; Kinlaw, W. B.; Wong, N. C. W.; and H. C. Freaque. 1987. Advances in our understanding of thyroid hormone action at the cellular level. *Endocrine Rev.* 8:288-308.
- Padgett, C. S.; and W. D. Ivey. 1960. The normal embryology of the Coturnix quail. *Anat. Rec.* 137:1-11.
- Prati, M.; Calvo, R.; and G. Morreale de Escobar. 1992. L-thyroxine and 3,5,3'-triiodothyronine concentrations in the chicken egg and in the embryo before and after the onset of thyroid function. *Endocrinology* 130(5):2651-2659.
- Robbins, J.; and L. Bartalena. 1986. Plasma transport of thyroid hormones. In *Thyroid hormone metabolism*. G. Hennemann (ed). pp. 3-38. New York: Marcel Dekkar, Inc.
- Robbins, J.; and H. Edelhoeh. 1986. Thyroid hormone transport proteins: their nature, biosynthesis, and metabolism. In *Werner's The Thyroid: a fundamental and clinical text*, fifth edition, S. H. Ingbar and L. E. Braverman (eds). pp. 116-127. Philadelphia: J. B. Lippincott Co.
- Romanoff, A. L.; and A. J. Romanoff. 1949. *The avian egg*. New York: John Wiley & Sons.
- Romanoff, A. L. 1960. *Avian embryo*. New York: The Macmillan Co.
- Romanoff, A. L. 1967. *Biochemistry of the avian embryo - a quantitative analysis of prenatal development*. New York: John Wiley & Sons.
- Scavo, L.; Alemany, J.; Roth, J.; and F. de Pablo. 1989. Insulin-like growth factor I activity is stored in the yolk of the avian egg. *Biochem. Biophys. Res. Comm.* 162(3):1167-1173.

- Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Neurobiology* 90:11446-11450.
- Schwartz, H. L. 1983. Effects of thyroid hormone on growth and development. In *Molecular basis of thyroid hormone action*. Eds., J. H. Oppenheimer, and H. H. Samuels. pp. 413-44. New York: Academic Press.
- Sechman, A.; and S. Bobek. 1988. Presence of iodothyronines in the yolk of the hen's egg. *Gen. Comp. Endocrinol.* 69:99-105.
- Sigma Diagnostics. 1990. Alkaline phosphatase (procedure No. 245). Revised: February, 1990. St. Louis: Sigma Chemical Company.
- Singh, A.; Reineke, E. P.; and R. K. Ringer. 1967. Thyroxine and triiodothyronine turnover in the chicken and the bobwhite and Japanese quail. *Gen. Comp. Endocrinol.* 9:353-361.
- Southwell, B. R.; Duan, W.; Tu, G.; and G. Schreiber. 1991. Ontogenesis of transthyretin gene expression in chicken choroid plexus and liver. *Comp. Biochem. Physiol.* 100(B):329-338.
- Suvarna, S.; McNabb, F. M. A.; Dunnington, E. A.; and P. B. Siegel. 1993. Intestinal 5' deiodinase activity of developing and adult chickens selected for high and low body weight. *Gen. Comp. Endocrinol.* 91:259-266.
- Tagawa, M.; and T. Hirano. 1987. Presence of thyroxine in eggs and changes in its content during early development of Chum salmon, *Oncorhynchus keta*. *Gen. Comp. Endocrinol.* 68:129-135.
- Tagawa, M.; and T. Hirano. 1990. Changes in tissue and blood concentrations of thyroid hormones in developing chum salmon. *Gen. Comp. Endocrinol.* 76:437-443.
- Thommes, R. C. 1987. Ontogenesis of thyroid function and regulation in the developing chick embryo. *J. Exp. Zool. Suppl.* 1:273-79.
- Thommes, R. C.; and V. W. Hylka. 1978. Hypothalamo-adenohypophyseal-thyroid interrelationship in the chick embryo. I. TRH and TSH sensitivity. *Gen. Comp. Endocrinol.* 34:193-200.

- Thommes, R. C.; Vieth, R. L.; and S. Levasseur. 1977. The effects of hypophysectomy by means of surgical decapitation on thyroid function in the developing chick embryo. *Gen. Comp. Endocrinol.* 31:29-36.
- Van Middlesworth, L. 1974. Metabolism and excretion of thyroid hormones. In *Handbook of Physiology*, section 7: *Endocrinology III: Thyroid*. M. A. Greer, and D. H. Solomon (eds). pp. 215-231. Baltimore: Williams and Wilkins.
- Vieira, A. V.; Sanders, E. J.; and W. J. Schneider. 1995. Transport of serum transthyretin into chicken oocytes. *J. Biol. Chem.* 270(7):2952-2956.
- Vieira, A. V.; and W. J. Schneider. 1993. Transport and uptake of retinol during chicken oocyte growth. *Biochim. Biophys. Acta* 1169:250-256.
- Wentworth, B. C.; and R. K. Ringer. 1986. Thyroids. In *avian physiology*, P. D. Sturkie (ed). pp. 452-65. New York: Springer-Verlag.
- Wollman, S. H.; and E. Zwillig. 1953. Radioiodine metabolism in the chick embryo. *Endocrinology* 52:526-535

**TABLE 1**

<b>HEN</b>	<b>DYE PATTERN IN EGG YOLK</b>
<b>A</b>	BL, RD, BL, RD, BL, YELLOW CENTER
<b>B</b>	RD, BL, RD, BL, RD
<b>C</b>	BL, RD, BL, RD, BL
<b>D</b>	BL, RD, BL, RD, BL
<b>E</b>	BL, RD, BL, RD, BL, RD

Dye patterns in the yolks of longitudinally sectioned eggs from hens which laid one egg each day during the time period of dye dosing. Dosing was done by alternating days with the lipid soluble dyes, Sudan Red and Sudan Black (RD = red ring of yolk, BL = black ring of yolk).

**TABLE 2**

	<b>Adult Hen Plasma T4 (ng/ml)</b>	<b>Adult Hen Plasma T3 (ng/ml)</b>	<b>Yolk T4 of Unincubated Eggs (ng/g)</b>	<b>Yolk T3 of Unincubated Eggs (ng/g)</b>
<b>This Study ‡</b>	10.9 ± 1.0	2.1 ± 0.2	6.0 ± 0.3	2.5 ± 0.1
<b>Hilfer and Searls (1980) ¥</b>	-----	-----	15.0 - 20.0	-----
<b>Sechman and Bobeck (1988) ¥</b>	-----	-----	6.0 - 10.0	1.5 - 2.3
<b>Prati, et al., (1992) ¥</b>	-----	-----	3.8 ± 0.33	1.5 ± 0.16

Comparison of yolk thyroid hormones in chicken and Japanese quail eggs. Data from control hens in the current study (Japanese quail, ‡), Hilfer and Searls (1980; chickens ¥), Sechman and Bobeck (1988; chickens, ¥), and Prati et al. (1992; chickens, ¥).

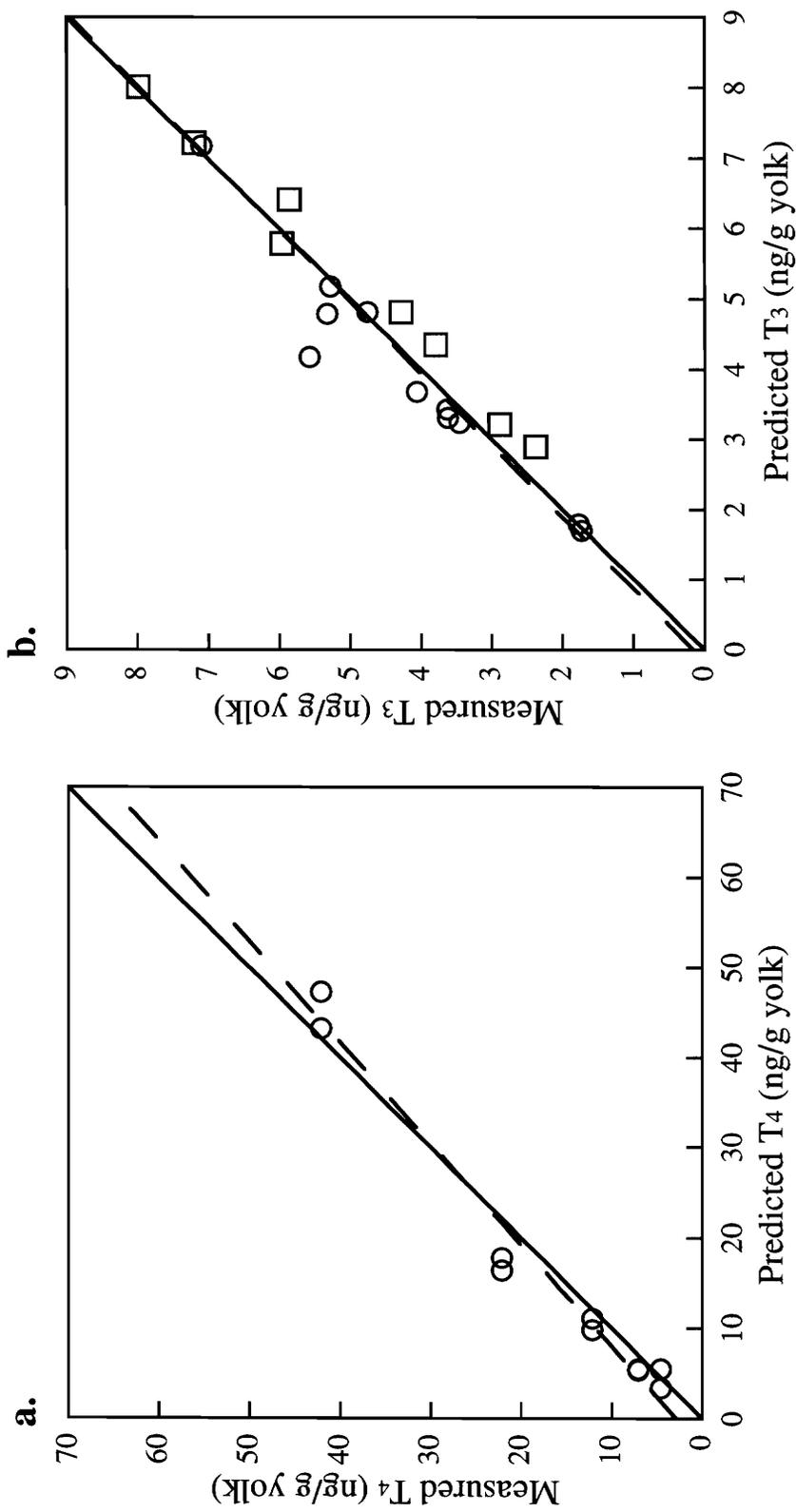


FIG. 1. Verification of radioimmunoassays for measuring thyroid hormones in extracts of egg yolk. **a.** T<sub>4</sub>: Open circles indicate measured values of yolk extract spiked with known amounts of T<sub>4</sub>, the dashed line is a Theil-Sen regression line of the measured values (slope = 0.87), and the solid line has a slope of 1.0. There is no significant difference between the slopes of the solid and dashed lines ( $k = 3$ ;  $p = 0.431$ ). **b.** T<sub>3</sub>: Open circles indicate measured values of yolk extract spiked with known amounts of T<sub>3</sub>, open squares indicate serial dilutions of yolk extract, the dashed line is a Theil-Sen regression line of the measured values (slope = 0.99), and the solid line has a slope of 1.0. There is no significant difference between the slopes of the solid and dashed lines ( $k = 9$ ;  $p = 0.376$ ).

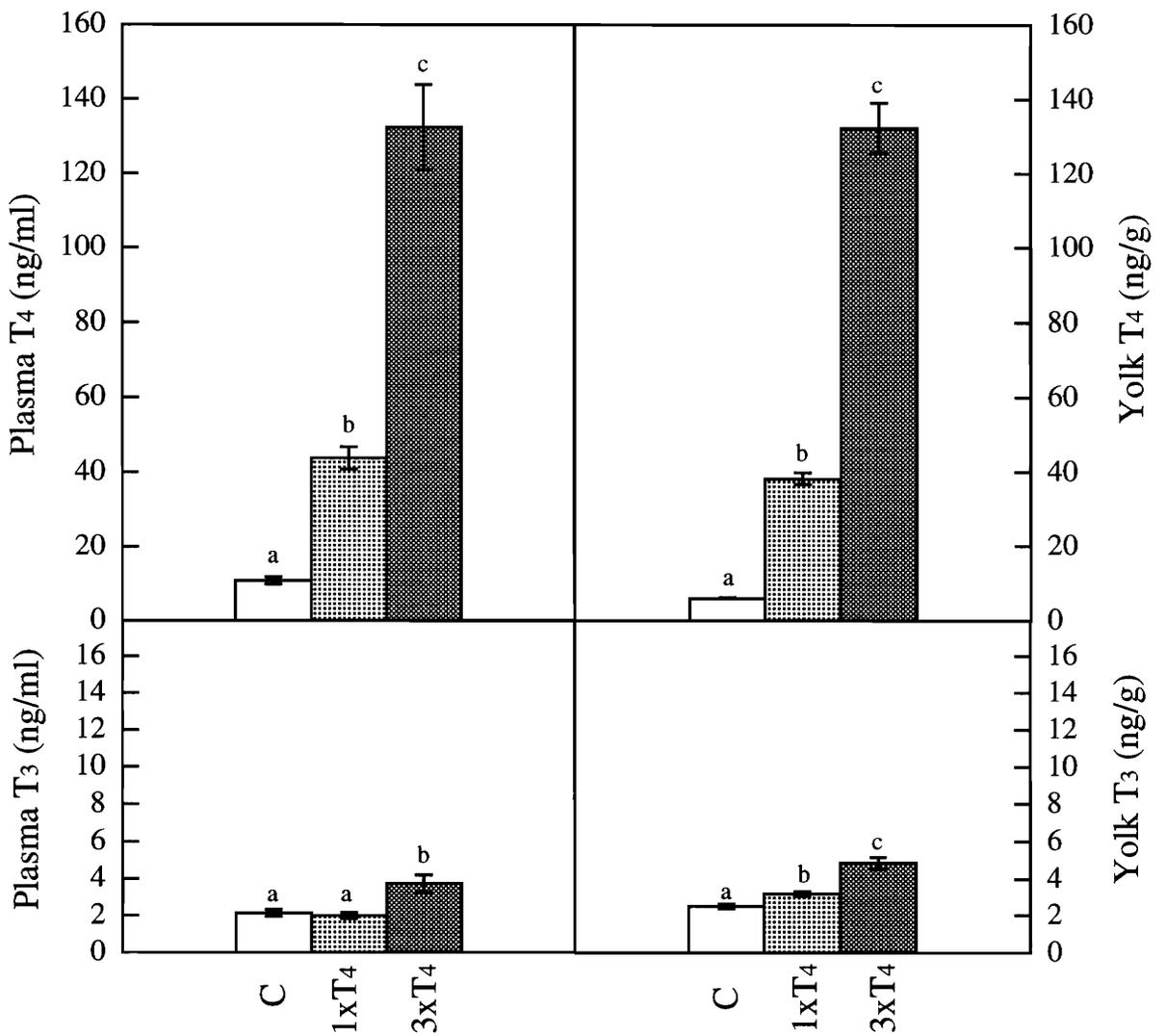


FIG. 2. Hen plasma and egg yolk thyroid hormone concentrations. **Top panels:** T4. **Bottom panels:** T3. C, control hens; 1xT4, hens dosed with 1x TSR T4; 3xT4, hens dosed with 3x TSR T4. Bars represent mean +/- SE, and different lower case letters above the bars indicate significant differences between means.

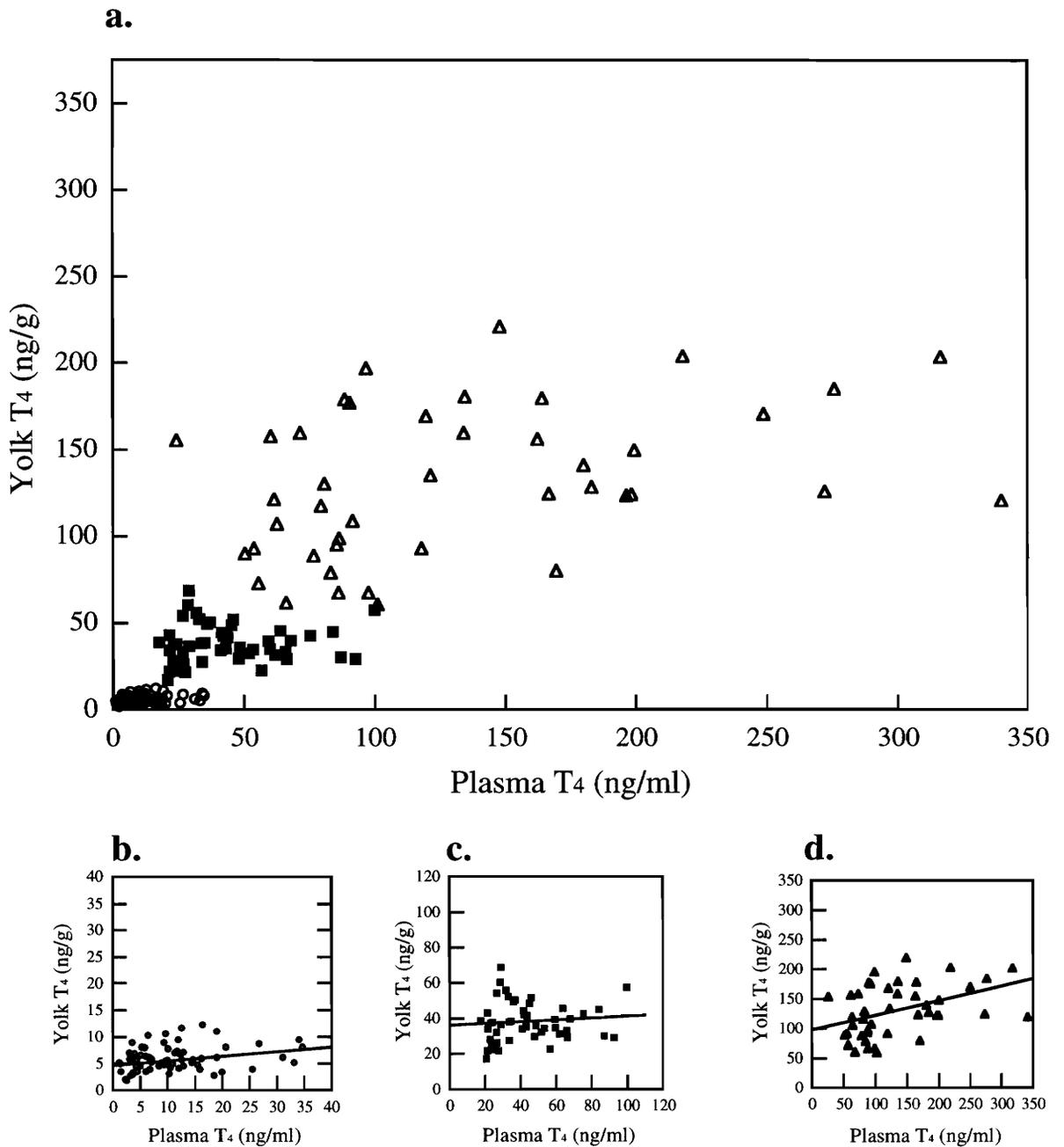


FIG. 3. The relationship between egg yolk T<sub>4</sub> concentrations and plasma T<sub>4</sub> concentrations of the hen that laid each egg. **a.** Controls, open circles; 1x TSR T<sub>4</sub> dose, closed squares; 3x TSR T<sub>4</sub> dose, open triangles. Data are represented with a Theil-Sen regression line: **b.** Controls (slope = 0.08), **c.** 1x TSR T<sub>4</sub> dose (slope = 0.05), **d.** 3x TSR T<sub>4</sub> dose (slope = 0.025).

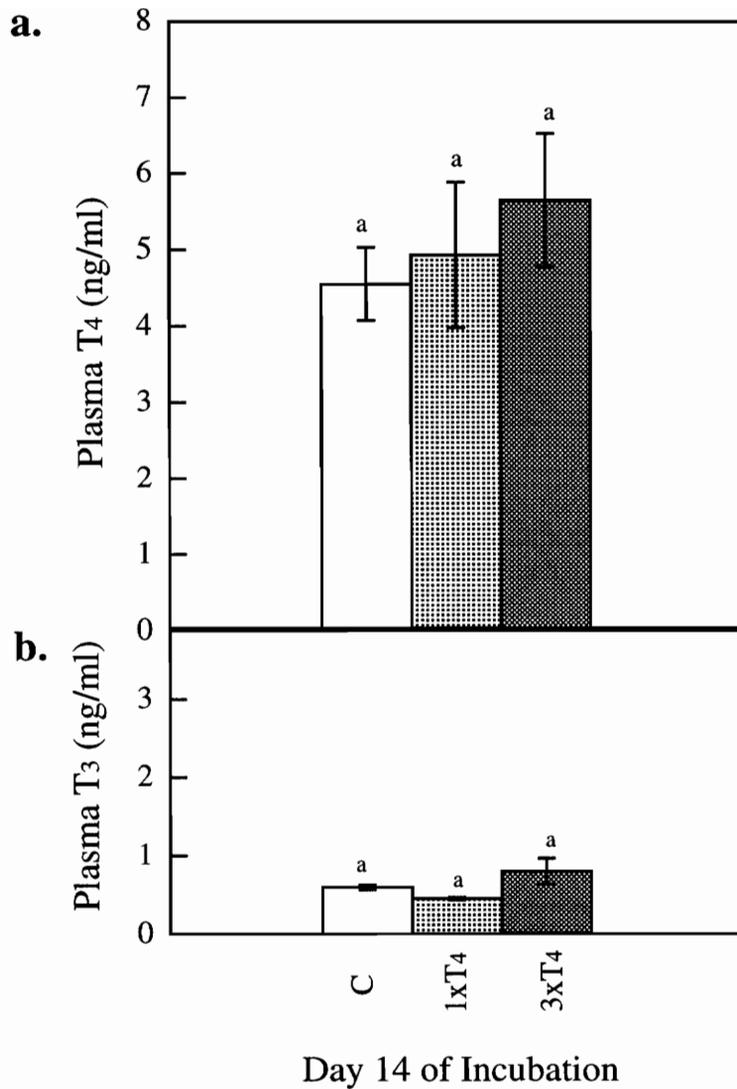


FIG. 4. Embryonic plasma thyroid hormone concentrations on day 14 of incubation. **a.** T<sub>4</sub>. **b.** T<sub>3</sub>. C, embryos from eggs of control hens; 1xT<sub>4</sub>, embryos from eggs of 1x TSR T<sub>4</sub> dosed hens; 3xT<sub>4</sub>, embryos from eggs of 3x TSR T<sub>4</sub> dosed hens. Bars represent mean  $\pm$  SE; n = 4 to 6 for each mean.

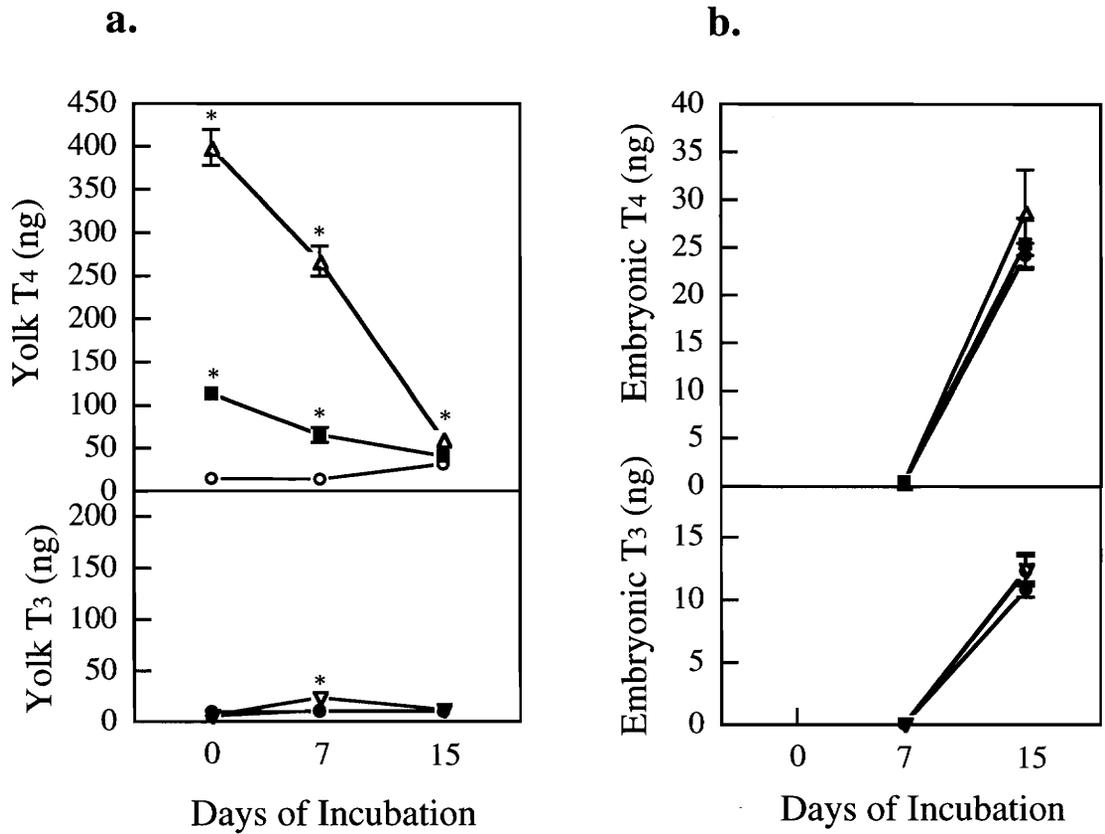


FIG. 5. Thyroid hormone content of egg yolk and embryos (carcass without thyroid gland). **a.** Egg yolk. **b.** Embryos (without thyroid gland). Upper panels, T<sub>4</sub>; lower panels, T<sub>3</sub>. Open circles, data from control hens; closed squares, data from 1x TSR T<sub>4</sub> dosed hens; open triangles, data from 3x TSR T<sub>4</sub> dosed hens. Symbols represent mean  $\pm$  SE; an asterisk indicates a significant difference from control on the same day of incubation; n = 4 to 7 for each mean.

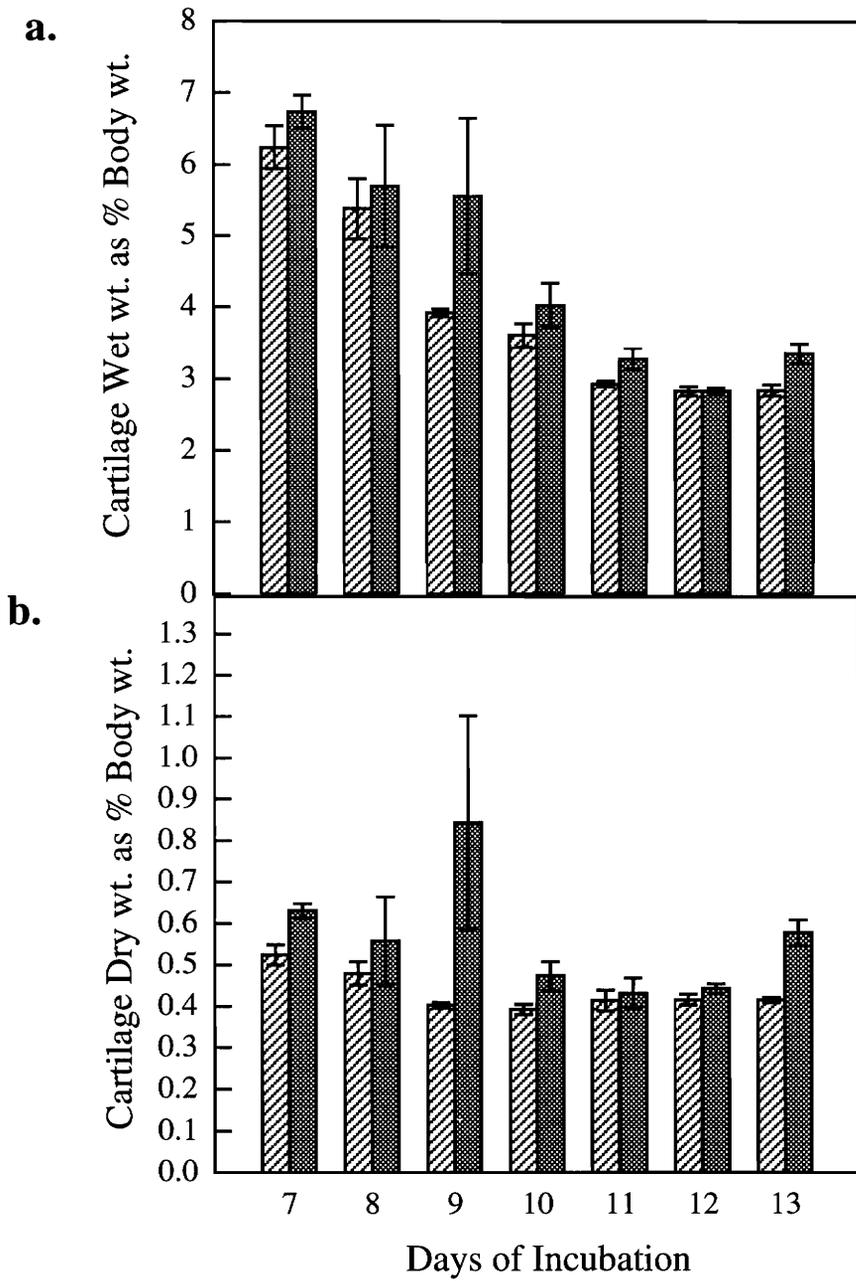


FIG. 6. Embryonic pelvic cartilage weights. **a.** Wet weight as a percentage of embryonic body weight. **b.** Dry weight as a percentage of embryonic body weight. Embryos from eggs of control hens, light hatched bars; embryos from eggs of 3x TSR T4 dosed hens, dark stippled bars. Bars represent mean +/- SE; n = 4 to 6 for each mean. Analysis of variance showed a significant elevation in pelvic cartilage wet and dry weights as percentages of embryonic body weight in embryos from eggs of 3x TSR T4 dosed hens compared to controls (p = 0.013 and 0.002, respectively).

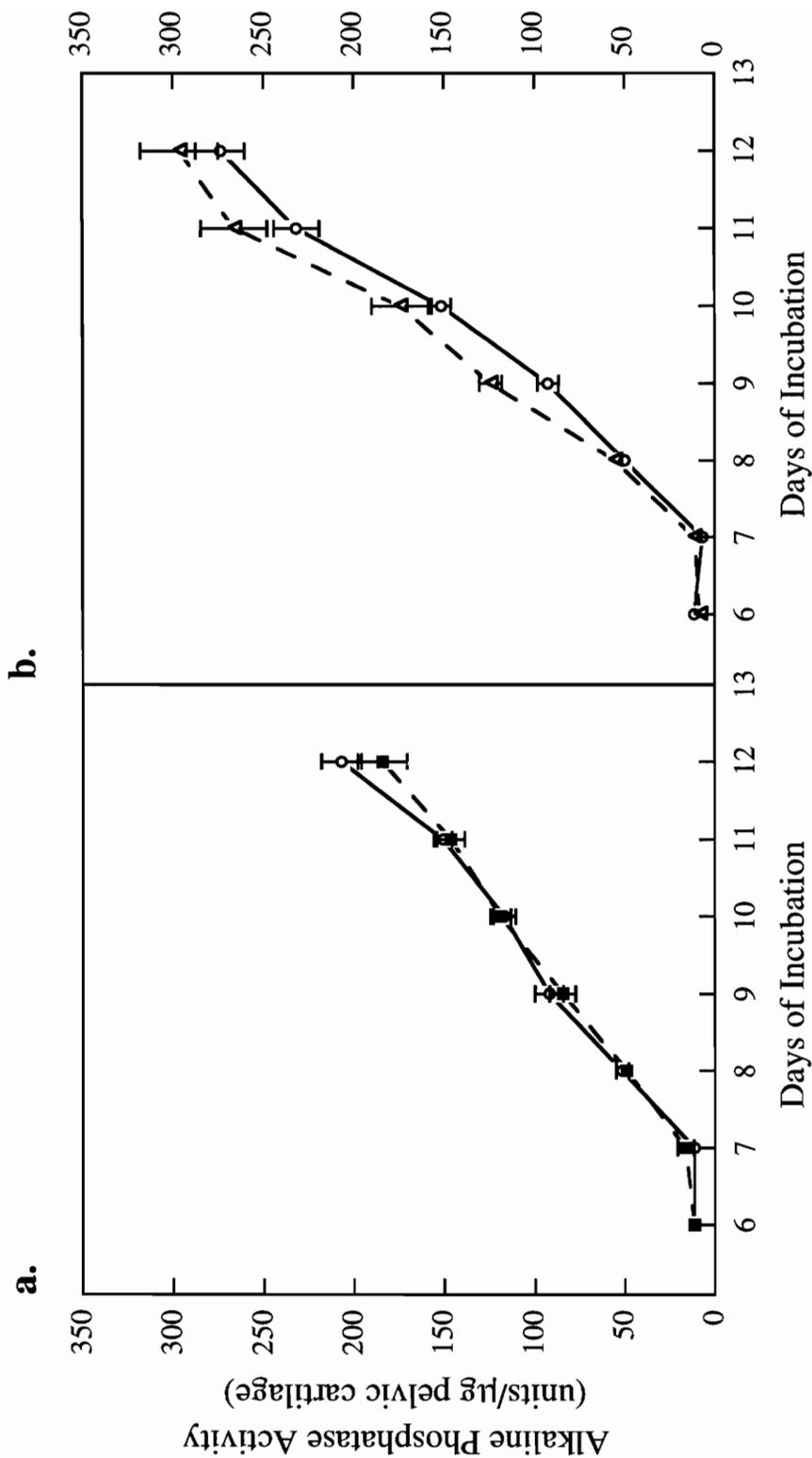


FIG. 7. Alkaline phosphatase activity in embryonic pelvic cartilage (units of activity/ $\mu$ g pelvic cartilage wet weight). One unit is that amount of enzyme which will produce one  $\mu$ -mol of p-nitrophenol per minute at 25 C (Sigma Diagnostics, 1990). Symbols represent mean  $\pm$  SE; n = 6 for each mean. **a.** Open circles, embryos from eggs of control hens; closed squares, embryos from eggs of 1x TSR T4 dosed hens. **b.** Open circles, embryos from eggs of control hens; open triangles, embryos from eggs of 3x TSR T4 dosed hens. Analysis of variance showed a significant elevation in pelvic cartilage alkaline phosphatase activity in embryos from eggs of 3x TSR T4 dosed hens ( $p = 0.0045$ ).

# CURRICULUM VITA

## CHARLES MORGAN WILSON

### DATE AND PLACE OF BIRTH

December 31, 1970  
Winchester, Virginia

### EDUCATION

**Master of Science, Zoology.** July, 1996.  
Virginia Polytechnic Institute and State University (VPI&SU)  
Blacksburg, Virginia  
QCA: 3.82/4.0  
Thesis: Maternal Thyroid Hormones in Japanese Quail Eggs.

**Bachelor of Science, Biology.** May, 1993.  
Hampden-Sydney College, Hampden-Sydney, Virginia  
QCA: 3.79/4.0  
Graduated Summa Cum Laude  
Honors in Biology  
Senior Honors Thesis: Gravitational Effects on the Bone Morphology and Histology of Mice.

### PROFESSIONAL EXPERIENCE

- Graduate Teaching Assistant (VPI&SU):
  - *General Biology Laboratory* (for non-majors). Spring 1996, Fall 1994, 2nd Summer 1994; Fall 1993; three, two-hour laboratories per week. Responsible for laboratory lecture, supervision of laboratory proceedings, testing, and grade assignments.
  - *Principles of Biology Laboratory* (for Life Science majors). Fall 1995; three, two-hour laboratories per week. Responsibilities same as General Biology Laboratory.
  - *Ornithology Laboratory*: Summer 1995; two, three-hour laboratories per week. Responsible for taking students into the field each laboratory to identify avian species, songs, and habitats.
  - *Human Anatomy and Physiology Laboratory*: Spring 1995, Spring 1994; two, three-hour laboratories per week. Responsible for laboratory lecture, laboratory preparation, supervision of laboratory proceedings, testing, and grade assignments.
- Assistant to Biology Laboratory Coordinator (VPI&SU). 2nd Summer, 1996. Assisted in the revision of the Fall Semester General/Principles of Biology Laboratory text.
- Guest Lecturer (VPI&SU): *Introduction to Animal Physiology* (Biol. 3404) Fall 1994; taught several 1.25 hour lectures to college juniors and seniors.

- Laboratory Technician (VPI&SU): Supervisor, Dr. F.M. Anne McNabb. Summer, 1993.
  - Tissue thyroid hormone extraction
  - Evaluation of tissue thyroid hormone content by radioimmunoassay.
  - Dissection, imbedding, histological sectioning, and staining of avian tissue.
  - Routine monitoring of laboratory after radioactive isotope work.
  - Routine ordering of laboratory supplies.
- Undergraduate Teaching Assistant (Hampden-Sydney College):
  - *General Biology Laboratory*; September, 1991 to May, 1992; assisted professor in teaching laboratory and grading assignments.

## HONORS AND AWARDS

- Phi Beta Kappa (National Honor Society)
- Sigma Xi, Associate Member (National Scientific Research Society)
- Phi Sigma (National Biological Science Honor Society)
- Omicron Delta Kappa (National Leadership Honor Society)
- Chi Beta Phi (National Natural Science Honor Society)
- Pre-Health Society Senior Award (Hampden-Sydney College)
- H. B. Overcash Premedical Award (Hampden-Sydney College)
- Samuel P. Jones Phi Beta Kappa Scholarship (Hampden-Sydney College)

## MEMBERSHIP IN PROFESSIONAL AND STUDENT ORGANIZATIONS

- Society for Integrative and Comparative Biology (1994 to present; formerly American Society of Zoologists)
- Virginia Academy of Science (1994 to present)
- Biology Graduate Student Association (BGSA) of VPI&SU (1993 to present); member of the Executive Committee (1993 to 1995).
- Graduate Student Assembly Delegate, Department of Biology (VPI&SU; 1993 to 1995)

## ABSTRACTS / PROFESSIONAL ACTIVITIES

- Wilson, C. M. Thyroid hormones in the yolk of Japanese quail eggs. Poster Presentation at the Graduate Student Assembly Graduate Research Symposium (VPI&SU, March, 1995).
- Wilson, C. M. 1995. Thyroid hormones in the yolk of Japanese quail eggs. *Va. J. Sci.* 46(2):100. (poster presentation at The Virginia Academy of Science Annual Meeting, May 24-26, 1995).
- Wilson, C. M.; and F. M. A. McNabb. 1995. Thyroid hormones in the yolk of Japanese quail eggs. *Amer. Zool.* 35(5):46A. (paper presentation at the American Society of Zoologists National Meeting; December 26-30, 1995)..
- Wilson, C. M.; and F. M. A. McNabb. In Press. Maternal thyroid hormones in quail eggs. *Poult. Avian Rev.* (poster presentation at the Sixth International Symposium on Avian Endocrinology, Alberta, Canada. April, 1996; presented by Dr. F. M. Anne McNabb)

## **GRANT AWARDS**

- Sigma Xi grant proposal funded July 1994. (\$600; matched to \$500 by the Department of Biology, VPI&SU).
- Graduate Research Development Project (GRDP) of the Graduate Student Assembly (VPI&SU); grant proposal funded September 1994. (\$300; matched to \$300 by the Department of Biology, VPI&SU).
- Sigma Xi grant proposal funded January 1996. (\$500; matched to \$500 by the Department of Biology, VPI&SU).

## **RESEARCH INTERESTS**

- endocrinology
- physiological ecology
- cellular and organismal biology

*Charles Morgan Wilson*