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Sex Steroid and Thyroid Hormone Receptor Expressions in the Thyroid of the American Alligator (*ALLIGATOR MISSISSIPPIENSIS*) During Different Life Stages

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ABSTRACT The expression of estrogen receptors, ESR1 (ER α) and ESR2 (ER β), and androgen receptors (AR) in the thyroid gland has been reported in few vertebrate species other than a few mammals. This study reports the presence of sex steroid hormone receptors and thyroid receptors (ER α , ER β , AR, TR α , and TR β) in the thyroid gland of the American alligator at several life stages. It provides a semiquantification and distribution of ER α in the thyroid follicle cells using an immunohistochemical approach as well as reports quantitative differences in mRNA expression of ER α , ER β , TR α , TR β , and AR in the same tissue using quantitative real time-PCR (Q-PCR) with primers designed specifically for alligators. The thyroid tissue of the American alligator expresses ER α , ER β , and AR at all of the life stages examined here although no statistically significant differences were observed between male and female in thyroid mRNA expression for any of the genes analyzed. No sexual dimorphism was observed in ER α immunostaining. No statistical analysis across life stages were performed due to confounding factor of season. *J. Morphol.* 272:698–703, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: alligator; ER α ; thyroid

INTRODUCTION

The thyroid and its hormones play essential roles during development and growth of numerous tissues, such as the central nervous system and skeletal system (Styne, 1998; Cayrou et al., 2002; Bernal et al., 2003; Norris, 2007). It has been shown to play various roles in homeostasis, cellular metabolism, and reproduction (Cooke et al., 1991; Arambepola et al., 1998; Norris, 2007). In juvenile American alligators, seasonal variations in plasma thyroxine concentrations parallel seasonal

variations in sex steroid concentrations (Bermudez et al., 2005). This pattern suggest that the thyroid axis could have a role in regulating gonadal activity and vice versa. Sex steroid hormone receptors in the thyroid are thought to be of the nuclear super family of receptors, which regulate target gene expression involved in metabolism, development, and reproduction (McKenna and O'Malley, 2001). The role that these sex steroid hormones, and their receptors, play in the regulation of the thyroid is not currently well understood.

The presence of estrogen receptors, ESR1 (ER α) and ESR2 (ER β), and androgen receptors (AR) in cells of the thyroid has been reported for mammals, namely humans, rats and livestock (Fujimoto et al., 1992; Giani et al., 1993; Madej et al., 2002; Kawabata et al., 2003). In humans, thyroid disorders are more frequently diagnosed in the female compared with the male population (approximately

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3:1; Paterson et al., 1999; Manole et al., 2001; Arain et al., 2003; Chen et al., 2008). Further, neoplastic tissues from human thyroid glands express a higher number of ERs when compared with normal tissue (Manole et al., 2001; Chen et al., 2008), suggesting a potential relationship between sex and susceptibility to thyroid abnormalities. Gonadal steroid hormones have been shown to influence thyroid function by altering clearance of thyroxine-binding globulin (TBG), with estrogens increasing serum TBG concentrations and androgens decreasing concentrations of TBG (Tahboub and Arafah, 2009). Testosterone and estradiol up-regulated their respective receptors in the thyroids of gonadectomized rats (Banu et al., 2002). These findings suggest that ER signaling could play a significant role in thyroid regulation.

This study describes the presence and distribution of sex steroid hormone (ER α , ER β , and AR) and thyroid hormone receptors (TR α and TR β) in the thyroid gland obtained from alligators at several life stages and provides a semiquantification of ER α protein using an immunohistochemical approach. Quantitative differences in mRNA expression of ER α , ER β , TR α , TR β , and AR also were determined in the same tissue using quantitative real time-PCR (Q-PCR) with primers designed specifically for alligators. This study examines the potential sexual dimorphism in hormone receptor expression in the alligator thyroid gland. Because of the presence of both ER α and ER β throughout life in the thyroid gland of humans (Kawabata et al., 2003), we hypothesized that other vertebrates, such as alligators, would also express both ERs in the thyroid gland although this has not been documented in any vertebrate other than mammals.

The thyroid gland plays a role in sex steroid hormone regulation, and because hormone production changes with an animal's developmental stages from neonate to adult, it is possible that steroid hormone receptor expression changes with developmental maturity. To investigate whether sex steroid hormone receptor expression changes ontogenically, we examined alligators from neonate, juvenile, and adult life stages.

MATERIALS AND METHODS

Animals

Five male and five female neonatal, juvenile, and adult alligators were collected from Lake Woodruff (latitude 29°06'N, longitude 81°25'W), Florida, USA. In June of 2003, the juvenile specimens were captured at night from an airboat by a hand-restraint technique. These juvenile American alligators (*A. mississippiensis*) ranged from 84.6–137.6 cm in total length with a mean length of 110.2 cm and were captured between 2100 and 0100 h. On capture, they were sexed and placed in a cloth bag for transport back to the University of Florida where they were euthanized and tissues obtained within 10 h of initial capture.

In late June 2003, 12 eggs were collected from Lake Woodruff and transported to the University of Florida. Because the sex of alligators is temperature dependent, six eggs were incubated at 33.5°C, a temperature that generates 100% males, 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 obtained. The neonates ranged from 23.5 to 26 cm in total length with a mean length of 24.9 cm. In September of 2003, adult alligators were captured at night using a standard noosing technique. The adult alligators ranged from 178 to 333 cm in total length with a mean length of 225.4 cm were captured during the 2300–0200 h. The specimens were sexed in the field and transported to the University of Florida where the alligators were euthanized and tissues obtained within 7 h.

Euthanasia was performed by an overdose of sodium pentobarbital, injected intravenously into the postcranial vertebral vein, a protocol approved by the University of Florida IACUC. Thyroid glands were removed from all specimens and divided into two lobes. One thyroid lobe was preserved in cold Bouin's 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. For immunohistochemical analysis of ER α expression, each animal was represented by a set of slides that consisted of three slides (with multiple tissue sections): one control slide that was not treated with primary antibody, one experimental slide in which tissues were exposed to the primary antibody for ER α , and one normal hematoxylin and eosin stained slide. Experimental slides were treated using standard immunohistochemistry techniques (Vector Elite ICC kit) and antibodies specific for the AR (C-19, Santa Cruz Technology): sc-815 or ER α (MC-20, Santa Cruz Technology): sc-542.

Once the slides were stained, three intact thyroid follicles were analyzed. The total number of positive stained nuclei from the immunohistochemical slide was divided by the total number of nuclei from the same follicle. These ratio data were then transformed by arcsine conversion for use in statistical analyses. The average for the three follicles was used to represent the relative ER α protein expression in the thyroid gland of each alligator. This technique was used to provide a semiquantification of ER α protein expression levels in the thyroid gland. Comparisons between sexes at each life stage was analyzed using StatView software with a significance $\alpha = 0.05$ (version 5.0; SAS Institute, Cary, NC). 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 with the available antibody so that an analysis could be performed.

Isolation of RNA, Reverse Transcription, and Northern Blots

Quantitative real time-PCR (Q-PCR) was performed to quantify mRNA expression levels for ER α , ER β , AR, TR α , and TR β in neonatal, juvenile, and adult thyroid gland tissue. The techniques used were those that we have validated previously for alligator tissues (Katsu et al., 2004; Helbing et al., 2006).

Quantitative real time-PCR was performed using standard techniques. In short, total RNA was isolated with a RNeasy kit (QIAGEN, Chatsworth, CA). First strand cDNA synthesis was performed on 4 μ g of total RNA using SuperScript II RNase H-Reverse Transcriptase (Invitrogen, Gaithersburg, MD) and oligo (dT)12–18 (Invitrogen) to reverse transcribe polyA+ mRNA. Primer annealing was carried out at 70°C for 10 min, before reverse transcriptase was added. Conditions for

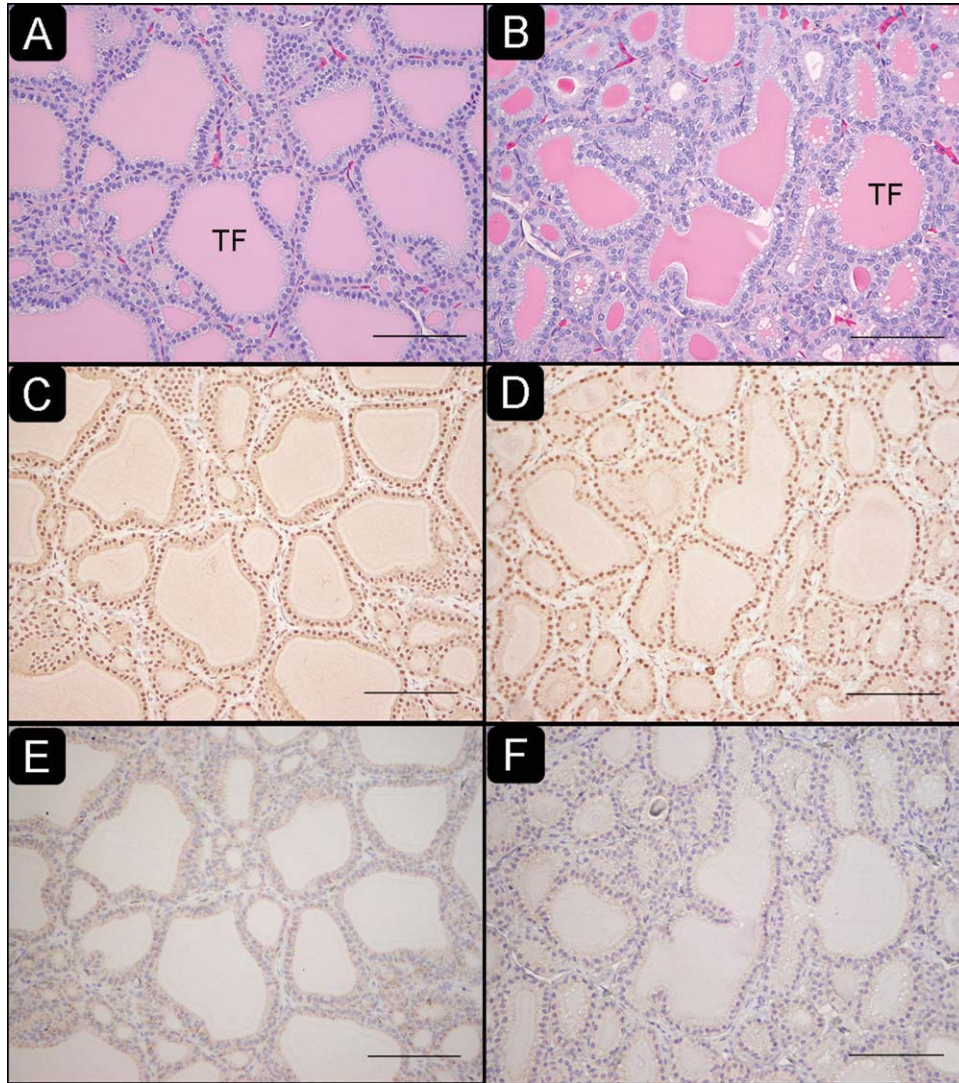


Fig. 1. Photomicrograph of juvenile (A,C,E) and adult (B,D,F) American alligator (*A. mississippiensis*) thyroid gland tissue. Tissues presented at stained with H&E (A,B), primary antibody MC-20 for ER α (C,D) or the negative control (E,F). Note the extensive positive staining in the nuclei of thyroid follicle (TF) cells. Scale bar represents 100 μ m.

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. 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. Quantitative real time-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 μ l per well was used. Quantitative real time-PCR conditions were 50°C for 2 min, 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 (ER α , ER β , TR α , TR β , and AR). Relative expression counts were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Northern analysis was performed using standard techniques to determine quality of the mRNA before Q-PCR; gels were loaded with 20 μ g 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.

RESULTS

Immunohistochemical Localization of ER α

Localization of ER α was visualized in the thyroid follicle cells using a mammalian polyclonal antibody (Fig. 1). ER α expression was observed at a high level, with the majority of the nuclei of the thyroid follicle cells displaying positive staining for the ER α antibody at all life stages examined. Using the ratio of IHC-ER α positive cell nuclei to nonpositive nuclei, an ANOVA revealed that sexual dimorphism was not detected in ER α protein expression, as determined by immunohistochemistry, at any of the life stages examined in this study (Fig. 2). Adult, juvenile, and neonate tissue samples were collected at different times of the year, which precluded evaluation of ER α across life stage due to confounding factor of season.

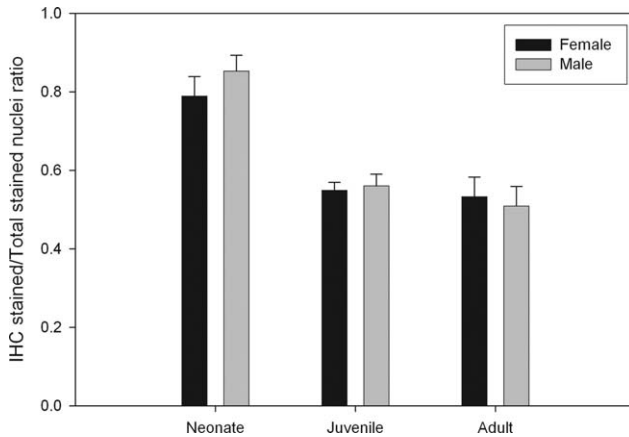


Fig. 2. Mean ratio for immunohistochemistry (IHC) ER α expression (measured by the ratio of IHC ER α positive staining to normal hemotoxylin and eosin staining) in the thyroid follicle at three life stages in the American alligator (*A. mississippiensis*). Error bars are ± 1 standard error from the mean. No sexually dimorphic pattern was observed.

Quantitative RT-PCR

Thyroid tissues from neonatal, juvenile, and adult alligators expressed mRNA for ER α , ER β , TR α , TR β , and AR. Using a Q-PCR approach, we examined whether a sexually dimorphic pattern existed in the expression of sex steroid hormone receptors or thyroid hormone receptors. No statistically significant differences were observed between male or female thyroid gland mRNA expressions for any of the genes analyzed in this study, although we did observe very high variance in expression of all of the genes studied (Fig. 3). Confounding factor of season precluded analysis of gene expression across life stages.

DISCUSSION

There is a dearth of literature describing the presence of ERs and ARs in cells of the thyroid from nonmammalian species. The data presented demonstrates that the thyroid of a nonmammalian vertebrate can express both forms of ERs, TRs, and AR throughout its life. Both forms of ER (ER α and ER β) are known to be expressed in the human thyroid gland (Kawabata et al., 2003). Further, we were able to observe that the mRNA for ER α is translated to protein, as we detected its presence in the thyroid follicle cells using an antibody specific for ER α .

When IHC-derived ER α expression in thyroid gland tissues were analyzed, we were unable to detect a sexually dimorphic pattern at any of the three life stages examined. These results are contrary to our initial hypothesis that females would display higher ER α expression in the thyroid gland. It is important to note that we were count-

ing positive nuclei per thyroid follicle and this would be but one measure of potential estrogen activity in a thyroid follicle; that is, an indication of the number of potential cells that could respond to an estrogenic signal. When we quantitatively measured ER mRNA expression for both isoforms of ER, in thyroid gland tissues from males and females at various life stages, no sexual dimorphism was detected in mRNA expression for either ER α or ER β . Both of these measurements, IHC and mRNA expression, are but estimates of potential ERs activity in a thyroid follicle and do not preclude that a sexually dimorphic pattern might exist. Recent studies examining the mammalian thyroid gland suggest that ER expression is not sexually dimorphic, but rather, the postligand binding response of ERs to E $_2$ is dimorphic (Correa da Costa et al., 2001; Lima et al., 2006; Marassi et al., 2007). Estrogens exert effects by activation of MAP kinases as well as by binding to ERs.

However, like other vertebrates, there clearly is a sexually dimorphic pattern in hormone expression, as female alligators exhibit elevated plasma concentrations of various estrogens when compared with males at all ages except neonates (Guillette et al., 1994; Guillette et al., 1997; Milnes et al., 2002; Milnes et al., 2005; Moore et al., 2010). As alligators sexually mature, the plasma concentrations of sex steroids increase. We

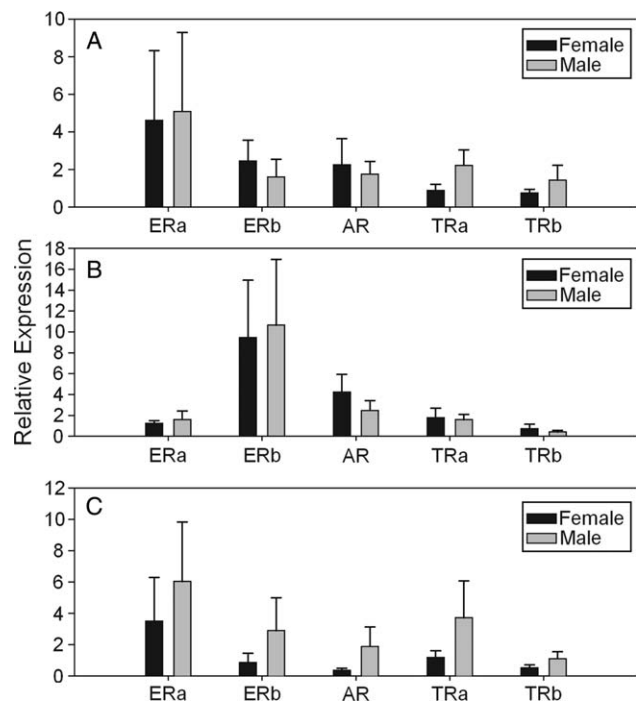


Fig. 3. mRNA gene expression in thyroid gland tissue from neonatal (A), juvenile (B), and adult (C) American alligator (*A. mississippiensis*). Genes were normalized to ribosomal L8 and relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method. Error bar represent ± 1 standard error from mean. No sexual dimorphic patterns were observed.

hypothesized that, because adults have higher plasma concentration of E₂ compared with the other life stages (Guillette and Milnes, 2000; Rooney et al., 2004), the adult alligators would show lower ER α ratios and, therefore, lower ER α expression due to a potential feedback loop that would downregulate the expression of the receptor. Our immunohistochemical data suggests that neonates have a significantly higher ratio of ER α compared with either the juvenile or adult alligators. The antibodies for ER α were an affinity purified rabbit polyclonal antibody rose against a peptide (12–20 amino acids) mapping between 550 and 599 of the carboxyl-terminus for ER α of mouse origin. The similarity of E/F domain of ER α between the American alligator and mouse is 86%. Although both mouse and alligator ER α display strong sequence homology at the E/F domain, caution should be taken when interpreting solely from immunohistochemical results.

The difference between sexes in circulating hormone concentrations, coupled with the active expression of ER in the thyroid, suggests that 1) estrogens likely play an important and largely unstudied role in the regulation of the thyroid of nonmammalian vertebrates, and 2) the thyroid gland could be a target of various ubiquitous environmental contaminants known to function as estrogen, androgen, or thyroid hormone receptor agonists or antagonists (McLachlan, 2001), again an area of research largely unexamined in all vertebrates. Given the growing list of environmental estrogens (Rooney and Guillette, 2000), antiandrogens, and now thyroid hormone receptor antagonists, this should be a growing concern, given the critical role, the thyroid gland plays in metabolism, thermoregulation, growth, nerve function, and reproduction.

Further, we observed that both forms of ER are expressed in the thyroid gland of the American alligators, and previous studies have shown that these receptor isoforms can have different physiological roles in cells. An ER α agonist can play a role in the proliferation of thyroid cancer cell growth, whereas an ER β agonist can reduce proliferation of cancer cells (Ceresini et al., 2006; Cho et al., 2007; Chen et al., 2008).

One confounding factor in comparing the life stages should be noted; although we have demonstrated that ER α protein and mRNA for ER α and ER β are expressed in the thyroid gland, the available tissues from each life stage came at different times in the year. Tissues from neonatal alligators were obtained immediately after hatching in late August, whereas those from juveniles were obtained during mid-summer (June) or during September for adults. We note this, as a previous study examining the tissue distribution of ER α in alligators done in collaboration with our group, observed no ER mRNA expression for juvenile

alligator thyroid gland tissue (Helbing et al., 2006) in contrast to our findings. Interestingly, the animals in that study were collected from the wild (Lake Woodruff NWR, Florida) 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., 2002) than those examined by Helbing et al. (2006). These data suggest possible life stage differences, but that is unlikely given that we observed ER α mRNA expression in the thyroid gland tissue of neonates, juveniles, and adults. We suggest that possible seasonal variation in the expression of ERs in the thyroid is more likely and this needs to be tested, although the protected status of this animal could preclude monthly sampling for such a test.

The alligator thyroid gland expresses AR mRNA. Both adult and neonatal stages displayed significantly lower AR mRNA expression levels when compared with juveniles. Juvenile alligators of the size we examined in this study display seasonal variation in plasma testosterone concentrations (Rooney et al., 2004) and respond dramatically with elevated androgen production when stimulated with FSH (Edwards et al., 2004). Further, juvenile alligators appear to display a multi-year period of puberty (Rooney et al., 2004), and these data suggest a hypothesis that AR function during the juvenile life stage could play a role during peripubertal maturation of the thyroid gland. 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). Future studies need to test this hypothesis.

This study has shown that mRNA for both forms of ER, both forms of TR and AR are found on the thyroid gland of the American alligator (*A. mississippiensis*). We did not observe sexual dimorphism in the mRNA expression of these genes in the thyroid gland nor did we note an obvious sexually dimorphic expression pattern for ER α based on immunohistochemical visualization. However, the presence of sex steroid hormone receptors provides a mechanism by which gonadal steroid hormones, or contaminants mimicking these ligands, could influence thyroid gland development and function.

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