THYROID-GONAD AXIS OF THE AMERICAN ALLIGATOR (*Alligator mississippiensis*): AN EXAMINATION OF PHYSIOLOGICAL AND MORPHOLOGICAL ENDPOINTS

By

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To my friends and family. Without your continuous support and inspiration, none of this would be possible. And to those kindred spirits lost in the struggle.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGMENTS</th>
<th>.................................................................</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>...........................................................................</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>...........................................................................</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>.................................................................</td>
<td>12</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>...........................................................................</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>...........................................................................</td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>...........................................................................</td>
<td>17</td>
</tr>
<tr>
<td>General Review</td>
<td>...........................................................................</td>
<td>17</td>
</tr>
<tr>
<td>Metabolic Effects</td>
<td>...........................................................................</td>
<td>19</td>
</tr>
<tr>
<td>Effects on Differentiation</td>
<td>...........................................................................</td>
<td>20</td>
</tr>
<tr>
<td>Permissive Actions</td>
<td>...........................................................................</td>
<td>21</td>
</tr>
<tr>
<td>Sexual Dimorphism in Thyroid Disease</td>
<td>...........................................................................</td>
<td>23</td>
</tr>
<tr>
<td>Thyroid and EDCs: An Emerging Field</td>
<td>...........................................................................</td>
<td>24</td>
</tr>
<tr>
<td>Thyroid and Gonadal Development</td>
<td>...........................................................................</td>
<td>26</td>
</tr>
<tr>
<td>Hypotheses</td>
<td>...........................................................................</td>
<td>28</td>
</tr>
<tr>
<td>2 SEASONAL VARIATION IN PLASMA THYROXINE, TESTOSTERONE AND ESTRADIOL-17(\beta) CONCENTRATIONS IN JUVENILE ALLIGATORS (Alligator mississippiensis) FROM THREE FLORIDA LAKES</td>
<td>.................................................................</td>
<td>33</td>
</tr>
<tr>
<td>Introduction</td>
<td>...........................................................................</td>
<td>33</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>...........................................................................</td>
<td>34</td>
</tr>
<tr>
<td>Study Sites</td>
<td>...........................................................................</td>
<td>34</td>
</tr>
<tr>
<td>Sample Collection</td>
<td>...........................................................................</td>
<td>35</td>
</tr>
<tr>
<td>Thyroxine Radioimmunoassay and Statistical Analysis</td>
<td>...........................................................................</td>
<td>37</td>
</tr>
<tr>
<td>Results</td>
<td>...........................................................................</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>...........................................................................</td>
<td>39</td>
</tr>
<tr>
<td>3 ESTROGEN RECEPTOR EXPRESSION IN THE THYROID FOLLICLE OF THE AMERICAN ALLIGATOR (Alligator mississippiensis) DURING DIFFERENT LIFE STAGES</td>
<td>.................................................................</td>
<td>48</td>
</tr>
<tr>
<td>Introduction</td>
<td>...........................................................................</td>
<td>48</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>...........................................................................</td>
<td>49</td>
</tr>
<tr>
<td>Animals</td>
<td>...........................................................................</td>
<td>49</td>
</tr>
<tr>
<td>Histological Analysis and Statistics</td>
<td>...........................................................................</td>
<td>50</td>
</tr>
<tr>
<td>Isolation of RNA, Reverse Transcription and Northern Blots</td>
<td>...........................................................................</td>
<td>51</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Immunohistochemical Localization of ERα</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Quantitative RT-PCR</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>4 EFFECTS OF IN OVO AND IN VIVO PROPYLTHIOURACIL EXPOSURE ON THYROID AND GONAD GENE EXPRESSION IN NEONATAL AMERICAN ALLIGATORS (Alligator mississippiensis)</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Animals</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>In Ovo PTU Treatment</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>In Ovo Dissections and Tissue Collection</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>In Vivo PTU Treatment</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>In Vivo Dissections and Tissue Collection</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Histological Analysis and Statistics</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Isolation of RNA, Reverse Transcription and Northern Blots</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Gene sequence and QPCR primer design</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Thyroid: In Ovo PTU Treatment</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Thyroid: In Vivo after Neonatal Acute PTU Exposure</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Gonad: In Ovo PTU Exposure</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Gonad: In Vivo after Acute PTU Exposure</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Thyroid</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Gonads</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>Summary</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>5 SUMMARY OF RESULTS</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Seasonal Thyroxine Variation</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Characterization of ERs on the Thyroid</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>PTU Exposure in the Thyroid and Gonad</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>APPENDIX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A STAINING PROTOCOL FOR ERα IHC</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>B PARTIAL SEQUENCES FOR CLONED THYROID GENES</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>BIOGRAPHICAL SKETCH</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>4-1</td>
<td>Primers used for Quantitative Real-time RT-PCR as markers for thyroid and gonad physiology in the American alligator (<em>A. mississippiensis</em>)</td>
<td>82</td>
</tr>
<tr>
<td>A-1</td>
<td>Immunohistochemistry staining protocol for ERα.</td>
<td>118</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1-1</td>
<td>Location, structure and basic function of the thyroid follicle in a representative reptile, such as the American alligator</td>
<td>30</td>
</tr>
<tr>
<td>1-2</td>
<td>Gonadal expression of alligator TRβ and TRα mRNAs as determined by quantitative RT-PCR</td>
<td>31</td>
</tr>
<tr>
<td>1-3</td>
<td>Thyroid-gonad axis of regulation. TSH secreted from pituitary has stimulatory role on thyroid and gonad</td>
<td>32</td>
</tr>
<tr>
<td>2-1</td>
<td>Average cloacal temperature (°C) for juvenile American alligators during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA</td>
<td>44</td>
</tr>
<tr>
<td>2-2</td>
<td>Mean (high and low) ambient air temperature (°C) during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA</td>
<td>45</td>
</tr>
<tr>
<td>2-3</td>
<td>Mean (± 1 SE) plasma thyroxine (T₄) concentration (ng/ml) for male juvenile American alligators during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA</td>
<td>46</td>
</tr>
<tr>
<td>2-4</td>
<td>Mean (± 1 SE) plasma thyroxine (T₄) concentration (ng/ml) for female juvenile American alligators during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA</td>
<td>47</td>
</tr>
<tr>
<td>3-1</td>
<td>Three types of slides used (control, experimental, and normal) and how the tissue was oriented to ensure the ease and accuracy of the analysis</td>
<td>57</td>
</tr>
<tr>
<td>3-2</td>
<td>Thyroid follicle from a juvenile alligator</td>
<td>57</td>
</tr>
<tr>
<td>3-3</td>
<td>Mean ratio for IHC ERα expression (measured by ratio of IHC ERα stained to normal hemotoxylin and eosin stain) in the thyroid at three life stages in the American alligator</td>
<td>58</td>
</tr>
<tr>
<td>3-4</td>
<td>Neonate mRNA gene expression in thyroid tissue from the American alligator, <em>A. mississippiensis</em></td>
<td>59</td>
</tr>
<tr>
<td>3-5</td>
<td>Juvenile mRNA gene expression in thyroid tissue from the American alligator, <em>A. mississippiensis</em></td>
<td>60</td>
</tr>
<tr>
<td>3-6</td>
<td>Adult mRNA gene expression in thyroid tissue from the American alligator, <em>A. mississippiensis</em></td>
<td>61</td>
</tr>
<tr>
<td>4-1</td>
<td>Thyroid axis of the American alligator, <em>Alligator mississippiensis</em></td>
<td>83</td>
</tr>
</tbody>
</table>
4-2 Gonad axis of the American alligator, *Alligator mississippiensis*. ........................................84

4-3 Estrogen receptor alpha (ERα) mRNA gene expression from *in ovo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ........................................85

4-4 Deiodinase type 2 mRNA gene expression from *in ovo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ..................................................86

4-5 Sodium-iodide symporter (NIS) mRNA gene expression from *in ovo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ...............................................87

4-6 Pendrin (PEN) mRNA gene expression from *in ovo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ..........................................................88

4-7 Deiodinase 2 (D2) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ..................................................89

4-8 Androgen receptor (AR) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ...............................................90

4-9 Estrogen receptor alpha (ERα) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ...............................................91

4-10 Estrogen receptor beta (ERβ) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ...........................................92

4-11 Thyrotropin receptor (TSHr) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ...........................................93

4-12 Pendrin (PEN) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ..........................................................94

4-13 Sodium-iodide symporter (NIS) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. ...............................................95

4-14 Androgen receptor (AR) mRNA gene expression from *in ovo* PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. ...........................................96

4-15 Estrogen receptor alpha (ERα) mRNA gene expression from *in ovo* PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. ...........................................97

4-16 Estrogen receptor beta (ERβ) mRNA gene expression from *in ovo* PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. ...........................................98

4-17 Steroidogenic acute regulatory protein (StAR) mRNA gene expression from *in ovo* PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. ...........99
4-18 Aromatase (AROM) mRNA gene expression from in ovo PTU treatment in gonad tissue from the American alligator, A. mississippiensis .......................................................... 100

4-19 Androgen receptor (AR) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, A. mississippiensis ........................................... 101

4-20 Estrogen receptor alpha (ERα) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, A. mississippiensis ...................................... 102

4-21 Estrogen receptor beta (ERβ) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, A. mississippiensis ........................................... 103

4-22 Deiodinase type 1 (D1) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, A. mississippiensis ............................................. 104

4-23 Deiodinase type 2 (D2) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, A. mississippiensis ............................................. 105

5-1 Thyroid-gonad axis of regulation revisited ..................................................................... 110

5-2 In Ovo PTU mRNA expression of genes analyzed for sexual dimorphism via QPCR in thyroid tissue of juvenile American alligators (A. mississippiensis) ...................... 111

5-3 In Ovo PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of male juvenile American alligators (A. mississippiensis) .................................................. 111

5-4 In Ovo PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of female juvenile American alligators (A. mississippiensis) .................................................. 112

5-5 In Vivo PTU mRNA expression of genes analyzed for sexual dimorphism via QPCR in thyroid tissue of juvenile American alligators (A. mississippiensis) ...................... 113

5-6 In Vivo PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of male juvenile American alligators (A. mississippiensis) .................................................. 114

5-7 In Vivo PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of female juvenile American alligators (A. mississippiensis) .................................................. 115

5-8 In Ovo PTU mRNA expression of genes analyzed for sexual dimorphism via QPCR in gonad tissue of juvenile American alligators (A. mississippiensis) ...................... 116

5-9 In Ovo PTU mRNA expression of genes analyzed for treatment effects via QPCR in gonad tissue of juvenile American alligators (A. mississippiensis) ...................... 116

5-10 In Vivo PTU mRNA expression of genes analyzed via QPCR in gonad tissue of female juvenile American alligators (A. mississippiensis) .................................................. 117
LIST OF ABBREVIATIONS

AR  Androgen receptor involved in receptor-ligand interactions.
AROM  Aromatase. Major enzyme needed to convert testosterone into estradiol-17β

CDNA  Complementary DNA is synthesized from mRNA template in a reverse transcription reaction.

CIP/KIP  One of two families of cyclin-dependant kinase inhibitors and well characterized for their role as negative regulators of G-phase cell cycle progression.

DDE  1,1-Dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) is a breakdown product of DDT and a known EDC.

DDT  Dichloro-diphenyl-tricloroethane is one of the first modern pesticides and a common synthetic. It was developed early in WWII and initially used to combat mosquitoes from spreading malaria, typhus and other insect-borne human diseases. It is known as an organochlorine insecticide and EDC.

DIT  Two linked iodinated tyrosine molecules are diiodotyrosine. It is a component of thyroid hormones.

DNA  Deoxyribonucleic acid is a molecule that contains the genetic code used in the development and functioning of all living organisms.

E2  Estradiol-17β. Major estrogen hormone studied in this dissertation.

EDC  Endocrine disrupting contaminants. Chemicals known to have an affect on the endocrine system.

ER  Estrogen receptors involved in receptor-ligand interactions. Focus was on estrogen receptor alpha (α) and beta (β) of the American alligator.

ICC  Immunocytochemistry is a technique used to localize and stain specific proteins in cells of a tissue. Interchangeable with IHC.

IGF  Insulin-like growth factors. These are peptide growth stimulators that are structurally related to insulin and have some insulin-like activity in addition to their growth promoting actions.

IHC  Immunohistochemistry. A technique used to localize and stain specific proteins in cells of a tissue. This technique exploits the principles of antibodies binding to specific antigens.

LH  Luteinizing hormone.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIT</td>
<td>One iodinated tyrosine molecule is termed monoiodytyrosine. It is a component of thyroid hormones.</td>
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<tr>
<td>NIS</td>
<td>Sodium-iodide symporter. Iodide pump in the thyroid.</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid is a molecule of RNA encoding for a specific protein. mRNA is transcribed from a DNA template.</td>
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<tr>
<td>p27Kip1</td>
<td>p27Kip1 is a member of the CIP/KIP family of cdk inhibitors that negatively regulates cyclin- cdk complexes. A cyclin-dependent kinase (cdk) inhibitor, it plays important roles in cell cycle progression in normal cells.</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyls are a class of organic compounds known to be EDC. Most PCBs were manufactured as cooling and insulating fluids for industrial transformers and capacitors.</td>
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<td>PCR</td>
<td>Polymerase chain reaction is a molecular biology technique for isolating and amplifying a fragment of DNA.</td>
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<td>PEN</td>
<td>Pendrin. Cloride-iodide pump on the thyroid.</td>
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<td>PTU</td>
<td>Proplythiouracil, an anti-thyroid compound used to treat hyperthyroidism pharmaceutically.</td>
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<tr>
<td>Q-PCR</td>
<td>Quantitative PCR is a molecular biology technique used to quantify relative gene expression.</td>
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<td>RNA</td>
<td>Ribonucleic acid is a polymer composed of nucleic monomers that play various important roles in the processes that translate genetic information from DNA into proteins.</td>
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<td>RT-PCR</td>
<td>Reverse transcription PCR is a technique used to amplify, isolate or identify a known sequence from RNA.</td>
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<td>StAR</td>
<td>Steroidogenic acute regulatory protein.</td>
</tr>
<tr>
<td>T3</td>
<td>Triiodothyronine is a thyroid hormone. It is a combination of MIT and DIT. This form of thyroid hormone is considered the active form in tissue.</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine or tetraiodothyronine is a thyroid hormone. It is commonly considered the transport and non-active form of the thyroid hormones. It is considered a prohormone but nonetheless is known to be functional/active in tissues.</td>
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<td>Tg</td>
<td>Thyroglobulin. Large protein used in the thyroid to make thyroid hormones.</td>
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<tr>
<td>Tp</td>
<td>Thyroperoxidase. Enzyme used in the thyroid for the organification of iodide.</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TR</td>
<td>Thyroid hormone receptors involved in receptor-ligand interactions. Focus was on thyroid hormone receptor alpha (α) and beta (β) of the American alligator.</td>
</tr>
<tr>
<td>TRE</td>
<td>Thyroid response elements, play a role in the molecular mechanism for transcription.</td>
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<td>TRH</td>
<td>Thyrotropin releasing hormone or thyroid hormone releasing hormone.</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone also known as thyrotropin.</td>
</tr>
<tr>
<td>TSHr</td>
<td>Thyrotropin receptor.</td>
</tr>
</tbody>
</table>
Thyroid hormones are known to have a cooperative role in gonadal development and function. There is a growing body of work demonstrating that thyroid hormones play a crucial role in the development of Sertoli and Leydig cells in the testis. Thyroid hormones at proper levels are necessary for ovulation and severe hypothyroidism can cause ovarian atrophy and amenorrhea. Thyroid receptors are found in various parts of the ovary such as granulosa cells, oocytes and cumulus cells of the follicle, and corpora lutea, indicating that thyroid hormones can play a role in various cells of the ovary. The mechanisms of action are still not well understood.

In many vertebrate species, including humans, thyroid disorders are more frequent in the female population. In addition, studies have shown that neoplastic thyroids have a higher number of estrogen receptors (ER) compared to normal tissue, suggesting a relationship between the sex of an individual and susceptibility to thyroid abnormalities.

Recently, it has been shown that thyroid hormone concentrations parallel sex steroid patterns in American alligators. We investigate the mechanism of communication between the thyroid and gonad axis of the American alligator. Previous studies have demonstrated a one directional endocrine pathway from the thyroid to the gonad. We describe a possible new avenue of communication from the gonad to thyroid via the estrogen receptor located on alligator
thyroid follicles. Through the use of genetic markers for thyroid and gonad physiology, we describe a novel mechanism of communication between these two axes.
CHAPTER 1
INTRODUCTION

General Review

The thyroid has been studied for thousands of years. The first description of thyroid disease was of abnormal enlargement of the thyroid in humans, recognized by Chinese physicians about 3000 B.C. Since then, thyroid-associated problems have been recognized and even became fashionable at one time; the painting of ‘The Mona Lisa’, with her goiter, is a famous example. In 1896, Bauman discovered that an organic iodine-containing compound could be extracted from the thyroid. The iodine-containing hormone, thyroxine (T₄) was isolated and crystallized by Edward C. Kendall in 1915. This discovery was a milestone in endocrine research, since it was the first hormone isolated in pure form. The importance of the thyroid and its functions can be grasped simply by observing that the incidence of thyroid disease in humans is exceeded only by the incidence of diabetes mellitus (Norris 1997).

In amphibians, reptiles (including alligators), birds and mammals, the thyroid gland is a bilobed organ that lies ventrally to the trachea in the mid-throat region (Fig.1-1). Histologically, the thyroid is composed of many follicles surrounded by connective tissue. The follicles are filled with a proteinaceous fluid called colloid that is secreted by the single layer epithelium that comprises the wall of the follicle (Fig. 1-1). Within this colloid, several important precursor molecules accumulate that will be used to form the thyroid hormones.

In the simplest terms, thyroid hormones are iodinated tyrosine molecules. One iodinated tyrosine molecule is termed monoiodotyrosine (MIT). Two linked iodinated tyrosine molecules are diiodotyrosine (DIT). When a MIT and DIT bind, they form the active form of the thyroid hormone triiodothyronine (T₃) whereas two bound DIT molecules form thyroxine (T₄) (Fig. 1-1).
Thyroxine and T₃ are present in all vertebrates as well as annelid worms and various other invertebrates such as cnidarians, arthropods and echinoderms (Eales 1997; Norris 1997). Thyroid hormones influence many aspects of reproduction, growth, differentiation, and metabolism (Lynn 1970; Bentley 1982; Eales 1997; Norris 1997). The thyroid is possibly the most highly vascularized endocrine gland in mammals and appears to be one of the oldest endocrine glands phylogenetically (Dickhoff et al. 1983).

The hypothalamus-pituitary-thyroid axis regulation of thyroid hormone synthesis is well known (Fig.1-3). Thyrotropin releasing hormone (or corticotropin releasing hormone in some non-mammalian species) from the hypothalamus stimulates the production of pituitary thyrotropin (TSH, thyroid stimulating hormone)(Norris 1997; Denver 1999). Thyrotropin stimulates the thyroid to produce and secrete thyroid hormones (mostly thyroxine, T₄). Thyroid hormones (THs) are transported to target tissues/cells where T₄ is converted to T₃ via iodothyronine deiodinases (Norris 1997). Following the binding of thyroid hormones to nuclear or mitochondrial receptors, THs initiate genomic gene transcription ultimately leading to synthesis of new proteins. Thyroid hormone receptors (TRs) recognize specific thyroid response elements (TREs) and bind predominantly as heterodimers with the retinoid X receptors but may also form homodimers in the promoters region of targeted genes (Bassett et al. 2003). Non-genomic actions and binding to TH receptors have been shown at the plasma membrane, cytoplasm and cellular organelles. TRs are members of the nuclear receptor superfamily and act as hormone inducible transcription factors (Evans 1988; Bassett et al. 2003). Two major isoforms of TRs have been well described in the literature, TRα and TRβ.
Collaboration with Caren Helbing, of the University of Victoria, has recently produced cloned TR$\alpha$ and TR$\beta_2$ from the American alligator. Using quantitative RT-PCR (Q-PCR), we have observed that both TR$\alpha$ and TR$\beta_2$ are expressed in the gonads of juvenile alligators (Helbing et al. 2006), with greatly elevated levels of TR$\beta_2$ relative to TR$\alpha$ (Fig. 1-2). Further, there appears to be a differential response to TSH treatment, with no effect on TR$\beta_2$ mRNA levels in either gonad 24 or 48 hr after treatment (Fig. 1-2). In contrast, TR$\alpha$ mRNA levels were elevated in the testis but not the ovary 24 hr after treatment (Fig. 1-2). These data suggest that, like the rodent gonad, cells in the alligator gonad express TR, suggesting that this tissue is responsive to the actions of thyroid hormones. Further, given the differential response in TR$\alpha$ future studies are needed to determine if this response could play a role in gonad development.

There appears to be sparse data in the literature indicating whether or not TRs are expressed in a sexually dimorphic manner and data on the topic suggest that sexual dimorphism is absent in TRs gene expression (Helbing et al. 2006; Bermudez in press; Bermudez unpublished data).

**Metabolic Effects**

Metabolic effects of the thyroid hormones in mammals have been well documented. Thermogenic actions, as well as specific effects on carbohydrate, protein, and lipid metabolism, are among some of the well-studied effects of T$_3$ and T$_4$. Thyroid hormones increase synthesis of several mitochondrial respiratory proteins such as cytochrome c, cytochrome oxidase, and succinoxidase (Stevens et al. 1995; Norris 1997). A decrease of basal metabolic rate would be advantageous to animals during a period of hibernation or low caloric intake. Many non-hibernating mammals, such as the beaver and the muskrat, have depressed thyroid activity during the winter period. Hypothyroidism has been shown to occur in hibernating ground squirrels and badgers (Silva 1993). The shark embryos of *Squalus suckleyi* show an increase in oxygen

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19
consumption following T₃ and T₄ treatment (Blaxter 1988). Increased oxygen consumption is also demonstrated with tissue obtained from the frog, *Rana pipiens*, when treated with T₄ *in vivo* (May et al. 1976). A study examining the lizard, *Dipsosaurus dorsalis* found that thyroid hormones (T₄/T₃) influence locomotory endurance, suggesting an essential activity on muscular energetics (Eales 1985b).

**Effects on Differentiation**

Thyroid hormones affect differentiation, including growth, development, and metamorphosis. Thyrotoxicosis, Grave’s disease, Hashimoto’s disease, cretinism and juvenile myxedema in humans are examples of disorders in growth and development caused by altered thyroid hormone action (Norris 1997; Kilpatrick 2002). The thyroid is known to influence metabolic rate and inhibit calcium loss in bones (Gu et al. 2001). These two actions are necessary for development and normal growth (Segal 1990; Norris 1997; Kisakol et al. 2003). Thyroid hormones also are necessary for the normal development of the nervous system. Thyroid hormone treatment of early *Xenopus* larvae promotes neurogenesis in the spinal cord, where thyroid receptor TRₐ is expressed from early larval stages onward and results in precocious up-regulation of several other genes (Schlosser et al. 2002). Shark (*S. suckleyi*) embryos treated with T₄ and T₃ have accelerated differentiation of the hypothalamic neurosecretory centers, which suggest thyroid hormones play a role in differentiation and maturation of the hypothalamo-hypophysial system (Blaxter 1988).

Replacement of hair in adult mammals is stimulated by the thyroid hormones. The postnuptial molt cycle in harbor seals, *Phoca vitulina*, gray seals (*Halichoerus grypus*) and the molt cycles in the red fox, badger (*Meles meles L.*) and mink are examples of thyroid hormone influenced hair replacement (Maurel et al. 1987; Boily 1996; Norris 1997). Molting in
amphibians, reptiles and birds is also stimulated by thyroid hormones (Kar et al. 1985b; Sekimoto et al. 1987; Norris 1997).

Metamorphosis in amphibians and fish and smoltification in salmonid fishes are probably the best-known effects of thyroid hormones in non-mammalian vertebrates. Thyroid hormones play crucial roles in the metamorphosis of a frog from a tadpole (Denver 1998; Wright et al. 2000). During flounder metamorphosis, T4 concentrations increase and are associated with the migration of the eye and attendant neural structure to one side and the mouth and associated structures to the other side of the head (Blaxter 1988). Behavioral changes are associated with this alteration as well. Another example of thyroid-regulated metamorphosis is smoltification in many salmonids like the Atlantic salmon (Salmo salar) (Kulczykowska et al. 2004) and Coho salmon (Oncorhynchus kisutch) (Sweeting et al. 1994). Smoltification is the transformation from freshwater parr to smolt with pre-adapted osmoregulation for salt water.

**Permissive Actions**

Thyroid hormones also play a role in modifying the action of other cell signals, generally termed “permissive actions”. Many of the actions of thyroid hormones occur cooperatively with different hormones or cell signaling agents (paracrines or autocrines). This cooperative role or permissive action is common, where the thyroid hormone enhances the effectiveness/sensitivity of the other hormones or neural stimuli. The permissive actions of THs may be related to events such as the stimulation of the synthesis of components of second-messenger systems, up-regulation of receptors for another regulator, effects on structural components, etc. (Norris 1997). For example, several of the non-genomic actions of thyroid hormones include the modulation of Na+, Ca+, and glucose transport, activation of protein kinase C, protein kinase A and estrogen receptor kinases/mitogen activated protein kinases and regulation of phospholipid metabolism by activation of phospholipase C and phospholipase D (Kavok et al. 2001). In addition, many
thyroid mediated metabolic actions occur in cooperation with other hormones such as epinephrine and growth hormone. Thyroid hormones alter nitrogen balance and are either protein anabolic or catabolic (Kawaguchi et al. 1994; DeFeo 1996; Rendakov et al. 2003). These actions are related to an enhancement of the effects normally regulated by other hormones. Thyroid hormones, for example, can stimulate insulin-like growth factors or IGF production, which augments the action of growth hormone (Nanto-Salonen et al. 1993).

Thyroid hormones also have a cooperative role in gonadal development and function. Cycles in the plasma concentrations of thyroid hormones are positively correlated with reproductive cycles in various vertebrate species. For example, thyroid hormone serum concentrations of the sheath-tailed bat, *Taphozous longimanus* were higher during gonadal recrudescence and the breeding period during late winter dormancy but were minimal during gonadal quiescence and the initial stages of pregnancy (Singh et al. 2002a). Ovarian T4 concentrations have been shown to increase during vitellogenesis and oocyte final maturation but decrease during embryogenesis in the viviparous rockfish, *Sebastes inermis* (Kwon et al. 1999a). Serum T4 concentrations also fluctuated seasonally in Kemp’s ridley sea turtles (*Lepidochelys kempi*), with elevated concentrations observed in females during vitellogenesis when plasma E2 concentrations are elevated (Rostal et al. 1998a). Thyroid hormones are increased in many teleost fishes during periods when they are exhibiting spawning, pre-migratory, and migratory behaviors (Blaxter 1988). The thyroid hormones are hypothesized to have a permissive role as opposed to a causative role in these behaviors. The behavioral changes that occur during and after metamorphosis in vertebrates are also thought to be permissive roles of thyroid hormones. During metamorphosis in amphibians, thyroid hormones act to augment the effects of corticotrophins, thus providing a permission action (Denver 1998).
Sexual Dimorphism in Thyroid Disease

In many vertebrate species, including humans, thyroid disorders are more frequent in the female population (Arain et al. 2003). In addition, studies have shown that neoplastic thyroids have a higher number of nuclear estrogen receptors (ER) compared to normal tissue (Manole et al. 2001), suggesting a relationship between the sex of an individual and susceptibility to thyroid abnormalities. ERs are part of a family of nuclear receptors that act as transcription factors, response for significant changes in gene expression following exposure to such hormones as sex and stress steroids. Additionally, since the thyroid plays a role in hormone regulation, and hormone production changes during an animal’s development from neonate to juvenile through adulthood, it is possible that estrogen receptor expression changes with developmental maturation. Adults are expected to have greater estrogen receptor expression and seasonal variation in receptor expression since they have elevated circulating sex hormone concentrations due to reproductive activity. For example, our laboratory has reported dramatic changes in circulating concentrations of estradiol-17β in female alligators throughout the reproductive cycle (Guillette et al. 1997). We have also reported that peri-pubertal alligators show seasonal changes in plasma concentrations of E₂, but these levels are 10 to 100 fold lower than those reported in adult females (Rooney et al. 2004) and yearling alligators have further reduced, but detectable plasma E₂ concentrations (Guillette et al. 1994). Our initial study in juvenile alligators demonstrated that exogenous E₂ would depress expression of ERα but not ERβ in the ovary suggesting that as with other species, ER expression can be influenced by changing plasma concentrations of E₂ (Katsu et al. 2004).

Is the phenomenon of greater thyroid disease in females due to a sexually dimorphic pattern in the expression of steroid receptors? Could there be differences in the expression of
estrogen and androgen receptors (ER and AR, respectively) or even TRs in the thyroid at different life stages that might explain these observed differences in disease rates? One study on human thyroid tissue showed no significant difference in ER incidence (Hiasa et al. 1993). These questions, however, have been poorly studied in vertebrates and will be addressed in this dissertation.

**Thyroid and EDCs: An Emerging Field**

Endocrine disrupting contaminants (EDCs) have been shown to modify or impair function in various endocrine organs, including the thyroid (Zoeller 2003). DDT (an organochlorine used as a pesticide), its metabolites and various other environmental contaminants exert an effect on the thyroid by disrupting one of several possible steps in the biosynthesis and/or secretion of thyroid hormones (Fig. 1-1). These steps include: (1) inhibition of the iodine trapping mechanism (thiocyanate or perchlorate have been shown to exhibit this mode of action), (2) blockage of organic binding of iodine and coupling of iodothyronines to form thyroxine (T₄) and triiodothyronine (T₃) (sulfonamides, thiourea, methimazole, aminotiazole act at this stage), or (3) inhibition of T₃/T₄ secretion by affecting proteolysis of active hormone from the colloid (methimazole, propylthiouracil and flavanoids are known to affect secretion)(Capen 1992; Capen 1994; Hamann et al. 2006; Moriyama et al. 2007).

Contaminants can also alter thyroid hormone action by other mechanisms. For example, DDT has been shown to disrupt thyroid hormone availability by increasing the peripheral metabolism of thyroid hormones through an induction of hepatic microsomal enzymes (Capen 1992; Capen 1994). Male juvenile alligators from Lake Apopka, that are exposed to a wide array of environmental chemicals and have elevated organochlorine pesticide residues in their tissues and blood (especially p,p’-DDE), exhibit elevated plasma T₄ concentrations when compared to male juvenile alligators from Lake Woodruff, FL, a reference site and National Wildlife Refuge.
(Crain et al. 1998). DDT-treatment in rats increased thyroid mass as well as plasma T₃ and T₄ concentrations. Rats also displayed decreased thyroid iodine, serum iodine and protein-bound iodine levels (Seidler et al. 1976; Goldman 1981). A metabolite of DDT, p,p’-DDE has been shown to have similar effects on thyroid hormones. There is a positive correlation between serum concentrations of DDE and T₄/FreeT₄ in polar bears (Skaare et al. 2001). Another DDT metabolite, o,p’-DDD has been shown to increase T₃, T₄ and free T₄ concentrations in dogs. This compound can be used to treat hyperadrenocorticism in canines as well, as it suppresses adrenal steroidogenesis (Ruppert et al. 1999). Japanese quails exposed to DDT displayed a slight decrease in T₄ but a moderate increase in T₃ (Rattner et al. 1984). Ring doves (Streptopelia risoria) fed a diet dosed with DDE and PCB (Aroclor 1254) had plasma T₄ increase in a dose dependant manner that caused a doubling in the birds exposed to the highest doses (McArthur et al. 1983). In freshwater catfish (Clarias batrachus), endosulfan (an insecticide used on various crops) decreases T₃ but increases T₄, whereas malathion (an insecticide, used in mosquito control) induces a decrease in T₃ and no change in T₄, and carbaryl (a broad spectrum insecticide used in forestry) increases T₃ and provokes a decrease in T₄ (Sinha et al. 1991). The mechanisms that induce these varying effects are unknown. Other known EDCs, such as the polychlorinated biphenyls (PCBs; used as coolants and lubricants in transformers, capacitors and other electrical equipment), PBDEs and dioxin inhibit thyroid hormone binding to plasma transport proteins, such as transthyretin, resulting in more rapid clearance and decreased plasma thyroid hormone concentrations (Brouwer et al. 1998).

Nitrogen pollution, in the form of nitrates, has recently emerged as another area of concern as they appear to have the potential to disrupt the thyroid axis. Bulls administered nitrates orally within environmentally relevant ranges had depressed thyroid activity with a decrease in plasma
T4 concentrations as well as suppressed hypothalamic function with non-detectable levels (< 0.001 μg/ml) of the pituitary hormone thyrotropin (TSH) following a challenge test with the hypothalamic releasing hormone TRH (Zraly et al. 1997). Elevated nitrates in the diet also has been shown to depress thyroid function in humans and are associated with goiter in some nitrate-exposed children (Gatseva et al. 1998a; Gatseva et al. 2000a).

Nitrates have been shown to depress circulating thyroid hormones in other mammals and some fishes (Lahti et al. 1985; Katti et al. 1987; Gatseva et al. 1992; Brunigfann et al. 1993; Kursa et al. 2000). Animals exposed to nitrates also exhibit altered thyroid morphologies, including hypertrophy of the thyroid, increased cell height of the thyroid follicle cells, vacuolation in the periphery of the folliculi, and reduction of colloid (van Maanen et al. 1994). Nitrate contamination has also been shown to decrease iodide uptake (Lahti et al. 1985; Katti et al. 1987). The inability to take up iodide at adequate levels by the thyroid would alter thyroid action if this effect were chronic.

**Thyroid and Gonadal Development**

There is a growing body of work demonstrating that thyroid hormones play a crucial role in the development of Sertoli (cell assisting spermatozoa production) and Leydig cells (steroid producing cells) in the testis. Manipulation of the thyroid environment can be used to produce increases in testis size, Sertoli cell number, and sperm production (Cooke et al. 2004). Neonatal hypothyroidism is shown to impair testicular development (Jannini et al. 1995). However, hypothyroidism in neonatal rats, which is followed by a recovery to euthyroidism, leads to an increase in testis size and daily sperm production in adult rats (Cooke et al. 1991a). This body of work, in conjunction with other studies indicating that thyroid hormone receptors (TRs) are present in high quantities in the neonatal testis, led to the hypothesis that thyroid hormones could have key roles in testicular development (Palmero et al. 1988; Jannini et al. 1990).
Cooke et al. (1994) state that it appears T3 normally inhibits Sertoli cell proliferation directly while stimulating differentiation. These actions are observed in neonatal hypothyroid animals. Also, neonatal Sertoli cells express both TRα and TRβ although the relative contribution of these receptors in thyroid signaling remains unclear (Jannini et al. 1994; Palmero et al. 1995; Buzzard et al. 2000). Developmental hypothyroidism and an increase in adult testis size is not solely described in rats but also in mice (Joyce et al. 1993), humans (Jannini et al. 2000), bulls (Majdic et al. 1998), roosters (Kirby et al. 1996) and fish (Matta et al. 2002).

Additionally, recent work indicates that the mechanism of Sertoli cell proliferation in hypothyroidism is through regulation of p27Kip1, a member of the Cip/Kip family of cyclin-dependant kinase inhibitors and a critical regulator of proliferation of many cell types (Cooke et al. 2004). Thyroid hormones increase p27Kip1 expression in developing Sertoli cells (Buzzard et al. 2003; Holsberger et al. 2003) and hypothyroidism leads to a down regulation of p27Kip1 expression (Holsberger et al. 2003). This recent work provides a mechanistic template for further molecular studies in this area.

Thyroid hormones also play an active role with Leydig cells during development and adulthood. Several studies demonstrate how hypothyroidism decreases testosterone concentrations in adults and is attributed to a decrease in response to tropic hormones like luteinizing hormone (LH) (Hoffman et al. 1991; Anthony et al. 1995; Maran et al. 2001). Recently, it was demonstrated that thyroid hormones influence steroidogenic acute regulatory protein (StAR). Lack of thyroid hormone causes a down regulation of StAR mRNA and protein, resulting in impaired testosterone production in these cells (Manna et al. 2001b).

The literature on the role of thyroid hormones on ovarian function and development is sparse compared to studies on testis. Thyroid hormones at proper levels are necessary for
ovulation (Maruo et al. 1992). Doufas et al. (2000) demonstrated that severe hypothyroidism can cause ovarian atrophy and amenorrhea. TRs are found in various parts of the ovary such as granulosa cells (Maruo et al. 1992; Zhang et al. 1997), oocytes and cumulus cells of the follicle (Zhang et al. 1997), and corpora lutea (Bhattacharya et al. 1988), indicating that thyroid hormones can play a role in various cells of the ovary. The mechanisms of action are still not well understood.

Recent evidence also suggest that thyrotropin receptors found on gonadal tissue play a direct role in reproductive physiology of several teleost species (Goto-Kazeto et al. 2003; Rocha et al. 2007). Recent work on the American alligator also suggest that the gonads are being stimulated by thyrotropin and upregulating expression of TRs in the gonad (Helbing et al. 2006). The literature on thyroid-gonad interaction details pathways from the thyroid axis to the gonad (Fig.1-3) (Norris 1997; Johnson et al. 2000; Senger 2003). Regulation via estrogen receptors to the hypothalamus and pituitary has also been documented but no pathway from the gonad to the thyroid has been shown in the literature. Is there a regulatory pathway from the gonad directly to the thyroid?

**Hypotheses**

This study will examine the thyroidal/gonadal axis of the American alligator. We will examine two major areas of thyroidal and gonadal activity; the affect of the thyroid axis on the development of the gonad and a mechanism of communication between the thyroid and gonad. In particular, I will attempt to address whether the thyroid plays a role in the sexual differentiation of the gonads and reproduction in alligators. The role of the thyroid axis in the development and functioning of the gonad during the neonatal and peripubertal periods will also be investigated. The experiments performed are divided into two groups, developmental studies and juvenile studies. The developmental studies examine gonadal differentiation and
development following exposure to an antithyroid-agent during the window of sexual
differentiation. In the studies of adolescent alligators (juvenile peripubertal individuals ranging
100 – 150 cm in length), I will describe normal physiology and morphology of the thyroid/gonad
axis. Does the thyroid axis influence seasonal reproductive hormone variation? We will
ultimately attempt to describe a novel mechanism of communication between the thyroid and
gonad axis. This mechanism will include the characterization of ER and AR on the thyroid
follicle as well as expression levels of these receptors to manipulations. I propose to test several
hypotheses stated below.

• **Hypothesis 1**: Plasma thyroxine concentrations display seasonal variation that parallels
  seasonal variation in sex steroid concentrations, not seasonal activity patterns.

• **Hypothesis 2**: ER, AR and TR expression on the thyroid will vary among life stages and
  show sexual dimorphism.

• **Hypothesis 3**: Treatment of the thyroid with proplythiouracil (PTU), and anti thyroidal
  pharmaceutical agent, will alter the expression of genes related to gonadal physiology.

• **Hypothesis 4**: By blocking the thyroid with PTU during the temperature dependant sexual
  differentiation period of the alligator embryo, an alteration in the development of the testis or
  ovary will be observed.
Figure 1-1: Location, structure and basic function of the thyroid follicle in a representative reptile, such as the American alligator. The thyroid is a bi-lobed structure, composed of follicles that accumulate iodine, and form iodinated tyrosine molecules that are used to make the thyroid hormones T3 and T4.
Figure 1-2: Gonadal expression of alligator TRβ and TRα mRNAs as determined by quantitative RT-PCR. Juvenile male and female alligators were treated with ovine TSH by i.v. injection and tissues were obtained 24 or 48 hr after treatment. (Helbing et al. 2006)
Figure 1-3: Thyroid-gonad axis of regulation. TSH secreted from pituitary has stimulatory role on thyroid and gonad. FSH secreted from pituitary has stimulatory role on gonads. E₂ secreted from gonads plays an inhibitory role in pituitary on FSH secretion. E₂ possibly plays a regulatory role on thyroid.
CHAPTER 2
SEASONAL VARIATION IN PLASMA THYROXINE, TESTOSTERONE AND ESTRADIOL-17β CONCENTRATIONS IN JUVENILE ALLIGATORS (Alligator mississippiensis) FROM THREE FLORIDA LAKES1.

Introduction

The thyroid hormones influence many aspects of reproduction, growth, differentiation, and metabolism in vertebrates. Metabolic effects of these thyroid hormones have been well documented (Lynn 1970; Eales 1985a; Eales 1988). Thermogenic action, such as positive and negative effects on carbohydrate, protein, and lipid metabolism, are among the actions of these hormones. Further, thyroid hormones increase synthesis of several mitochondrial respiratory proteins, such as cytochrome c, cytochrome oxidase, and succinoxidase (Norris 1997). These compounds are necessary for normal development of the nervous system and influence molting in amphibians, reptiles and birds as well as smoltification in many salmonids (Lynn 1970; Norris 1997; Shi 2001).

Circulating concentrations of thyroxine (T4) have been observed to fluctuate during the year in various species (Kar et al. 1985a; Kuhn et al. 1985; Gancedo et al. 1997). For example, the frog Rana ridibunda has a plasma T4 cycle which peaks during the months of February through April, T4 plasma concentrations then drop and peaks again during October\November. The two peaks occur during periods of changing photoperiod and rainfall (Kuhn et al. 1985). A similar pattern in plasma concentrations of T4 is found in a reptile, the Indian garden lizard, Calotes versicolor, from the same geographical region (Kar et al. 1985a). The first peak is found prior to reproduction and the second prior to hibernation or a period of low metabolic activity. Decreased basal metabolic rate would be advantageous to animals during a period of hibernation or low caloric intake. Reduced food intake in mammals and fish has been shown to reduce

1 Part of this chapter is published in Comparative Biochemistry and Physiology A (Bermudez et al., 2005).
thyroid hormone production (Eales 1988; MacKenzie et al. 1998). Thyroxine concentration
decreases prior to winter months and is lowest during hibernation in the Chinese cobra, *Naja

Although alligators in Florida do not exhibit true hibernation, they do endure a period of
low caloric intake and inactivity during the winter months. Do alligators exhibit seasonal
variation in circulating T₄ concentration similar to that observed in other vertebrates
experiencing winter inactivity? Is an abiotic environmental factor, such as temperature correlated
with plasma concentrations of T₄? For example, stress can influence thyroid hormone
concentrations in humans, mice, birds, and fish (Bau et al. 2000; Davis et al. 2000; Kioukia et al.
2000; Morgan et al. 2000; Steinhardt et al. 2002; Coleman et al. 2003). Our laboratory has
previously reported that contaminants can alter hormone concentrations in alligators and fish,
including sex steroids and thyroid hormones (Crain 1997; Guillette et al. 2000; Orlando et al.
2002; Toft et al. 2003). Thyroxine concentrations have been shown to be elevated in male
juvenile alligators from a contaminated site when compared to reference juveniles (Crain et al.
1998). That study, however, only examined animals for a single period in time. Would the
pattern of plasma T₄ concentration found in alligators from a contaminated site mimic that found
in alligators from reference sites or would it be different? Further, would the alterations, if
present, be consistent throughout the year?

**Materials and Methods**

**Study Sites**

This study examined seasonal variation in plasma concentrations of T₄ in juvenile
American alligators from three populations in central Florida, USA. One site, Lake Woodruff
National Wildlife Refuge, is considered a reference site whereas the other two lakes, Lake
Apopka and Orange Lake, are significantly impacted by human activity. Lake Woodruff (lat.
29°06’N, long. 81°25’W) is a relatively pristine environment with little modern agricultural activity in its watershed and little discharge of nutrient-laden agricultural or storm water discharge. For example, alligators from this lake have lower concentrations of various organochlorine (OC) pesticides or their metabolites in their blood than lake Apopka (Heinz et al. 1991; Guillette et al. 1999b). Animals from Orange Lake (lat. 29°26’N, long. 82°11’W) have similar low levels of OC pollutants as those from Lake Woodruff (Guillette et al. 1999c) but is eutrophic. The third population (Lake Apopka) is a historically contaminated site, receiving city effluent until 1970’s as well as direct agricultural runoff until 1998 (Woodward et al. 1993; Guillette et al. 2000). Lake Apopka (lat. 28°40’N, long. 81°38’W) is the fourth largest lake in Florida and 1.5 miles downstream from an EPA Superfund site (EPA 1994). Lake Apopka was directly connected via a freshwater stream to the site of a major pesticide spill of dicofol (composed of 15% DDT) and sulfuric acid in 1980 (EPA, unpublished report). Animals and eggs from this lake environment exhibit elevated concentrations of OCs and the lake is highly eutrophic relative to other areas (Heinz et al. 1991; Sengal et al. 1991; Schelske et al. 1992; Guillette et al. 1999b).

**Sample Collection**

Juvenile American alligators (*A. mississippiensis*) ranging from 75cm - 150cm in total length were hand captured at night during the hours (h) of 8 pm – 1 am. The majority (80 - 90 %) of the samples where collected during the period of 9 pm – 11 pm. Alligators of this size, range from 2 - 6 years of age (Milnes et al. 2002). A majority of juveniles collected were first time captures with a small percentage (approximately 10%) of recaptures. All animals captured conformed to the same size and age class. Approximately 30 alligators were collected each night with a minimum of 10 males and 10 females obtained from each lake. Collections occurred
during the middle 2 weeks of each month and all samples were collected within a week of each other for all three sites. Samples from juvenile alligators living in Orange Lake were collected from November 2000 - April of 2002, except during March 2002. No collections of juvenile alligators where possible on Orange Lake during May and June of 2001 because of a drought that lowered water levels enough to prevent entry with boats. Blood samples were collected from juvenile alligators from Lake Woodruff between March 2001 - April 2002, except during March 2002. Finally, samples from the alligators living in Lake Apopka were collected between February 2001 - April 2002, except March 2002.

An immediate blood sample (within 3 min of capture) was obtained from the postcranial supravertebral blood vessel once the animals were secured. Approximately 10 ml of blood was taken from each animal (depending on size). Blood was collected in a heparinized Vacutainer® and stored on ice for 8 - 10 h until centrifugation at 1,500 g for 20 min. Plasma T₄ concentrations do not change in whole and clotted blood stored for 72 h at 4°C or room temperature (22 - 26°C) (Reimers et al. 1982). Plasma was stored at -80°C. On site water and air temperature was collected as well as body temperature within the first 5 min of capture. Figure 2-1 displays the average cloacal temperature for the juvenile alligators from each lake during the months of this study. The average (high, low) air temperature for each month from all three lakes is displayed in Figure 2. Other morphometric measurements were then obtained. These measurements included total length, snout-vent length, weight, sex, and if male, phallic tip and cuff length using predefined criteria (Allsteadt et al. 1995; Guillette et al. 1996). Animals were released in the vicinity of capture once all measurements were recorded.
Thyroxine Radioimmunoassay and Statistical Analysis

Total thyroxine (T₄) was analyzed using a radioimmunoassay (RIA) previously validated for alligator plasma (Crain et al. 1998). A previous study from our laboratory (Crain et al. 1998) demonstrated that body length of juvenile alligators was a covariate of plasma T₄ concentrations. Thus, a subset of all the samples collected, based on juvenile snout vent length, was used for RIA analysis. That is, 7 to 10 males and 7 to 10 females of a matched size were selected from each lake for each month to remove the possible confounding effects of body size. Animals ranged in length from 79 cm to 122.5 cm with a mean of 104.1 cm. Juvenile alligators sampled ranged in weight from 1.7 kg to 9.7 kg and had a mean weight of 3.2 kg. Hormone concentrations were determined from raw CMP (counts per min) data using a log-linear cubic spline standard curve generated by Microplate Manager PC 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA). Interassay variance was 16.3% whereas intraassay variance was 6.6%. Intraassay variation was determined by calculating the average variation between duplicate samples in every assay (n = 1466). Interassay variation was determined by calculating the average variation in interassay sample from each assay (n = 17) of plasma created from a pool of juvenile plasma. Values were corrected for interassay variation. Briefly, the assay most median in variation was chosen as the “base”. The other assays and their respective T₄ concentrations where then corrected by multiplying the percentage of variation from the “base assay”. This procedure was applied to all assays until interassay variation was not present. Analysis of variance (ANOVA) was performed to determine if differences in T₄ concentrations occurred among months, lake or between sexes for animals in the three alligator populations. All statistical tests were performed with Statview 5.0 (SAS Institute Inc., Cary, NC). Statistical significance was considered if p ≤ 0.05.
Results

To determine if ANCOVA analyses were required, we examined if a relationship existed between plasma T₄ concentrations and weight, snout-vent length (SVL), or cloacal temperature, using linear regression analyses with data for all months combined or each month separately. Significant relationships were not observed between plasma T₄ concentration and either weight ($r^2 = 0.001$; $p = 0.47$) or SVL ($r^2 < 0.001$; $p = 0.92$) when all months were examined together. A relatively weak relationship, however, was detected between plasma T₄ concentration and cloacal temperature ($r^2 = 0.074$; $p < 0.0001$).

Plasma T₄ concentration and weight, SVL and cloacal temperature were then regressed for each month; no relationships were significant. Figure 2-1 displays the average cloacal temperature for the juvenile alligators from each lake during the months of the study. The average (high, low) air temperature for each month from all three lakes is displayed in Fig. 2-2.

We examined plasma T₄ concentrations in juvenile alligators using a 3 way ANOVA, with lake of capture, month of capture and sex as variables. The effect of month of capture on plasma T₄ concentrations was highly significant ($F = 58.8$; $df = 12$, $P < 0.0001$: Fig. 2-3, 2-4). Although not consistent every month, in spring and fall male and female alligators from lake Apopka had higher concentrations of T₄ whereas in winter the concentrations were lower than those observed in animals from lake Woodruff and Orange. Likewise, the lake from which the animals were obtained also influenced plasma T₄ concentrations ($F = 7.94$; $df = 2$, $P = 0.0004$: Fig. 2-3, 2-4). Sex of the individual had no influence on plasma T₄ concentrations alone ($P = 0.82$) but the interaction between sex and date of capture was significant ($F = 2.68$; $df = 36, 569$; $P < 0.0001$). Although a difference was noted in plasma T₄ concentration when males and females were examined, no consistent pattern of sexual dimorphism was noted, as females had elevated levels compared to males in some months whereas males had the higher concentrations
in other months or no difference was noted (Fig. 2-3, 2-4). The one major difference seen between males and females was a dramatic peak in plasma T₄ concentrations in females captured in September, whereas males showed no change from the previous month.

**Discussion**

Plasma T₄ concentrations in juvenile alligators exhibit seasonal variation that are not driven by ambient temperature alone, as we obtained a poor correlation between body temperature and plasma T₄ concentration. The poor correlation and lack of significance when plasma T₄ concentrations were regressed against weight and SVL also were expected as the subset of samples examined in this study was selected for conformity for these variables. However, by constructing our samples sets in this way, we removed the possible confounding effects of SVL and weight as variables influencing the analysis. The significant relationship found between cloacal temperature and plasma T₄ concentration had a relatively low r² value (less than 0.07 – 0.2 for a given month of capture) suggesting that variation in plasma thyroxine concentration is apparently induced by additional biotic and abiotic factors such as water level, nutritional level, behavior or contaminants. We observed that ambient and body temperatures were highest during spring and summer months with an expected drop during the fall and winter months. Our data reveal that plasma T₄ concentrations in both male and female juvenile alligators were increased during the transition from winter to spring months and late fall and winter. The increase in plasma concentrations in spring coincides with increasing ambient temperature but the greatest variation occurs during the fall and winter months when temperatures drop precipitously from October – November. However, we observed a highly significant increase in plasma T₄ concentrations during the period when ambient temperatures were lowest, the period of December – February.
Thyroid cycles are positively correlated with reproductive cycles in various vertebrate species. Thyroid hormone concentrations in serum of the sheath-tailed bat, *Taphozous longimanus* were elevated during gonadal recrudescence and the breeding period, during late winter dormancy, and minimal during gonadal quiescence and the initial stages of first pregnancy (Singh et al. 2002b). Ovarian thyroxine concentrations have been shown to increase during vitellogenesis and oocyte final maturation and decrease during embryogenesis in the viviparous rockfish, *Sebastes inermis* (Kwon et al. 1999b). Serum thyroxine also fluctuated seasonally in Kemp’s ridley sea turtles (*Lepidochelys kempi*), with elevated levels observed in females associated with the period of vitellogenesis (Rostal et al. 1998b). Thyroid hormones are increased during spawning, premigratory, and migratory behaviors of many teleost fishes (Blaxter 1988).

Plasma T4 concentrations in juvenile alligators exhibit a pattern similar to that seen in plasma testosterone (T) and estradiol-17β (E2) concentrations reported by our group for a different set of plasma samples obtained several years earlier from juvenile alligators (these animals are of a size and age reported to be non sexually mature) (Rooney et al. 2004). We have suggested, based on these and other data (Edwards et al. 2004) that alligators exhibit a multi-year onset of puberty and that ‘juvenile’ animals, of the size studied by our group previously and in this study, are actually peripubertal. Juvenile males display a peak in plasma T concentrations in March, followed by a decline and then a rise again in August (Rooney et al. 2004). Females showed a rapid rise in plasma E2 concentration during the spring, with a peak in June (Rooney et al. 2004). In the present study, plasma T4 concentrations peaked during April. Given these patterns, we hypothesize that thyroid hormones could play a cooperative role with T and E2 in juveniles, helping stimulate important events in puberty.
Ando et al. (2001) has shown that prolonged exposure to T₃ in neonatal rats is a mechanism by which thyroid hormone can down regulate aromatase activity in Sertoli cells. Also, work with Meishan boars found that transient neonatal hyperthyroidism during late gestation was associated with a decline in proliferation and early maturation of Sertoli cells, followed by early onset of puberty (McCoard et al. 2003). These observations indicate a possible role for thyroid hormone in modification of Sertoli cell development, thereby influencing growth and differentiation of the testis. Precocious puberty has been reported as a complication of severe acquired hypothyroidism in children (Chattopadhyay et al. 2003).

Additionally, T₄ plasma concentration increased during prepubertal and peripubertal periods in rhesus monkeys and appear to occur in concert with the peripubertal increase in testicular size (Mann et al. 2002). The changes in T₄ during the peripubertal period suggest that thyroid status could be a significant contributor to the process of sexual development.

We also observed a significant rise in plasma T₄ concentrations between November and December in males and females; a pattern similar to that found in vertebrates that hibernate (Kar et al. 1985a; Kowalczyk et al. 2000). Animals captured in December also display an increase in cloacal temperature. This peak could be attributed to the rise in plasma T₄ seen in December since thyroid hormones are potent stimulators of thermogenesis and metabolism. Although Florida has short and relatively mild winters compared to more northern temperate regions, this peak could be ‘prehibernatory’ for alligators, a subtropical species. Alligators do not exhibit hibernation but do display cold temperature torpor, involving relatively low body temperature, reduced or no food intake and greatly reduced activity levels (Mellhenny 1987; Grenard 1991; Levy 1991).
Juveniles from Lake Woodruff appear to exhibit a seasonal pattern in plasma T₄ concentration that is significantly different from that seen in animals from Lake Apopka. Animals from Orange Lake appeared to display a pattern intermediate to that observed on the other two lakes. We noted that the seasonal patterns between lakes Woodruff and Apopka were reasonably similar although the concentrations of T₄ in any given month could vary significantly. The populations of alligators in these three lakes were chosen as they represented three unique environments as discussed earlier, but also represented populations with many similarities. Samples were obtained each month on consecutive nights to minimize weather, photoperiod and temperature difference. These lakes are less than 75 miles apart on a north–south axis with Lake Apopka being the southernmost lake and Orange Lake being the northernmost (for map of lake locations see (Guillette et al. 1999a). A recent population genetics study indicated that the animals from these three lakes are similar; a panel of molecular markers could not distinguish animals taken from these three lakes (Davis et al. 2002). A number of studies have shown that xenobiotic contaminants, such as organochlorine (OC) pesticides (or their metabolites), PCBs (polychlorinated biphenyls) and PBDEs (polybrominated diphenyl ethers) influence the thyroid axis (Brucker-Davis 1998; Zoeller et al. 2000; Zoeller et al. 2002). Further, additional studies have begun to document the role of nitrates in the disruption of the thyroid axis (Guillette et al. 2005). Nitrates/nitrites have recently been shown to depress thyroid function and are associated with goiter in some nitrate-exposed children (Gatseva et al. 1998b; Gatseva et al. 2000b). They also alter gene expression for the thyroid receptor in an amphibian (Barbeau et al. 2007). Many Florida lakes exhibit nitrate contamination. Our lab has previously shown altered T₄ concentrations in juvenile alligators living in Lake Apopka and Lake Okeechobee, both eutrophic lakes (Crain et al. 1998). The fact that plasma T₄ concentrations from alligators in
Lake Apopka seem to vary from the pattern displayed in the reference lake, Lake Woodruff, could be due to the elevated exposure to pollutants in Lake Apopka; that is, both elevated OCs and NO₃/NO₄, whereas the primary pollutant in Orange Lake is NO₃/NO₄. Plasma concentrations of T₄ are but one measure of thyroid action and future studies need to reexamine other aspects of the thyroid axis before we can determine if the differences we have observed among the animals from these lakes are biologically significant.

In conclusion, we have observed that juvenile American alligators display seasonal variation in circulating T₄ concentrations. Plasma T₄ concentrations peak in March or April but the pattern observed does not parallel that of ambient or body temperature. Although we have detected significant differences in the basic pattern, especially when month and plasma concentrations are compared among the animals from the three lakes, the general seasonal patterns observed for both sexes for the three lakes are generally similar. Future studies are required to determine if the differences observed among the populations are related to contaminants found in these wetlands or if other factors contribute to the observed differences. Further, comparing the seasonal pattern observed in plasma concentrations of T₄ with the seasonal patterns in other hormones, such as testosterone, estradiol-17β and corticosterone could provide insight into the endocrinology of the multiyear puberty this species appears to exhibit.
Figure 2-1: Average cloacal temperature (°C) for juvenile American alligators during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA. The sample size for each cloacal temperature ranged from 11 – 20 data points per month. Only temperatures from samples for which thyroxine concentrations were obtained are presented.
Figure 2-2: Mean (high and low) ambient air temperature (°C) during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA. The temperature information obtained from the cities nearest the lakes as listed by the Weather Channel®.
Figure 2-3: Mean (± 1 SE) plasma thyroxine (T₄) concentration (ng/ml) for male juvenile American alligators during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA. a = statistically significant difference between juveniles from Lakes Woodruff and Apopka for a given month, \( p \leq 0.05 \), b = statistically significant difference between juveniles from Lakes Woodruff and Orange for a given month, \( p \leq 0.05 \), and c = statistically significant difference between juveniles from Lakes Apopka and Orange for a given month, \( p \leq 0.05 \).
Figure 2-4: Mean (± 1 SE) plasma thyroxine (T4) concentration (ng/ml) for female juvenile American alligators during the months of March 2001 through April 2002 from Lakes Woodruff, Apopka, and Orange, Florida, USA. a = statistically significant difference between juveniles from Lakes Woodruff and Apopka for a given month, p ≤ 0.05, b = statistically significant difference between juveniles from Lakes Woodruff and Orange for a given month, p ≤ 0.05, and c = statistically significant difference between juveniles from Lakes Apopka and Orange for a given month, p ≤ 0.05.
CHAPTER 3
ESTROGEN RECEPTOR EXPRESSION IN THE THYROID FOLLICLE OF THE AMERICAN ALLIGATOR (Alligator mississippiensis) DURING DIFFERENT LIFE STAGES.

Introduction

The thyroid and its hormones play essential roles during development and growth of numerous tissues such as the central nervous system and skeleton (Norris 1997; Styne 1998; Cayrou et al. 2002; Bernal et al. 2003). Additionally, this axis has been shown to play various roles in homeostasis, cellular metabolism and reproduction (Cooke et al. 1991b; Norris 1997; Arambepola et al. 1998). Recently, it has been shown that seasonal variations in plasma thyroxine concentrations parallel seasonal variations in sex steroid concentrations in juvenile alligators (Bermudez et al. 2005). This observed pattern suggests that the thyroid axis could have a role in regulating gonadal activity and vice versa. Sex steroid receptors on the thyroid are thought to be nuclear receptors, which regulate target gene expression involved in metabolism, development, and reproduction (McKenna et al. 2001). The role these sex steroids, and their receptors, play in the regulation of the thyroid is not currently well understood.

The presence of estrogen receptors (ER) and androgen receptors (AR) in the thyroid has been reported for only a couple of vertebrates, namely humans and rats (Fujimoto et al. 1992; Giani et al. 1993; Kawabata et al. 2003). Additionally, many vertebrate species, including humans, have thyroid disorders more frequently diagnosed in the female than the male population (approximately 3:1)(Paterson et al. 1999; Manole et al. 2001; Arain et al. 2003). Studies have shown that neoplastic thyroids have a higher number of ERs compared to normal tissue (Manole et al. 2001), suggesting a potential relationship between sex and susceptibility to thyroid abnormalities. These findings also suggest that ER signaling could play a larger role in the thyroid than the AR.
This study describes the presence and distribution of sex steroid and thyroid receptors (ERα, ERβ, AR, TRα, and TRβ) in the thyroid gland obtained from alligators at several life history stages and provides a semi-quantification of the sex steroid receptor types using an immunocytochemical approach. Quantitative differences in mRNA expression of ERα, ERβ, TRα, TRβ and the AR was determined on the same tissue using quantitative real time PCR (Q-PCR) with primers designed specifically for alligators. This study examines the potential sexual dimorphism in receptor expression in the alligator thyroid. Due to the presence of both ERα and ERβ nuclear receptors throughout life in the thyroids of humans (Kawabata et al. 2003), we expected other vertebrates, such alligators, would also express both estrogen receptors in the thyroid.

The thyroid axis plays a role in hormone regulation, and since hormone production changes during an animal’s development from neonate to adult, it is possible that steroid receptor expression changes with developmental maturity. To investigate whether sex steroid receptor expression changes throughout an animal’s life, we examined alligators from three different life stages.

**Materials and Methods**

**Animals**

Five male and five female neonatal, juvenile, and adult alligators were collected from Lake Woodruff (lat. 29°06’N, long. 81°25’W), Florida, USA. In June of 2003, the juvenile specimens were captured at night from an airboat by a hand-restraint technique. The juvenile American alligators (*A. mississippiensis*) ranged from 84.6 cm – 137.6 cm in total length with a mean length of 110.2 cm and were hand captured during the hours (h) of 9 pm – 1 am. Upon capture, these alligators were sexed and placed in a cloth bag for transport back to the University
of Florida. The specimens were euthanized and tissues dissected within 10 h of the capture. In mid July of 2003, 12 eggs were collected from Lake Woodruff and transported to the University of Florida, Florida, USA. Since the sex of alligators is temperature dependent, six eggs were incubated at 33.5°C, the male determining temperature, and six eggs were incubated at 30°C, the female determining temperature for alligators from central Florida, USA. In mid August, as each egg hatched, the neonate was euthanized and tissues dissected. The neonates ranged 23.5 cm – 26 cm in total length with a mean length of 24.9 cm. In September of 2003, the adult specimens were captured at night using a standard noose technique. The adult alligators ranged from 178 cm – 333 cm in total length with a mean length of 225.4 cm and were captured during the hours of 11 pm- 2 am. The specimens were sexed in the field and transported to the University of Florida. Within 7 h of capture, alligators were euthanized and tissues dissected.

In all cases, alligator euthanasia was performed by an overdose of sodium pentabarbital, injected intravenously into the post-cranial vertebral vein, a protocol approved by the University of Florida IACUC. Thyroids were removed from all specimens and divided into two lobes. One thyroid lobe was preserved in cold Bouins fixative (fixative was on ice), whereas the other lobe was flash frozen in liquid nitrogen for molecular studies. Additional tissues (gonad, liver, heart, phallus, and brain) were harvested for use in other ongoing studies.

**Histological Analysis and Statistics**

Thyroid tissues from each age group were prepared using standard histological techniques. Each animal was represented by a set of slides and each set contained three slides: one control slide, one experimental slide, and one normal Hemotoxylin and Eosin stain slide. The control slides contained sections 1, 4, and 7; the experimental slides included sections 2, 5, and 8; the normal slides included sections 3, 6, and 9 (Fig. 3-1). After the tissues were mounted, the
sections on the control and experimental slides were treated using immunocytochemistry (ICC) techniques and an antibody specific for ERα (Appendix A) to visualize the presence of estrogen receptors. The two slides differ in that experimental slides received antibody specific for a receptor and control slides did not. Detection was performed using the Vector Elite ICC kit and antibodies from Santa Cruz Biotechnology, Inc.: androgen receptor AR (C-19): sc-815 and the estrogen receptor ERα (MC-20): sc-542. Recently, Japanese collaborators (Ohta, Y. unpublished data) have validated the use of these antibodies for alligator ERα and AR. The third slide of the set was stained with Hemotoxylin and Eosin stain (Fig. 3-2).

Once the slides were stained, sections through three intact thyroid follicles were analyzed. The total number of counted stained nuclei from the experimental slide was divided by the total number of counted stained nuclei from the same follicle in the normal slide. These data were then converted to the arcsine of the ratio obtained. This figure was used to represent the relative ERα protein expression in the thyroid of that specific alligator. This technique was used to semi-quantify ERα protein expression levels in the thyroid. Comparisons between the sex was analyzed using StatView software with a significance $\alpha = .05$ (version 5.0; SAS Institute Inc., Cary, NC, USA). We had very limited success with AR immunostaining on alligator thyroid follicles. Although the presence of an AR-like protein was localized, staining was never consistent enough so that we could perform a distribution analysis.

**Isolation of RNA, Reverse Transcription and Northern Blots**

Quantitative real time-PCR (Q-PCR) was performed to quantify mRNA expression levels for ERα, ERβ, AR, TRα, and TRβ in neonatal, juvenile and adult thyroid tissue. The technique used was that which validated previously for alligator tissues (Katsu et al. 2004; Helbing et al. 2006).
Q-PCR was performed using standard techniques. In short, total RNA was isolated with an RNeasy kit (QIAGEN, Chatsworth, CA). First strand cDNA synthesis was performed on 4 μg of total RNA using SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Gaithersburg, MD) and oligo (dT)12-18 (Invitrogen, Gaithersburg, MD) to reverse transcribe polyA+ mRNA. Primer annealing was carried out at 70°C for 10 min, before reverse transcriptase was added. Conditions for first-strand synthesis were 42°C for 60 min, followed by 10 min at 70°C. Primers for Q-PCR were designed from the alligator coding sequences (chapter 4, Table 4-1). A sequence also was previously obtained for alligator β-actin and ribosomal L8 for the purpose of normalization; primers have been designed based on alligator sequences. Q-PCR was carried out in a BioRad MyiQ single color real-time PCR detection system according to the manufacturer’s protocol, with the exception that 15 µL per well was used. Q-PCR conditions were 2 min at 50°C, 95°C for 10 min and 40 cycles at 95°C for 15 sec, and 60°C for 1 min. To normalize data, the mean Ct (threshold cycle) for ribosomal L8 was used on the mean Ct of the genes of interest (ERα, ERβ, TRα, TRβ and AR). Relative expression counts were calculated using the $2^{-ΔΔCt}$ method (Livak et al. 2001). Northern analysis was preformed using standard techniques to determine quality of the mRNA prior to Q-PCR; gels were loaded with 20 µg total RNA. Labeling of cDNA probes was achieved by random priming (Prime-It II, Stratagene, La Jolla, CA) using (ATP-32P) dCTP (SA 3,000 Ci/mmol; New England Nuclear) according to the manufacturer’s protocol.

Results

Immunohistochemical Localization of ERα

Localizations of ERα was visualized in the thyroid follicle using a mammalian polyclonal antibody (Fig. 3-2). An ANOVA revealed that no sexual dimorphism was detected in ERα.
protein expression, as determined by immunocytochemistry, at any of the life stages examined in this study. The ratio of ICC ERα stained to normal hemotoxylin and eosin stain is displayed in Fig. 3-3.

**Quantitative RT-PCR**

Relative expression of thyroid tissue mRNA for ERα, ERβ, TRα, TRβ and AR were analyzed using QPCR to determine whether sexual dimorphism existed. An ANOVA was performed on the genes of interest with sex as the independent factor. No statistically significant difference was observed between male or female thyroid mRNA expression for any of the genes analyzed. Thyroid relative mRNA expression for genes analyzed in neonate, juvenile and adult alligators are displayed in Figs. 3-4, 3-5 and 3-6 respectively.

**Discussion**

Thyroid disorders are approximately three times more prevalent in females across species (Paterson et al. 1999; Manole et al. 2001; Arain et al. 2003). Our data demonstrate that the thyroid expresses both forms of ER in the alligator thyroid. Both forms of ER (α and β) are known to be expressed in the human thyroid (Kawabata et al. 2003). Our data demonstrate that mRNA for both ER and AR is expressed in the thyroid as well as for both forms of TR. Further, we observed that the mRNA for ERα is translated to protein as we were able to detect its presence in the thyroid follicle cells. When thyroid tissues from the American alligator were analyzed histologically, no sexually dimorphic pattern was observed for ERα staining when tissues from all three life stages were examined. These results are contrary to our hypothesis that females would show higher ERα expression. Quantitative PCR data from these tissues supports this conclusion as well as the results from another study that examined potential sexually dimorphic patterns of ERα expression in humans (Manole et al. 2001). Recent studies
examining the mammalian thyroid suggest that ER expression is not sexually dimorphic, but rather, the post-ligand binding response of ERs to E₂ in the thyroid cell is dimorphic (Correa da Costa et al. 2001; Lima et al. 2006; Marassi et al. 2007). That is, estrogens enhance expression of cyclin D₁ protein, which plays a role in regulation of transition from G₁ to S phase in the cell cycle. Estrogens exert effects by activation of MAP kinases as well as by binding to ERs.

As alligators sexually mature, the plasma concentrations of sex steroids increase. We hypothesized that, as adults have higher plasma concentration of E₂ compared to the other two life stages (Guillette, 2000; Rooney et al. 2004; Milnes, M. R. personal communication), the adult alligators would show lower ERα ratios and, therefore, lower ERα expression due to potential feedback loops down regulating the expression of the receptor. Our data suggest that neonates have a significantly higher ratio of ERα compared to both the juvenile and the adult specimens. Our Q-PCR data for mRNA expression for ERα and ERβ, however, did not support this observation, suggesting that differential translation could occur at different life stages.

One observational difference of note, is that we have demonstrated that ERα protein and mRNA for ERα and ERβ are expressed in the thyroid of juvenile alligators obtained immediately after hatching in late August, during mid summer (June) in juveniles and during September in adults. A previous study examining the tissue distribution of ERα observed no ER mRNA expresssion for juvenile alligator thyroid tissue (Helbing et al. 2006). Interestingly, the animals in that study were collected from the wild (Lake Woodruff NWR, Florida, USA) in September, the same location from which we obtained the animals for this study in June. Further, the animals used in the present study are approximately 20 cm longer in snout vent length, suggesting that they are approximately 1-2 years older (Milnes et al. 2000) than those examined by Helbing et al (2006). These data suggest possible life stage differences, but that is...
unlikely given that we observed mRNA ER expression in the thyroid tissue of neonates, juveniles and adults. We suggest that possible seasonal variation in the expression of the ER in the thyroid is more likely and this needs to be tested, although the protected status of this animal may preclude monthly sampling for such a test.

In addition to the expression of both forms of ER, we demonstrate that the alligator thyroid expresses mRNA for the AR. Both adult and neonatal stages displayed significantly lower mRNA expression levels when compared to juveniles. Androgens have been found in circulation in both juvenile male and female alligators (Rooney et al. 2004; Bermudez, D. S. unpublished data). Further, juvenile alligators of the size we examined in this study display seasonal variation in plasma testosterone concentrations (Rooney et al. 2004). Further, juvenile alligators appear to display a multiyear period of puberty and these data suggest a hypothesis that AR function during the juvenile life stage could play a role during peripubertal maturation of the thyroid. Androgens have been suggested to increase thyroid function by up regulating expression of genes such as thyroperoxidase and thyroglobulin (Correa da Costa et al. 2001). The increase in androgen concentration in the blood during puberty could up-regulate thyroid function, which consequentially, would increase activity and growth in tissues responsive to thyroid hormones. Future studies need to test this hypothesis.

TRα as well as TRβ display mRNA expression in the thyroid. TRs mRNA expression in thyroid tissue is seen in neonate, juvenile and adult life stages. TRs exert a regulatory role on the thyroid axis to maintain proper thyroid hormone balance (Norris 1997; Helbing et al. 2006) previously showed TRs mRNA expression in the thyroid and our data support those findings.

This study has shown that mRNA for both forms of the ER, both forms of TR and AR are found on the thyroid of the American alligator (A. mississippiensis). No sexual dimorphism was
observed in the mRNA expression of these genes in the thyroid tissue examined. However, the presence of sex steroid receptors provides a potential mechanism by which the gonadal steroid could influence thyroid development and function. This is the first study to describe ERs in the thyroid a none-mammalian species and to characterize the expression with mRNA expression and protein expression. Further studies are required to determine if such a regulatory pathway exists via ERs in the thyroid.
### Figure 3-1: Three types of slides used (control, experimental, and normal) and how the tissue was oriented to ensure the ease and accuracy of the analysis. The numbers descend in order from the earliest section of thyroid used to the latest.

<table>
<thead>
<tr>
<th>Control</th>
<th>Experimental</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 4 7</td>
<td>2 5 8</td>
<td>3 6 9</td>
</tr>
</tbody>
</table>

### Figure 3-2: Thyroid follicle from a juvenile alligator. (A) Control, (B) experimental, and (C) normal stained. The control and experimental tissues underwent the same IHC protocol; however, only the experimental tissues were treated with the ERα antibody. The normal follicle underwent a Hemotoxylin and Eosin stain. This stains for every nucleus present on the follicle.
Figure 3-3: Mean ratio for IHC ERα expression (measured by ratio of IHC ERα stained to normal hemotoxylin and eosin stain) in the thyroid at three life stages in the American alligator. Error bars are 1 standard error from mean. No sexually dimorphic pattern is observed.
Figure 3-4: Neonate mRNA gene expression in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. No sexual dimorphic pattern was observed.
Figure 3-5: Juvenile mRNA gene expression in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. No sexual dimorphic pattern was observed.
Figure 3-6: Adult mRNA gene expression in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta CT}$ method. Error bar represents 1 standard error from mean. No sexual dimorphic pattern was observed.
CHAPTER 4
EFFECTS OF **IN OVO** AND **IN VIVO** PROPYLTHIOURACIL EXPOSURE ON THYROID AND GONAD GENE EXPRESSION IN NEONATAL AMERICAN ALLIGATORS (*Alligator mississippiensis*)

**Introduction**

The thyroid axis plays diverse roles and functions in vertebrates. Metabolic effects of thyroid hormones, such as increases in the synthesis of several mitochondrial respiratory proteins such as cytochrome c, cytochrome oxidase, and succinoxidase, are well known (Stevens et al. 1995; Norris 1997). Also, shark embryos of *Squalus suckleyi* and tissue obtained from the frog, *Rana pipiens*, have been shown to increase oxygen consumption following triiodothyronine (T₃) and/or thyroxine (T₄) treatment (Blaxter 1988; May and Packer 1976). Effects on growth and development such as the human disorders thyrotoxicosis, Grave’s disease, Hashimoto’s disease, cretinism and juvenile myxedema are caused by imbalances in the thyroid axis (Norris 1997; Kilpatrick 2002). In amphibians and fish, metamorphosis and smoltification are classic examples of roles played by the thyroid axis (Denver 1998; Wright et al. 2000; Kulczykowska et al. 2004).

The general permissive/cooperative roles played by the thyroid axis on the reproductive axis is yet another demonstration of the diversity of functions by this axis. Cycles in the plasma concentrations of thyroid hormones are positively correlated with reproductive cycles in various vertebrate species such as the sheath-tailed bat, *Taphozous longimanus* (Singh et al. 2002), the viviparous rockfish, *Sebastes inermis* (Kwon et al. 1999) and the American alligator, *Alligator mississippiensis* (Bermudez et al. 2005, this dissertation). Thyroid hormones play a crucial role in the development of testicular Sertoli (cell assisting spermatozoa production) and Leydig cells (steroid producing cells). Manipulation of the thyroid environment can be used to produce an
increase in testicular size, Sertoli cell number, and sperm production (Cooke et al. 2004). Neonatal hypothyroidism has been shown to impair testicular development (Jannini et al. 1995). Plasma concentrations of thyroid hormones, at proper levels, are necessary for ovulation (Maruo et al. 1992). Doufas and Mastorakos (2000) demonstrated that severe hypothyroidism causes ovarian atrophy and amenorrhea. Thyroid receptors (TRs) are found in various parts of the ovary such as granulosa cells (Maruo et al. 1992; Zhang et al. 1997), oocytes and cumulus cells of the follicle (Zhang et al. 1997), and corpora lutea (Bhattacharya et al. 1988). Recent evidence also suggests that thyrotropin receptors found in gonadal tissue play a direct role on the reproductive physiology of several teleost species (Goto-Kazeto et al. 2003; Rocha et al. 2007). Recent work from our group, examining the American alligator, also suggest that the gonads are capable of being stimulated by thyrotropin as we observed an up regulation of expression of TRs in the gonad following treatment (Helbing et al. 2006). These studies, in conjunction with other available data, indicate that TRs are present in high quantities in the testis and ovary, leading to the hypothesis that thyroid hormones could have key roles in gonadal development and function.

This study examines the potential role the thyroid plays on the developing reproductive axis of the American alligator. This investigation will focus on the thyroid axis and address what happens to the reproductive axis if the thyroid axis is depressed with a pharmaceutical agent. Alligators were treated with proplythiouracil (PTU) in ovo during the window of sexual differentiation of the gonad in developing embryos and in vivo in neonates. PTU is a commonly used anti-thyroid agent for the treatment of hyperthyroidism. PTU functions as an inhibitor of gap-junction-intercellular communication in the thyroid follicular cells. Two thyroid hormones, manufactured by the thyroid gland, T₄ and T₃, are formed by combining iodine and the protein thyroglobulin with the enzymatic assistance of peroxidase. PTU inhibits the normal interaction
of iodine and peroxidase on thyroglobulin, thus blocking the formation of T₄ and T₃. PTU also interferes with the conversion of T₄ to T₃, and, since T₃ is more active than T₄ at the cellular level, this also reduces the activity of the thyroid axis. By blocking the thyroid with PTU during the temperature dependant sexual differentiation period of the alligator embryo, we predict an alteration in the development of the testis or ovary and change in gene expression. We also predict a change in gene expression on both gonad and thyroid tissue treated with PTU as neonates.

This study examines the thyroid axis primarily through changes in gene expression using quantitative RT-PCR (QPCR) of markers of thyroid and reproductive steroid hormone function. We examined markers such as the nuclear receptors for estrogens (ERα, ERβ), androgens (AR), thyroid hormones (TRα, TRβ), plasma membrane receptors for thyrotropin (TSHr), deiodinases (D1, D2), sodium-iodide symporter (NIS), pendrin (PEN), thyroglobulin (Tg) and thyroperoxidase (Tp) (Fig. 4-1). ERs¹ and ARs² are believed to play a possible regulatory role on the thyroid axis. TRs³ are known regulatory agents of the thyroid axis. Both deiodinase 1 and 2 help convert T₄ to T₃, which is believed to be the more active thyroid hormone in tissues. The last four endpoints play roles in the synthesis of thyroid hormones. The sodium-iodide symporter⁶ (NIS) is located in the basal membrane of an epithelial cell of thyroid follicles. It pumps sodium (Na⁺) and iodide (I⁻) ions into the epithelial cell where it is then transported to the apical surface and released into the lumen of the follicle. Pendrin⁷ (PEN) is a co-transporter found at the apical membrane of a thyroid epithelial cell. Pendrin pumps iodide (I⁻) from the epithelial cell into the thyroid follicular lumen and chloride (Cl⁻) from the follicular lumen into the epithelial cells. Thyroglobulin⁸ is a large protein that plays a role in the coupling of iodinated tyrosine molecules to form thyroid hormones T₃ and T₄. Lastly, thyroperoxidase⁹ is an enzyme that helps convert
inorganic iodide to active iodide, which then readily binds to a tyrosine molecule leading to an organically bound iodine.

The mRNA expression of several additional biomarkers of gonadal function were examined as well using QPCR. Gene expression in gonadal tissue was examined for the nuclear receptors for estrogens (ERα, ERβ), androgens (AR), the plasma membrane receptor for thyrotropin (TSHr), deiodinases (D1, D2), P450 aromatase (AROM) and steroidogenic acute regulatory protein (StAR) (Fig. 4-2). ERs¹ and ARs² are known to regulate the gonadal axis as well as other tissues. Likewise, TRs³ actively modulate the physiology of the gonads. Thyroid stimulating hormone, presumably acting via its receptor⁴ has been recently shown to increase the expression of TRs the alligator gonad (Helbing, Crump et al. 2006). Steroidogenic acute regulatory protein⁵ is known to shuttle cholesterol into the mitochondria for conversion in the steroidogenic pathway. Both deiodinase⁶ 1 and 2 help convert T₄ to T₃, which is the more active thyroid hormone in tissues. Aromatase⁷ is an enzyme necessary for the conversion of testosterone to estradiol-17β.

Materials and Methods

Animals

Alligator clutches from Lake Woodruff National Wildlife Refuge (lat. 29°06’N, long. 81°25’W), Florida, USA were collected during late June 2003 for the in ovo study. Alligator clutches from Lake Woodruff were collected during late June 2004 for the in vivo study. Alligator Eggs from these clutches were candled and staged. Eggs were then systematically sorted into groups with an N = 10. One set of eggs was incubated at 30°C (female determining temperature) whereas the other set was incubated at 33.5°C (male determining temperature).
Each set had five subsets: control, ethanol vehicle control, low dose PTU, medium dose PTU, and high dose PTU

**In Ovo PTU Treatment**

Given that no previous studies of embryonic exposure to PTU had been done in alligators, we created doses *de novo* with suggestions taken from toxicology. The high dose was to be 50% of the LD$_{50}$ for rats. The LD$_{50}$ for PTU in the laboratory rat is 1,250 mg/kg. The average weight (n = 10) of an alligator egg was 90 g. The high dose for an egg was calculated to be approximately 56 mg PTU/egg. However, given the solubility of this compound in our vehicle 95% ethanol (90 g/100 ml), we treated eggs with a topical dose of 100 μl. Thus, the high dose was 900 μg/egg with a medium dose 100 fold less at 9 μg/egg and a low dose of 90 pg/egg. Eggs were dosed each day for five consecutive days starting when eggs were at embryonic stage 19, just prior to the period of sex determination. Vehicle controls received 100 μl of ethanol as did each treatment group where as the non-vehicle control received no treatment.

**In Ovo Dissections and Tissue Collection**

Embryos were allowed to incubate and gestate to hatching. Once neonates hatched, they were immediately euthanized with an overdose of pharmaceutical grade sodium pentobarbitol, injected intravenously into the post-cranial vertebral vein, a protocol approved by the University of Florida IACUC. Approximately 2-3 ml of blood was extracted, centrifuged and plasma collected for analyses of plasma hormone concentrations by validated RIA. Thyroid and gonadal tissues where immediately removed, partitioned into separate lobes (thyroid) or pieces (gonad) and flash frozen with liquid nitrogen and stored at -80°C until processed for QPCR. One piece of thyroid tissue was fixed in chilled Bouin’s fixative and stored in 75% ETOH for standard
histology and ICC of ER. Snout vent length (SVL), Total length (TL), body mass, and thyroid and gonad weight were also collected.

**In Vivo PTU Treatment**

Treatment was administered 14 days post hatch to allow for the absorption of the yolk sac. High dose treatment was at 5 ng PTU/g body weight of neonate, whereas the medium dose treatment was 0.05 ng/g neonate and low dose treatment was 0.005 ng/g neonate. The average neonate weighed 65 g yielding a high dose of approximately 325 ng, medium dose of 3.25 ng, and low dose of 0.325 ng. PTU was dissolved in 95% ethanol and injections involved a volume of 50 μl each, intravenously into the post-cranial vertebral vein. Control groups received no treatment or 50 μl ethanol injections. After the initial treatment, a second identical treatment was given 6 h later. After a total 12 h since the initial treatment, animals were euthanized with an overdose of sodium pentobarbital and tissues collected.

**In Vivo Dissections and Tissue Collection**

Immediately prior to euthanasia, 2-3 ml of blood was obtained from the supravertebral blood vessel with a sterile needle and syringe. Neonates then were euthanized with an overdose of sodium pentobarbital, injected intravenously into the supravertebral vein, a protocol approved by the University of Florida IACUC. Thyroid tissue was immediately removed, weighed, partitioned into two distinct lobes and fixed or flash frozen with liquid nitrogen and stored at -80°C. Gonadal tissue was handled in a similar manner. Snout vent length, TTL, and body mass were also collected.

**Histological Analysis and Statistics**

Thyroid tissues from *in ovo* PTU experiment were prepared using standard histological techniques (Humason 1972). Tissues were stained using Hemotoxylin and Eosin. Once the slides were stained, sections through six intact thyroid follicles were analyzed. Briefly, tissue
slides from each individual were examined and the six largest intact thyroid follicles were selected for analysis. Follicle diameter and epithelial cell height were measured. Four epithelial cells were randomly selected from the chosen follicles and epithelial cell height was measured from the basal membrane to the apical membrane. Follicle diameter was measured from the apical membrane. Morphometric measurements were taken using Scion Image analysis software. Data were analyzed using StatView software with a significance $\alpha = .05$ (version 5.0; SAS Institute Inc., Cary, NC, USA).

**Isolation of RNA, Reverse Transcription and Northern Blots**

Quantitative real time-PCR (Q-PCR) was performed to quantify mRNA expression levels for ER$\alpha$, ER$\beta$, AR, TR$\alpha$, TR$\beta$, D1, D2, Arom, StAR, Tg, Tp and TSHr in neonatal thyroid and gonadal tissues. The technique used was that which has been previously validated for alligator tissues (see Katsu et al., 2004; Helbing et al., 2006).

In short, total RNA was isolated with an RNeasy kit (QIAGEN, Chatsworth, CA). First strand cDNA synthesis was performed on 4 $\mu$g total RNA using SuperScript II RNase H-Reverse Transcriptase (Invitrogen, Gaithersburg, MD) and oligo (dT)12-18 (Invitrogen, Gaithersburg, MD) to reverse transcribe polyA+ mRNA. Primer annealing was carried out at 70°C for 10 min, before reverse transcriptase was added. Conditions for first-strand synthesis were 42°C for 60 min, followed by 10 min at 70°C. Primers for Q-PCR were designed from the alligator coding sequences (Table 4-1). A sequence also was previously obtained for alligator $\beta$-actin and ribosomal L8 for the purpose of normalization; primers designed based on alligator sequences. Q-PCR was carried out in a BioRad MyiQ single color real-time PCR detection system according to the manufacturer’s protocol, with the exception that 15 $\mu$L per well was
used. Q-PCR conditions were 2 min at 50°C, 95°C for 10 min and 40 cycles at 95°C for 15 sec, and 60°C for 1 min. To normalize data, the mean Ct (threshold cycle) for ribosomal L8 was used on the mean Ct of the genes of interest. Relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Northern analysis was performed using standard techniques to determine quality of the mRNA prior to Q-PCR; gels were loaded with 20 µg of total RNA. Labeling of cDNA probes was achieved by random priming (Prime-It II, Stratagene, La Jolla, CA) using (ATP-$^{32}$P) dCTP (SA 3,000 Ci/mmol; New England Nuclear) according to the manufacturer’s protocol.

**Gene Sequence and QPCR Primer Design**

Several partial clones of the genes in the thyroid axis were created for this study. These genes include deiodinases (D1,D2), thyroglobulin (Tg), thyroperoxidase (Tp), Pendrin (PEN) and sodium-iodide symporter (NIS). There partial sequences can be found in appendix A. Using the NCBI search browser a “protein search” was performed for candidate gene. Once a sequence from an animal close to alligators on the phylogenetic tree was selected, candidate gene from various animals close to alligators were selected. CLUSTALX program was used to align the various sequences. Conserved regions with minimal degenerative sequences were selected for the upstream and downstream primers. Forward and reverse sequences were created and sent to Operon for degenerate primer creation. Degenerate primers were then used to get candidate gene full sequence and to determine proper sequence for quantitative PCR primers. First, degenerative primer PCR and gel electrophoresis was run to visualize if primer set was binding and amplifying the correct DNA sequence but checking if the correct base pair length for the primer set was seen in gel. Once correct band was visualized, the band was cut out of the gel and QIAquick DNA gel extraction kit was used to extract the DNA from the gel. The protocol
described in the kit manual was used with slight modifications. The DNA from the gel was then inserted into an *E.coli* vector through TA cloning. Petri dish cultures where made and clones with the insert were picked up for culturing. After culturing, we used Wizard Plus SV Minipreps DNA Purification System to extract DNA from the cell cultures. The “quick” centrifugation protocol was used. The plasmid DNA was then checked for insert DNA through gel electrophoresis. Once inserts where confirmed, samples were prepared for sequencing reactions. After sequencing, ABI Prism software for Mac was used to remove vector inserts of SP6 and T7 from the produced sequence. Then GENETYX-MAC software was used to check sequence homology and correct any unpaired nucleotide.

**Results**

**Thyroid: In Ovo PTU Treatment**

Relative expression of mRNA for ERα, ERβ, TRα, TRβ, D1, D2, TSHr, Tg, Tp, NIS and PEN were analyzed using QPCR to determine whether sexual dimorphism or differences among treatment groups existed. A 2-way ANOVA was performed on the genes of interest with sex and treatment as independent factors. No statistically significant sexual dimorphism was observed in thyroid tissue for mRNA expression of ERβ, TRα, D1, Tg, and Tp. A statistically significant difference in expression for D1 mRNA in males was observed between vehicle treatment and high and low dose *in ovo* PTU treatment (p < 0.001). Sexual dimorphism was observed for expression of Tp mRNA in the vehicle treatment groups (p < 0.001) but this pattern of sexual dimorphism was lost with PTU treatment. TSHr mRNA expression in the thyroid tissue from females displayed differences between vehicle treatment and high dose PTU exposure (p = 0.05) whereas males displayed no differences following treatment. ERα mRNA displayed sexually dimorphic expression in thyroid tissue in the vehicle control treatment (p = 0.045) that was lost
following in ovo PTU treatment (Fig. 4-3). Expression of ERα mRNA was significantly increased in females following high dose PTU exposure in ovo (p = 0.011) whereas males displayed differences following exposure to low dose (p = 0.046) and medium dose (p = 0.05) PTU (Fig. 4-3). Sexual dimorphism was observed in the mRNA expression of D2 in vehicle (p = 0.007) and medium PTU exposed neonates (p = 0.029). Treatment with PTU in ovo had no effect on D2 mRNA expression in females, however males exhibited statistically different expression between vehicle and high dose (p = 0.028) and low dose (p = 0.045) PTU exposure (refer to Fig. 4-4). NIS displayed a sexually dimorphic expression pattern in vehicle treated thyroids (p = 0.001). No treatment effect was found for thyroids from females for NIS mRNA expression. NIS mRNA expression in males displayed differences following PTU treatment with low dose PTU exposed thyroids exhibiting different expression that either vehicle (p = 0.001) and medium dose (p = 0.009) treatment groups (Fig. 4-5). PEN mRNA expression displayed sexual dimorphism following PTU exposure in ovo at all doses: low dose (p = 0.05), medium dose (p = 0.05) and high dose (p < 0.001) (Fig. 4-6). Interestingly, this sexual dimorphism is due to an increase in PEN mRNA expression in males, not females. No PTU treatment effect was found in PEN for either males or females.

**Thyroid: In Vivo after Neonatal Acute PTU Exposure**

Relative expression of mRNA for ERα, ERβ, TRα, TRβ, AR, D1, D2, TSHr, Tg, Tp, NIS and PEN were analyzed in thyroid tissue to determine whether sexual dimorphism or differences between treatment groups existed. A 2-way ANOVA was performed on the expression levels of genes of interest with sex and treatment as independent factors. No statistically significant difference in treatment or sexual dimorphism was found in TRα, TRβ, D1, Tg, and Tp. We did observed statistically significant sexual dimorphism in D2 mRNA expression in the vehicle
treatment group (p = 0.022) (Fig. 4-7). Females showed differences between high PTU exposure and either vehicle and medium dose (p = 0.05) treatments for D2 mRNA expression. Males exposed to either high dose (p = 0.008) or low dose (p = 0.023) PTU exhibited differences in D2 mRNA thyroid expression. No sexual dimorphism was found in AR mRNA expression in the neonatal thyroid (Fig. 4-8). When AR was examined following PTU treatment, males showed differences between vehicle and high dose PTU treatment (p = 0.031) (Fig. 4.8). Females displayed differences between control and all treatment groups: vehicle (p = 0.025), low dose (p = 0.002), medium dose (p = 0.025) and high dose (p < 0.001) (Fig. 4-8). ERα mRNA expression in the thyroid exhibited sexual dimorphism in vehicle treated animals (p = 0.016). ERα mRNA expression also was difference in males when vehicle and medium PTU dose (p = 0.029) exposed animals were compared. ERα mRNA expression in thyroid tissue from females were different between controls and low, medium, or high dose (p = 0.001) PTU treatments as well as between vehicle and low dose (p = 0.031) or medium dose (p = 0.047) PTU treatment (Fig. 4-9). ERβ also is expressed in a sexually dimorphic pattern in vehicle exposed thyroid tissues (p = 0.001). ERβ expression in thyroids from females displayed differences between control and vehicle treatments (p = 0.0331). Likewise, we observed that ERβ expression in thyroids from males was difference between control and low dose (p = 0.045), medium dose (p = 0.014) or high dose (p = 0.011) PTU treatments as well as between vehicle and low dose, medium dose or high dose (p < 0.001) PTU treatments (Fig. 4-10).

TSHr displayed a sexually dimorphic pattern in both control and high PTU treatment (p = 0.05) (Fig. 4-11). Females exhibited no change in TSHr mRNA expression with PTU treatment whereas males treated with high dose PTU displayed a significant increase in TSHr expression in the thyroid (Fig. 4-11). PEN was expressed in a sexually dimorphic pattern in thyroid tissue
obtained from non-treatment control animals as well as those exposed to the medium PTU dose (p = 0.037). PEN had differences in females exposed to high PTU exhibited significantly increased PEN expression compared to control (p = 0.026), vehicle (p < 0.001) or medium dose PTU (p = 0.006) treatments. Likewise, a difference was observed between low dose PTU treatment and vehicle (p = 0.03) in female thyroid tissue (Fig. 4-12). No sexual dimorphism was seen in NIS mRNA expression except in those animals treated with high dose PTU (p = 0.05). No treatment effect was seen in NIS mRNA expression in females whereas males exhibited differences between high dose PTU and vehicle (p = 0.002) or medium dose (p = 0.042) treatments as well as between vehicle and low dose (p = 0.034) treatment (Fig. 4-13).

**Gonad: In Ovo PTU Exposure**

Relative expression in gonadal mRNA for AR, ERα, ERβ, StAR and Arom was analyzed to determine whether sexually dimorphic patterns and differences between treatments existed. A 2-way ANOVA was performed on the mRNA expression of genes of interest with sex and treatment as independent factors.

No sexual dimorphism was observed in AR mRNA expression in control or vehicle exposed gonadal tissues whereas at low (p = 0.018), medium and high dose (p < 0.001) PTU treatments pronounced sexual dimorphism in AR expression is observed (Fig. 4-14). Interestingly, the pattern of AR expression changes creating this dimorphism (Fig. 4-14). For example, AR expression in ovarian tissue increased with medium and high dose PTU exposure in ovo when compared to controls (p = 0.009; p = 0.03, respectively) or vehicle (p < 0.001; p = 0.003, respectively) exposed tissues. AR mRNA expression in testicular tissue exhibited a complex pattern with low dose exposure (p = 0.012) increasing AR mRNA expression whereas
high dose exposure significantly decreasing (p = 0.05) AR mRNA expression compared to control or vehicle treated animals

Expression of ERα mRNA was sexually dimorphic in the gonadal tissue of vehicle (p = 0.05) exposed animals as well as in those exposed to the medium (p < 0.001) and high doses (p = 0.013) of PTU (Fig. 4-15). PTU treatment did not effect ERα mRNA expression in testicular tissue. In contrast, ovarian ERα expression changed with PTU treatment at medium (p < 0.001) and high (p = 0.005) doses compared to control and vehicle treatments. ERβ mRNA expression was also sexual dimorphic but only in those neonates exposed to the medium PTU dose in ovo (p < 0.001) (Fig. 4-16). No treatment effect was found for ERβ expression in males. In contrast, ERβ mRNA expression in the ovary changed with PTU treatment in ovo, as we observed differences in ovarian expression between females exposed to medium PTU dose and control (p = 0.039), vehicle (p = 0.005) and low dose (p = 0.022) as well as between vehicle and high dose (p = 0.019) exposure. Expression of StAR mRNA displayed sexual dimorphism in controls (p = 0.032), as well as those treated with vehicle (p = 0.033), low (p = 0.01) and high dose PTU (p = 0.011) (Fig. 4-17). Treatment in ovo with PTU at medium (p = 0.015) and high doses (p = 0.016) altered StAR mRNA expression in the testis with the medium dose depressing expression and the high dose increasing expression over that of the control. In ovarian tissue obtained from females exposed in ovo to PTU, treatment increased StAR mRNA expression following exposure to medium (p < 0.001) and high (p < 0.001) doses. Control tissues exhibited a highly significant pattern of sexual dimorphism in AROM mRNA expression, which was lost with exposure in ovo to the vehicle (Fig. 4-18). AROM expression was sexual dimorphic in tissues obtained from PTU treated animals at all dose: low dose (p < 0.012), medium dose (p = 0.007) and high dose (p
= 0.001) (Fig. 4-18). No treatment effects were found for AROM expression in either males or female tissues.

**Gonad: In Vivo after Acute PTU Exposure**

Male gonadal samples for the quantitative RT-PCR were lost due to degradation and poor mRNA quality, only ovary tissue were used and analyzed for this portion of the study. Relative expression mRNA for AR, ER\(\alpha\), ER\(\beta\), D1, D2, StAR, AROM was analyzed to determine whether differences between treatments existed. A 1-way ANOVA was performed on the genes of interest with treatment as the independent factor.

There were no treatment effects found for either StAR or AROM (figures not shown). AR mRNA expression decreased following high dose PTU exposure (\(p = 0.033\)) (Fig. 4-19). In contrast, treatment with PTU increased ER\(\alpha\) mRNA expression following exposure to the high dose (\(p = 0.007\))(Fig. 4-20) whereas ER\(\beta\) mRNA expression increased following treatment with either medium (\(p = 0.029\)) or high dose PTU (\(p = 0.014\)) (Fig. 4-21). Likewise, both deiodinases responded to PTU treatment, with D1 mRNA expression increasing following high dose exposure (\(p = 0.026\)) and (Fig. 4-22) as did mRNA expression for D2 (\(p = 0.003\)) (Fig. 4-23).

**Discussion**

We examined the potential role of thyroid hormones on the developing reproductive axis of the American alligator. We focused on the potential effects of depressing this axis with a pharmaceutical agent, PTU. This study examined both organizational effects with in ovo PTU treatment during the window of sexual differentiation of the gonad in developing embryos as well as activational effects with in vivo PTU treatment in neonates.
Thyroid

We examined gene expression for the same mRNAs in thyroids treated either *in ovo* and *in vivo* in neonate, and found a number of interesting patterns. As reported in Chapter 3, thyroid expresses mRNA for both ERs. ERα mRNA expression showed sexual dimorphism with females exhibiting higher concentrations than males following *in ovo* treatment with a vehicle. This dimorphism was lost with PTU treatment *in ovo*. Likewise, we observed that neonates exhibited a similar dimorphism in the expression of mRNA for both ERs, that was lost following acute treatment with PTU. Few studies have examined ER expression in the thyroid at any life stage (Fujimoto et al. 1992; Giani et al. 1993; Kawabata et al. 2003), and we know of no studies that have focused on steroid receptor expression in the thyroid of neonatal animals of any species. What is intriguing is that this study examining 12-24 h old neonates (Chapter 3) reported no sexual dimorphism in expression of either ER. However, in neonates 24-48 h old, a clear sexual dimorphism exists that is lost if the thyroid axis is pharmacologically perturbed with PTU. Two other genes, NIS and Tp examined, also exhibited sexual dimorphism in the vehicle treatment group at birth, which was lost when neonates were exposed to PTU *in ovo*. These data clearly demonstrate that our dosing altered the thyroid axis. In fact, we observed that all doses of PTU *in ovo* altered the expression of PEN in males, inducing a sexually dimorphic pattern that did not exist in vehicle treated hatchlings. Acute exposure to PTU in 14 day old neonates also altered gene expression profiles in the thyroid. We observed a sexually dimorphic pattern of mRNA expression for ERα, ERβ and D2 in the neonatal thyroid that was lost with acute treatment with PTU. In contrast, thyroid tissue from male neonates all showed an increase in TSHr, NIS and PEN following PTU exposure. Tp and NIS have been observed to increase activity in the thyroid of female rats exposed to E₂, suggesting a possible dimorphic pattern
(Lima et al. 2006). Also, in a study from the Netherlands measuring anti-Tp antibodies in a human population observed 8.6% males and 18.5% females had the anti-TP antibodies (Hoogendoorn et al. 2006). The presence of Tp antibodies was associated with abnormally high and low TSH concentrations and thyroid disorders.

One of the main questions we addressed with the current studies was to determine if altering the thyroid axis altered markers of the reproductive axis, such as steroid hormone receptors. Few studies directly examine the role of sex steroids on the thyroid and yet, this study and others have shown that the thyroid expresses sex steroid receptors (Chapter 3; Fujimoto et al. 1992; Giani et al. 1993; Kawabata et al. 2003). Current studies have shown that disruption of thyroid hormone synthesis in ovo alters the mRNA expression patterns for ERα in male and female thyroids. As predicted, various markers of thyroid function were altered such as TRα, Di, D2 and Tp expression in male thyroid tissue and yet the same pattern was not observed in thyroids removed from neonatal females treated with PTU in ovo. The basis for this difference in response is not obvious at this time, unless incubation temperature, cooler for females, could potential alter how the thyroid axis responds to PTU treatment, given that thyroid hormones are central to the regulation of metabolism (Blaxter 1988; Stevens et al. 1995; Norris 1997). We should note that we did see effects in the female, as expression for ERα, TSHr and Tp all exhibited a decrease in expression with in ovo PTU exposure. However, this initial study clearly demonstrates for the first time that steroid hormone receptor expression in the thyroid, at least estrogen receptor expression, is regulated in part by the thyroid axis.

This conclusion is further supported by our data from the acute PTU exposure study. We observed that ERα and ERβ mRNA expression decreased significantly in the thyroid obtained from males following PTU exposure. Interestingly, contrary to that observed with in ovo
treatment, females treated in vivo with PTU responded with an increase in the expression of ERα and ERβ. Again, these data provide support for the hypothesis that the thyroid axis appears to regulate the expression of ER in thyroid tissue. These data, along with data from Chapter 3, demonstrating mRNA and protein for ER are present in the thyroid indicate that significantly more work is needed to address the regulation of ER expression and its role in the thyroid. For example, a study examining whether ER expression varies seasonally in the thyroid coincident with changes in plasma T₃ and T₄ concentrations is needed.

In addition to changes in ER expression, we observed that in vivo treatment altered the expression of many of the markers of the thyroid axis, such as increased expression of TRα, D2, Tp and PEN in thyroid tissue from females and TSHr, NIS and PEN in male tissue. These data provide support that our doses were capable of altering thyroid hormone regulation and presumably feedback to the thyroid. A large literature exists in mammals demonstrating that PTU can alter many components of the thyroid axis (Moriyama et al. 2007; Gilbert and Paczkowski 2003; Diav-Citrin and Ornoy 2002 for review). However, similar studies are rare in wildlife and no previous study has examined this system in alligators. Further studies need to address the functioning of the thyroid axis following PTU treatment in vivo and in ovo by examining changes in circulating T₄ as well as other genes that are regulated by this axis.

Gonads

As we reported above, one aspect of this work was to address whether an alteration of the thyroid axis altered thyroid biology. We were also interested to determine of changes in thyroid physiology, following PTU exposure altered gonadal biology as well. The gonad of males and females express both ERs as well as the ARs. Likewise, they are steroid producing organs and thus, have the enzymes and proteins required for steroidogenesis. We noted that ERα mRNA
expression displayed a sexually dimorphic pattern with testicular tissue having higher levels than that observed in ovarian tissue. However, following in ovo PTU treatment, mid and high dose treatment induced a reversal. Expression of ERβ mRNA was not dimorphic in vehicle treated animals but following the mid PTU treatment in ovo it was dimorphic with females expressing greater levels. Similar complex responses were seen for StAR and the AR. In fact, the expression of the androgen receptor was decreased in testicular tissue following high dose PTU in ovo whereas it was increased in ovarian tissue. Previous studies have shown that altering the thyroid axis dramatically alters testis biology (Cooke et al. 2004; Jannini et al. 1995; Cooke et al. 1991). Manipulation of the thyroid environment can be used to produce increases in testis size, Sertoli cell number, and sperm production (Cooke et al. 2004). Neonatal hypothyroidism is shown to impair testicular development (Jannini et al. 1995). However, hypothyroidism in neonatal rats, which is followed by a recovery to euthyroidism, leads to an increase in testis size and daily sperm production in adult rats (Cooke et al. 1991). Cooke et al. (1994) state that it appears T3 normally inhibits Sertoli cell proliferation directly while stimulating differentiation. These actions are observed in neonatal hypothyroid animals. Developmental hypothyroidism and an increase in adult testis size is not solely described in rats but also in mice (Joyce et al. 1993), humans (Jannini et al. 2000), bulls (Majdic et al. 1998), roosters (Kirby et al. 1996) and fish (Matta et al. 2002).

In contrast, little is known about the ovarian response to altered thyroid physiology during the developmental or neonatal periods. Thyroid hormones at proper levels are necessary for ovulation (Maruo et al. 1992). Doufas and Mastorakos (2000) demonstrated that severe hypothyroidism can cause ovarian atrophy and amenorrhea. TRs are found in various parts of the ovary such as granulosa cells (Maruo et al. 1992; Zhang et al. 1997), oocytes and cumulus
cells of the follicle (Zhang et al. 1997), and corpora lutea (Bhattacharya et al. 1988), indicating that thyroid hormones can play a role in various cells of the ovary. The mechanisms of action are still not well understood.

We do know that hypothyroidism is associated with reduced fertility and the likelihood that a woman can not carry an infant to term (Buhling et al. 2007; Krassass 2000). Our data suggest that like the developing testis, the developing ovary is likely a target of the thyroid axis. Moreover, given the differential response to PTU treatment seen on testicular and ovarian tissues following in ovo or in vivo PTU treatment, it is unlikely that we can predict the ovarian response based on previous studies of the testis. For example, we noted that acute in vivo treatment with PTU increased AR mRNA expression at low doses and depressed expression at high doses. In contrast, PTU treatment in vivo, and thus a likely drop in thyroid hormone action induced an increased in ovarian mRNA expression for the AR, both ERs and StAR. Recently, it was demonstrated that thyroid hormones influence StAR. Lack of thyroid hormone causes a down regulation of StAR mRNA and protein (Manna et al. 2001b). Clearly, much further work is needed to examine the potential interaction between the developing thyroid and reproductive systems.

Summary

We predicted by blocking the thyroid with PTU during the temperature dependant sexual differentiation period of the alligator embryo, an alteration in the development of the testis or ovary and change in gene expression. We also predicted a change in gene expression on both gonad and thyroid tissue treated with PTU as neonates. Both predictions appear to be supported by these data.
In the thyroid we find that both ERα and D2 show a similar pattern suggesting influence by estrogens on D2 expression. We also note that ERβ may not play as large of a role during embryonic development and increases in function as neonate. AR data suggest that this might be a regulatory mechanism on the thyroid. Both NIS and PEN appear to be good candidate genes for regulation of the thyroid axis via sex steroids.

In the gonad, we find changes in gene expression caused by depressing the thyroid axis. AR shows possible organization changes from the in ovo PTU series. ERα and ERβ also appear to be influenced by treatment, especially in females. This trend is followed in AROM and StAR as well suggesting up regulation of the steroidogenic pathway in the ovary when thyroid is depressed.
Table 4-1: Primers used for quantitative real-time RT-PCR as markers for thyroid and gonad physiology in the American alligator (*A. mississippiensis*). Primer source are novel creations unless stated. Abbreviations represent the genes as follows: androgen receptor (AR); aromatase (Arom); deiodinases type 1, 2 (D1, D2); estrogen receptors α,β (ERα,β); ribosomal house keeping gene (L8); sodium-iodide symporter (NIS); pendrin (PEN); steroidogenic acute regulatory protein (StAR); thyroglobulin (Tg); thyroperoxidase (Tp); thyroid hormone receptors α,β (TRα,β); and thyrotropin receptor (TSHr).

<table>
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<tr>
<th>Gene</th>
<th>Primer 5' to 3'</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>AR</td>
<td>D TGTGTTCAGGGCATGACAACA U GCCCATTTCCACACATGCA</td>
<td>Gunderson et al. 2006</td>
</tr>
<tr>
<td>Arom</td>
<td>D CAGCCAGTTGAACTTGATCA U TTTGCCCTTCCTTCACAGAATAG</td>
<td>Kohno, unpublished data</td>
</tr>
<tr>
<td>D1</td>
<td>D CCACAAACTGGAATAAGGG U GCCTTGCAAACAGACGGATG</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>D CTGCGACCACACTGATCCATTG U CTGGCTTGGGTCCTGGAATAG</td>
<td></td>
</tr>
<tr>
<td>ER</td>
<td>D AAGCCTGGCCCTTCAACTTTTA U TGGCACTTCTTCTTCCG</td>
<td>Katsu et al. 2004</td>
</tr>
<tr>
<td>ER</td>
<td>D AAGACCAGGCGCAAAAGCT U GCGCAATTTTCATCCATTCAC</td>
<td>Katsu et al. 2004</td>
</tr>
<tr>
<td>L8</td>
<td>D ACGACGAGCAATAAGAC U GGTGTTGGCTATGAACTTC</td>
<td>Katsu et al. 2004</td>
</tr>
<tr>
<td>NIS</td>
<td>D CTCGGGAGTGATGGATACG U AGGTGTTCGTGATGCTCTC</td>
<td></td>
</tr>
<tr>
<td>PEN</td>
<td>D TACCAAACTGTAATCC U TGCAGGATGATGTGTTCC</td>
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<tr>
<td>StAR</td>
<td>D GTGGACCCGGAGATTTTGT U GTTGGAGCCGGGTCTTCTTAGT</td>
<td>Kohno, unpublished data</td>
</tr>
<tr>
<td>Tg</td>
<td>D ATCCCTTCTGAGTCCACACACC U AGCAGCACCACATCTTACATC</td>
<td></td>
</tr>
<tr>
<td>Tp</td>
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<tr>
<td>TR</td>
<td>D CAGAAGTGGGGATGGTGTG U TGCCAAAAACCTGCCCAT</td>
<td>Helbing et al. 2006</td>
</tr>
<tr>
<td>TR</td>
<td>D GTCTACTCTCGGGGTCATA U CACAAAGGAGCCCACTGGGA</td>
<td>Helbing et al. 2006</td>
</tr>
<tr>
<td>TSHr</td>
<td>D TTGTGACCCCTTGGCCTCC</td>
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Figure 4-1: Thyroid axis of the American alligator, *Alligator mississippiensis*. The thyroid is a bi-lobed organ nested ventrally to the trachea in the mid-throat region. The functional unit is the thyroid follicle. Numbers represent endpoints in thyroid function and physiology: 1) estrogen receptors α,β; 2) androgen receptor; 3) thyroid hormone receptors α,β; 4) thyrotropin receptor; 5) deiodinases 1, 2; 6) sodium-iodide symporter; 7) cloride-iodide co-transporter; 8) thyroglobulin; 9) thyroperoxidase.
Figure 4-2: Gonad axis of the American alligator, *Alligator mississippiensis*. The gonads are one of the primary sites for steroidogenesis. A basic steroidogenic pathway is depicted. Numbers represent endpoints in gonad function and physiology: 1) estrogen receptors α,β; 2) androgen receptor; 3) thyroid hormone receptors α,β; 4) thyrotropin receptor; 5) steroidogenic acute regulatory protein; 6) aromatase; 7) deiodinases 1, 2. 

C=cholesterol
Figure 4-3: Estrogen receptor alpha (ERα) mRNA gene expression from in ovo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Deiodinase type 2 mRNA gene expression from *in ovo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different lower case letter characters signify statistically different means in males.
Figure 4-5: Sodium-iodide symporter (NIS) mRNA gene expression from *in ovo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different lower case letter characters signify statistically different means in males.
Figure 4-6: Pendrin (PEN) mRNA gene expression from in ovo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*).
Figure 4-7: Deiodinase 2 (D2) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Figure 4-8: Androgen receptor (AR) mRNA gene expression from in vivo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males ($\alpha = 0.05$).
Figure 4-9: Estrogen receptor alpha (ERα) mRNA gene expression from in vivo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta \Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Figure 4-10: Estrogen receptor beta (ERβ) mRNA gene expression from in vivo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Figure 4-11: Thyrotropin receptor (TSHr) mRNA gene expression from in vivo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different lower case letter characters signify statistically different means in males.
Figure 4-12: Pendrin (PEN) mRNA gene expression from in vivo PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Figure 4-13: Sodium-iodide symporter (NIS) mRNA gene expression from *in vivo* PTU treatment in thyroid tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta CT}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different lower case letter characters signify statistically different means in males.
Figure 4-14: Androgen receptor (AR) mRNA gene expression from \textit{in ovo} PTU treatment in gonad tissue from the American alligator, \textit{A. mississippiensis}. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Figure 4-15: Estrogen receptor alpha (ERα) mRNA gene expression from in ovo PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females.
Figure 4-16: Estrogen receptor beta (ERβ) mRNA gene expression from *in ovo* PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females.
Figure 4-17: Steroidogenic acute regulatory protein (StAR) mRNA gene expression from *in ovo* PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*). Different capital letter characters signify statistically different means in females and lower case letter characters signify statistically different means in males.
Figure 4-18: Aromatase (AROM) mRNA gene expression from in ovo PTU treatment in gonad tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta C_t}$ method. Error bar represents 1 standard error from mean. Statistically significant ($\alpha = 0.05$) sexual dimorphism depicted by an asterisk (*).
Figure 4-19: Androgen receptor (AR) mRNA gene expression from *in vivo* PTU treatment in ovary tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta C_T}$ method. Error bar represents 1 standard error from mean. Different capital letter characters signify statistically different means in females ($\alpha = 0.05$).
Figure 4-20: Estrogen receptor alpha (ERα) mRNA gene expression from *in vivo* PTU treatment in ovary tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Different capital letter characters signify statistically different means in females ($\alpha = 0.05$).
Figure 4-21: Estrogen receptor beta (ERβ) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represents 1 standard error from mean. Different capital letter characters signify statistically different means in females ($\alpha = 0.05$).
Figure 4-22: Deiodinase type 1 (D1) mRNA gene expression from in vivo PTU treatment in ovary tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta\Delta C_t}$ method. Error bar represents 1 standard error from mean. Different capital letter characters signify statistically different means in females ($\alpha = 0.05$).
Figure 4-23: Deiodinase type 2 (D2) mRNA gene expression from *in vivo* PTU treatment in ovary tissue from the American alligator, *A. mississippiensis*. Genes were normalized to ribosomal L8 and relative expression counts were calculated using the $2^{-\Delta \Delta C_t}$ method. Error bar represents 1 standard error from mean. Different capital letter characters signify statistically different means in females ($\alpha = 0.05$).
CHAPTER 5
SUMMARY OF RESULTS

Introduction

This manuscript examined the thyroid/gonad axis of the American alligator. We investigated two major areas of thyroid/gonad activity; the affect of the thyroid axis on the development of the gonad and a mechanism of communication between the thyroid and gonad axes. In particular, the role of the thyroid axis in the development and functioning of the gonad during the neonatal and peripubertal periods was investigated. Developmental studies focused on gonadal differentiation and development following exposure to an antithyroid-agent during the window of sexual differentiation. In the studies of adolescent alligators, we described normal physiology and morphology of the thyroid/gonad axis as well as how these respective organs respond to hormonal challenges. Does the thyroid axis influence seasonal reproductive hormone variation? We also described a novel mechanism of communication between the thyroid and gonad axes. This mechanism included the characterization of ER and AR receptors on the thyroid follicle as well as expression levels of these receptors to manipulations. We proposed to test several hypotheses stated below.

- **Hypothesis 1**: Plasma thyroxine concentrations display seasonal variation that parallels seasonal variation in sex steroid concentrations, not seasonal activity patterns.

- **Hypothesis 2**: ER, AR and TR expression on the thyroid will vary among life stages and show sexual dimorphism.

- **Hypothesis 3**: Treatment of the thyroid with PTU will alter gene expression on the gonad to genes related to gonad physiology.

- **Hypothesis 4**: By blocking the thyroid with PTU during the temperature dependant sexual differentiation period of the alligator embryo, we predict an alteration in the development of the testis or ovary.
In addition, Chapter one began to elucidate on questions regarding the hypothalamus-pituitary-thyroid-gonad (H-P-T-G) axes of regulation. Collaboration with professor Caren Helbing, University of Victoria, has recently produced cloned TRα and TRβ2 from the American alligator. Using quantitative RT-PCR (Q-PCR), we have observed that both TRα and TRβ2 are expressed in the gonads of juvenile alligators (Helbing et al. 2006), with greatly elevated levels of TRβ2 relative to TRα. Further, there appears to be a differential response to TSH treatment, with no effect on TRβ2 mRNA after treatment, but elevation of TRα mRNA levels in the testis but not the ovary. These data suggest that, like the rodent gonad, cells in the alligator gonad express TR, suggesting that this tissue is responsive to the actions of thyroid hormones. We also answer whether TSH has an effect on the gonad (Fig. 5-1). We find that TSH up-regulates TR mRNA expression in the gonad, possibly through stimulation of the thyroid.

**Seasonal Thyroxine Variation**

In Chapter 2, we addressed hypothesis 1: whether or not plasma thyroxine concentrations display seasonal variation that parallels seasonal variation in sex steroid concentrations, not seasonal activity patterns. We observed that juvenile American alligators display seasonal variation in circulating T4 concentrations. Further, comparing the seasonal pattern observed in plasma concentrations of T4 with the seasonal patterns in other hormones, such as T and E2 we find that the thyroxine follows a similar pattern of variation to sex steroids in juvenile alligators. We hypothesized that thyroid hormones could play a cooperative role with T and E2 in juveniles, helping stimulate important events in puberty. We demonstrated that a relationship exist between the thyroid axis and the gonad axis. The relationship found with circulating levels of thyroxine and sex steroids led us to ask how are the thyroid and gonad axes communicating with one another?
Characterization of ERs on the Thyroid

In Chapter 3, we addressed hypothesis 2: ER, AR and TR expression on the thyroid will vary among life stages and show sexual dimorphism. The thyroid axis may have a role in regulating the gonads and vice versa. Sex steroid receptors on the thyroid are thought to be nuclear receptors, which regulate target gene expression involved in metabolism, development, and reproduction (McKenna and O-Malley, 2001). The role that these sex steroids and their receptors play in the regulation of the thyroid is not currently well understood.

This study demonstrated that mRNA for both forms of the ER, both forms of TR and AR are found on the thyroid of the American alligator (A. mississippiensis). No sexual dimorphism was observed in the mRNA expression of these genes in the thyroid tissue examined. However, the presence of sex steroid receptors provides a potential mechanism by which gonadal steroids could influence thyroid development and function. This also brings insights to how the gonad axis communicated back to the thyroid axis via the H-P-T-G axes (Fig. 5-1). This is the first study to describe ERs in the thyroid of a none-mammalian species and to characterize the expression with mRNA expression and protein expression. Further studies are required to determine if such a regulatory pathway exists via ERs in the thyroid.

PTU Exposure in the Thyroid and Gonad

Hypothesis 3 and 4 are addressed in Chapter 4. Treatment of the thyroid with PTU does alter gene expression on the gonad to genes related to gonad physiology (hypothesis 3). Also, by blocking the thyroid with PTU during the temperature dependant sexual differentiation period of the alligator embryo, we observed organization changes in mRNA expression in the thyroid, testis or ovary.

In the thyroid we find that both ERα and D2 show similar patterns suggesting that D2 could potentially, be influenced by estrogens. We also noted that ERβ may not play as large of
role during embryonic development and increases in function as neonate. AR data suggest that this might be a regulatory mechanism on the thyroid. Both NIS and PEN appear to be good candidate genes for regulation of the thyroid axis via sex steroids.

In the gonad we found changes in gene expression caused by depressing the thyroid axis. AR shows possible organization changes from the in ovo PTU series. ERα and ERβ also appear influenced by treatment, especially in females. This trend is followed in aromatase and StAR as well suggesting up regulation of the steroidogenic pathway in the ovary when thyroid is depressed. Results for mRNA expression in the thyroid or gonad are summarized in figures below. PTU in ovo sexual dimorphism and treatment effects in thyroid tissue for males or females are displayed in Figs. 5-2, 5-3 and 5-4 respectively. PTU in vivo sexual dimorphism and treatment effects in thyroid tissue for males or females are displayed in Figs. 5-5, 5-6 and 5-7 respectively. Figure 5-8 displays PTU in ovo sexual dimorphism in gonad tissue. PTU in ovo treatment effects in gonad tissue for males or females is displayed in Fig. 5-9. PTU in vivo treatment effects in gonad tissue for females are displayed in Fig. 5-10.

We examined the potential role of thyroid hormones on the developing reproductive axis of the American alligator. We focused on the potential effects of depressing this axis with a pharmaceutical agent, PTU. This study examined both organizational effects with in ovo PTU treatment during the window of sexual differentiation of the gonad in developing embryos as well as activational effects with in vivo PTU treatment in neonates. Further work is necessary to elucidate the mechanisms involved in the regulation of the thyroid from the gonad. These studies provide a good foundation to begin to understand the interactions between the thyroid and gonadal axes.
Figure 5-1: Thyroid-gonad axis of regulation revisited. TSH secreted from pituitary has stimulatory role on thyroid and gonad. FSH secreted from pituitary has stimulatory role on gonads. Estradiol secreted from gonads plays an inhibitory role in pituitary on FSH secretion. Estradiol possibly plays a regulatory role on thyroid.
Figure 5-2: *In Ovo* PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of juvenile American alligators (*A. mississippiensis*). This graphic represents whether sexual dimorphism existed.

Figure 5-3: *In Ovo* PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of male juvenile American alligators (*A. mississippiensis*). This graphic represents whether treatment effects existed. Intermediate expression not statistically different from either vehicle or treatment represented by fade effect.
Figure 5-4: *In Ovo* PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of female juvenile American alligators (*A.mississippiensis*). This graphic represents whether treatment effects existed. Intermediate expression not statistically different from either vehicle or treatment represented by fade effect.
Figure 5-5: *In Vivo* PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of juvenile American alligators (*A. mississippiensis*). This graphic represents whether sexual dimorphism existed.
Figure 5-6: *In Vivo* PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of male juvenile American alligators (*A. mississippiensis*). This graphic represents whether treatment effects existed. Intermediate expression not statistically different from either vehicle or treatment represented by fade effect.

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<th>Gene</th>
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- **Yellow**: No Change
- **Blue**: Decrease
- **Red**: Increase
Figure 5-7: *In Vivo* PTU mRNA expression of genes analyzed via QPCR in thyroid tissue of female juvenile American alligators (*A. mississippiensis*). This graphic represents whether treatment effects existed. Intermediate expression not statistically different from either vehicle or treatment represented by fade effect.
Figure 5-8: *In Ovo* PTU mRNA expression of genes analyzed via QPCR in gonad tissue of juvenile American alligators (*A. mississippiensis*). This graphic represents whether sexual dimorphism existed.

Figure 5-9: *In Ovo* PTU mRNA expression of genes analyzed via QPCR in gonad tissue of juvenile American alligators (*A. mississippiensis*). This graphic represents treatment effects existed in males or females.
Figure 5-10: *In Vivo* PTU mRNA expression of genes analyzed via QPCR in gonad tissue of female juvenile American alligators (*A.mississippiensis*). This graphic represents whether treatment effects existed. No males mRNA expression was examined.

<table>
<thead>
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<th>Gene</th>
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<th>Medium PTU</th>
<th>High PTU</th>
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![Color Key](image)

No Change | Decrease | Increase
APPENDIX A
APPENDIX STAINING PROTOCOL FOR ERα IHC

Table A-1: Immunohistochemistry staining protocol for ERα. Both the Vector Elite IHC kit and the ER-α antibody were obtained from the Santa Cruz Biotechnology Inc. (Santa Cruz, California).

<table>
<thead>
<tr>
<th>DAY ONE</th>
<th>SOLUTION</th>
<th>TIME</th>
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<tbody>
<tr>
<td>Deparaffinize and hydrate</td>
<td>Citrisolve X2 for 5 min, 100% EtOH X2 for 5 min, 95% EtOH for 5 min. Wash in deionized H2O for 1 min with stirring</td>
<td>30 min</td>
</tr>
<tr>
<td>Unmask antigens</td>
<td>0.02M Citrate Buffer (pH 6.0); Microwave (&gt;7000W)- High 3 min, Medium 3 min, Low 3 min, and cool to room temp ~20 min</td>
<td>35 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>PBS</td>
<td>2-3 times</td>
</tr>
<tr>
<td>Pap pen</td>
<td>Wipe away excess liquid around the sections and circle</td>
<td>dry 1-2 min</td>
</tr>
<tr>
<td>Soak</td>
<td>PBS</td>
<td>5 min</td>
</tr>
<tr>
<td>Block endogenous peroxidase</td>
<td>3% Hydrogen Peroxide</td>
<td>30 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>PBS</td>
<td>2 min</td>
</tr>
<tr>
<td>Block normal goat serum (~20 μl)</td>
<td></td>
<td>60 min</td>
</tr>
<tr>
<td>Aspirate</td>
<td>Aspirate serum from slides</td>
<td>&gt;1 min</td>
</tr>
<tr>
<td>Incubate</td>
<td>Primary antibody (dilute 1:400 with normal goat serum); negative control</td>
<td>Over night at 4C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DAY TWO</th>
<th>SOLUTION</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rinse</td>
<td>PBS</td>
<td>2 min</td>
</tr>
<tr>
<td>Incubate</td>
<td>Secondary Antibody (~20 μl)</td>
<td>30 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>PBS</td>
<td>2 min</td>
</tr>
<tr>
<td>Incubate</td>
<td>Peroxidase reagent (~20 μl)</td>
<td>30 min</td>
</tr>
<tr>
<td>Rinse</td>
<td>PBS</td>
<td>2 min</td>
</tr>
<tr>
<td>Make HRP substrate</td>
<td>In a mixing bottle, add 1.6 mL of deionized water, 5 drops 10X substrate buffer, 1 drop 50X DAB chromagen, and 1 drop 50X peroxidase substrate</td>
<td></td>
</tr>
<tr>
<td>Visualize</td>
<td>HRP substrate (1-3 drops)</td>
<td>8 min</td>
</tr>
<tr>
<td>Rinse and wash</td>
<td>deionized water</td>
<td>2 min</td>
</tr>
<tr>
<td>Dehydrate and mount</td>
<td>ethanol/dehydrate (2X 95% -10 sec; 2X 100% - 10 sec; Citrisolve until mounting with Permount)</td>
<td></td>
</tr>
</tbody>
</table>
### APPENDIX B

**APPENDIX PARTIAL SEQUENCES FOR CLONED THYROID GENES**

| Gene       | Accession Number | Length (bp) | Identity (%) | Sequence  
<table>
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<tbody>
<tr>
<td><strong>NIS</strong></td>
<td></td>
<td>180/2,070</td>
<td>8.7%</td>
<td>TGGACTGATGTGTTTCAGGTGTTCGTGATGCTCTCCGGGTTTCGTCGCCATCGCCAATCCAGGGCACGTGATGTGTTGGAGGAGGAGGTCTGGGCGGCCGCTACAACATCTTCTGGACCTTCGTA</td>
</tr>
<tr>
<td><strong>PEN</strong></td>
<td></td>
<td>780/2,349</td>
<td>33.2%</td>
<td>AATCAGGAGTTTTATTCATTTTGGGATGCAGCAATGTGCTTTCAGGAGCTTTTTCCTGTTTGTTGCTACAACTGCACTTTCACGTGCCCTGGATGGCGATGGCGACGAGCCGCCGATCCAGGATGTTCTTCGAT</td>
</tr>
<tr>
<td><strong>Tg</strong></td>
<td></td>
<td>561/8,322</td>
<td>6.7%</td>
<td>AATATCTTTGAGTATCAGGTGGAATCCAGCCTCTACGTCCATGTGAGCTTCGGAGAGAAAAGGCCTTTCTGGAAGGAGAAGATCATGTTCCCCAGTGCTCAGAAGATGGCCGTTCCGGACTGTGCAGTGCAGCAAGAACAACCTTTCCTGCTGGTGTGTAGATGACAGGGGAGCTGAAGTACCAGGCAGTAAACAGAATGGAGTTCCCATATCCTGTTTATCCTTTGTTCAGTGCAAATTTGTCAACATGACCGACATGATGATATTCGAT</td>
</tr>
<tr>
<td><strong>Tp</strong></td>
<td></td>
<td>519/2,700</td>
<td>19.2%</td>
<td>CACCCGGATAATATTGATGTGTTGGCTGGCTAGCAGAAATCCAGCCTCTACGTCCATGTGAGCTTCGGAGAGAAAAGGCCTTTCTGGAAGGAGAAGATCATGTTCCCCAGTGCTCAGAAGATGGCCGTTCCGGACTGTGCAGTGCAGCAAGAACAACCTTTCCTGCTGGTGTGTAGATGACAGGGGAGCTGAAGTACCAGGCAGTAAACAGAATGGAGTTCCCATATCCTGTTTATCCTTTGTTCAGTGCAAATTTGTCAACATGACCGACATGATGATATTCGAT</td>
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119
ATACAGGACTTTTCAGAAGTGCAGTTGATGCTTTTCAACTTGGGAAGTTTCTCAG
ACTTTGAGTCTGTGACAATAATACGAGGAAATAATTTGAGAGCAGGAGAAACT
ATGAGCAGAGAGAGCTGTGAGCCACCTTTACAGAAATGTGAGATGACTTTCTGA
TATTGTCAGAAGACTGCGAAATTCCATAGTGATTTATTCATGTCAATTTGGATTCAGC
TACATGGAAGAAGACATTACCTGTACAAATCTAAAGATGGAATTTCACCACCA
GTTTGATAGACGTCAACGAATGC

TSH-r  Thyrotropin receptor  550/2,475 bp  22.2%
3-5
CATTGAGTGCGATAATTAAGAAGAAAGGAGTAGTGCGGAATACCTGAT
GTGTAACCAGACAGCGATTATAACGTGCTTAAGAGAAGATCTGTAAGTGCTTCAA
TTGTCCTTTTTACCAAGACACTATGCAACGCACATACGTGAGGACTGTGACT
ATGACAAAAATCTCAAATTCAGGGGATTTTTATGCAATTTCCACTACTATGTCTTTTT
TGAAGAGCAAGAGGAGATGGTGAGTTGTGAGTTTGCCCAGAAATCTAAAGCGACTTG
AGGAAATGCCCAGATTTGCAAGGGATGTGACTATGCAATTCGTAATCGGACGAT
GAAGAAATAGTATGCGACCCAGAGCTGATGATTTATCTTGAGAAGACATTAAT
GGGATATTTTGTTCGTTCTACCTTCTACACGACCATTACAGTTGACTGTCCACAGT
TACAGAGCTTTACCTGCAGGACTCTGCAATGAG

D1  Deiodinase type 1  Helbing lab/Nik Veldhoen 10/20/2005  306/540 bp
5-3
CTTCTTTGAAATTGAGTCGACTTTAACACACTGTGACGTTCACTTCACTCCT
CTCAAGGAAACCTTCAAGGCCAGTTGCAATGTAACAGAT
TTCCCTTTAATCTACATGGAAAGCCTGATCGAACAGACGAGATGGGCTTTAAAAAT
AATATTGTTTATTTTTATACACCAAAAACCTTGAAGATCGAAAATGGCTGCACGGTTT
CTTCTTTTAAAGAAACCCCTTATAGGCATTTGGAATATCTTGCTGACGAGTTT
AGCTTAAAGTATGTCTGTCTTCGACACAGGACTTTACCTGCTTCAAGGAAGAAAGGT
GTTTATAAGGGTGAGAG

D2  Deiodinase type 2  Helbing lab/Nik Veldhoen 10/20/2005  526 bp
5-3
CTTCCTTTGCACTCTATGATTCTGTGACTCCCTCCTGAAAGCAGCAGCTTGTGCTTCTGTG
CGGTTCTAGCTCTGCGGGTGGTAGGGCGGAGATGCTGACTTCCAGGAGGCTGCG
TTGCCTGTGAGGATACTTCATCCCTGCTTCAAGAGATGGGAGGGACTGGGAGAA
AGGCCCAACTTCCAGAGTGAATCCATAACACAGTGGGACGCTGACGAGTTGACAC
AGATGGCAAGAATGTTGGGAGAGTGTGGGAGGAGGACTTGGGACGAGTTGCTGAT
 CCTTGTTGCTAATTAAAGGAGGCTCAGTAACCGGGCTGACTGCCGATCCG
GCCAGCTGTAGCCCTCAAGCGCAGCTGGAGGAGGACTTTGACGGTGATGAGGTGCGG
CTTGGTGTACAGAGGGATGTCAGTACTTCCAGCCTTACACCCATGGAATGATGAAT
CGCCCTTCTCATTGGTAGGAAGAACGACAAAGACGAGGACGAGGACTGTGACG
GCTCACCAGCTCCTG
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Brucker-Davis, F., 1998. Effects of environmental synthetic chemicals on thyroid function. Thyroid 8, 827-856.


Segal, J., 1990. Calcium is the first messenger for the action of thyroid hormone at the level of the plasma membrane: first evidence for an acute effect of the thyroid hormone on calcium uptake in the heart. Endocrinology 126, 2693-2702.


BIOGRAPHICAL SKETCH

Dieldrich Salomon Bermudez was born in the summer 1976 at 7:49 pm in Managua, Nicaragua. At age 2, his family moved to Los Angeles, California. He rejoined them 2 years later. Dieldrich attended elementary school in La Puente, CA. When he turned 10, he and his family moved to Miami, Florida. There he attended public school and graduated from Miami Coral Park Senior High in 1995. During high school, he volunteered at the Miami Museum of Science Falcon Batchelor Bird of Prey Center and was student body president. He then attended the University of Florida, Gainesville, FL. Dieldrich received a Bachelor in Science from the University of Florida in 1999, graduating with honors. He double-majored in psychology and zoology. During his tenure as an undergrad, he completed an undergraduate research project titled “Immunological effects of endocrine-disrupting contaminants on alligator (A. mississippiensis) spleen morphology” directed by Drs. Louis J. Guillette, Jr. and Andrew A. Rooney. Dieldrich was awarded a CLAS Undergraduate Research award for the project. After graduation, Dieldrich worked for the Florida Fish and Wildlife Conservation commission as a field biologist and alligator egg research technician.

In August 2000, Dieldrich began his graduate career at the University of Florida, Zoology department under the tutelage of Dr. Louis J. Guillette, Jr. In 2004, he completed the requirements for his Master’s (via bypass) and continued with his Ph.D. work. During his graduate tenure at the University of Florida, Dieldrich received a Florida-Georgia Louis Stokes Alliance for Minority Participation fellowship, a Sigma Xi Grants in Aid of Research, an NSF East Asia and Pacific Summer Institutes fellowship, a Delores A. Auzenne Graduate Scholars fellowship, a Science Partners in Inquiry-based Collaborative Education fellowship and an NIEHS Minority Predoctoral fellowship. Also while at the University of Florida he was employed as a graduate teaching assistant for Introductory Biology, Animal Physiology, and
Biology of Reproduction. Dieldrich also engaged in an active mentoring program directing 25 students in research projects. Eight students under his direction completed senior undergraduate research thesis or earned co-authorship on papers derived from this dissertation or peripheral project.