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Comparative thyroid hormone concentration in maternal serum and yolk of the bonnethead shark (*Sphyrna tiburo*) from two sites along the coast of Florida

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Abstract

Maternally provisioned yolk hormones have been determined to play critical roles in development across vertebrate taxa. This study ascertained the presence and concentration of thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) in the maternal serum and yolk of the developing placental viviparous shark *Sphyrna tiburo* from one site adjacent to Tampa Bay and another within Florida Bay, Florida, USA. The developmental profile of T_3 in yolk showed a steady increase from pre-ovulation to post-ovulation and peaked to its highest concentration during the pregnancy stage. There was an increase in the T_3/T_4 ratio in yolk during the pregnancy stage which suggests a possible increase in the conversion of T_4 to T_3 within yolk, possible embryonic endogenous production, or passive uptake of T_3 from uterine fluids. Similar to the pattern seen in yolk, maternal serum T_3 concentrations tended to increase as development progressed. The concentration of T_3 and T_4 in yolk from Tampa Bay was consistently higher than in yolk from Florida Bay. The differences in the patterns of thyroid hormones from these two locations may explain previously reported differences in the rate of embryonic development in the two locations.

Keywords: Development; Yolk; Serum; Elasmobranch; Bonnethead shark; *Sphyrna tiburo*; Thyroid hormone; Maternal–fetal transfer

1. Introduction

An emerging body of literature suggests that maternally derived hormones such as estrogen, testosterone, cortisol, corticosterone, and thyroid hormones are involved in the regulation of development, growth, sexual determination, and survival of offspring (Brown et al., 1987; Elf et al., 2002; Hayward and Wingfield, 2004; McCormick, 1999). The presence of thyroid hormone (TH) is of particular importance during early development of vertebrates (Brown and Bern, 1989; Hulbert, 2000; Power et al., 2001; Youson and Sower, 2001).

The transfer of TH from mother to embryos has been confirmed throughout the vertebrate classes, and some of the specific developmental consequences of this maternal provisioning have been investigated in mammals (Pickard et al., 1999), avians (Wilson and McNabb, 1997), and teleosts (Brown, 1997; Brown et al., 1987; Tagawa and Hirano, 1987). The concentration of TH in the yolk of teleost and avian eggs has been shown to affect development (Kobuke et al., 1987; Leatherland et al., 1989; Liu and Chan, 2002; McNabb and Wilson, 1997). The mechanism

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of teleost yolk TH sequestration has received some attention and the primary mode of uptake, at least in *Oreochromis* spp., appears to be via passive diffusion (Tagawa and Brown, 2001). Maternal TH is known to cross the placental barrier in mammals and has critically important influences on fetal development (Calvo et al., 1992). Deficiencies in maternal thyroid hormone concentrations in rats result in low birth weights, brain function impairment, and other irreversible developmental defects (Zertashia et al., 2002).

Elasmobranchs (sharks, skates, and rays) are the oldest jawed vertebrates known to possess TH, yet few investigations have addressed the role these hormones play in development (Volkoff et al., 1999). The concentrations of TH in the serum of several shark species have been measured, and correlated with reproductive and developmental events (Crow et al., 1999; Gash, 2000; Volkoff et al., 1999).

There are several modes of reproduction within elasmobranchs, including placental yolk-sac viviparity. This mode, as observed in the bonnethead shark (*Sphyrna tiburo*), is characterized by yolk-dependent embryos that undergo yolk-sac modification in which the fetal portion of a placenta attaches to the maternal uterine wall. The attachment, which occurs near mid-gestation, facilitates direct exchange of blood and nutrients between the mother shark and embryo (Schlernitzauer and Gilbert, 1966; Wourms, 1981). The shift from yolk to placental dependency in embryos presents a unique model for the study of both maternal investments in egg yolk and direct humoral hormone transfer across the elasmobranch placenta. Recent studies in *S. tiburo* have shown changes in steroid hormones found in yolk, including an elevation in estradiol that coincided with embryonic sexual differentiation (Manire et al., 2004).

Geographic variation in growth, reproductive, and developmental parameters has been reported among a variety of fishes (McCormick, 1999). Parsons (1993) reported that *S. tiburo* from a site in Tampa Bay attained a larger overall size, matured earlier, had larger embryos that developed faster, and were larger at birth than comparable cohorts found within a site in Florida Bay. The energy budgets of mature females in Tampa Bay were nearly double as compared with Florida Bay females, presumably reflecting distinctly different levels of reproductive investment in these two locations (Parsons, 1993). More recently, *S. tiburo* from three locations along the west coast of Florida were found to differ in adult maximum size, size and age at maturity, and length and weight of near term embryos (Lombardi-Carlson et al., 2003). The northernmost site had the largest adults and embryos while the southernmost site had the smallest, suggesting latitudinal variation in life-history traits of *S. tiburo* exists.

The first objective of this study was to determine concentrations of thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) in the maternal serum and egg yolk of *S. tiburo*, as related to pre-ovulatory, post-ovulatory, and yolk-dependent stage of development, prior to placentation. Second, a histological assessment was made on embryos to determine at which developmental stage a functional thyroid gland (possible endogenous TH production) emerged. Last, the relative abundance of thyroid hormones found during these developmental phases was measured in representative samples from Tampa Bay and Florida Bay to determine whether variation in maternal provisioning might help explain previously observed differences in growth during embryogenesis in these areas. Our working hypothesis was that differences in thyroid hormones may account for these variations in the rate and patterns of embryo development in two populations of bonnethead sharks.

2. Methods

2.1. Animals

Two locations were sampled: Anclote Key, a site adjacent to Tampa Bay (28°10′N, 82°48′W) and Long Key, within the Florida Keys (24°50′N, 80°49′W). Gill nets, long lines, and angling were used to capture sharks. Sampling was coincident with pre-ovulation, post-ovulation, and the embryonic yolk-dependent stage, described by Manire et al. (1995). Forty-eight mature female *S. tiburo* were collected between March 1998 and May 2003. All animals were measured, weighed, and the sex and stages of reproduction and maturity ascertained. Blood was collected via caudal venipuncture and allowed to clot on ice (0 °C). In the laboratory, serum was centrifuged at 1286*g* for 5 min and the resultant serum was stored at −40 °C until assayed. Ova were removed from the ovary or uterus, during post-ovulatory stages, and yolk was collected from the yolk sac of the developing embryo using a sterile syringe. Yolk was kept in cryovials on ice (0 °C) and then stored at −40 °C until assayed.

2.2. Yolk hormone extraction and radioimmunoassay

Radioimmunoassay (RIA) was used to measure the concentrations of triiodothyronine (T_3) and thyroxine (T_4) in yolk samples (ICN Pharmaceuticals Orangeburg, New York). Thyroid hormones were extracted from yolk using a modified methanol/chloroform extraction procedure used on salmon yolk by Greenblatt et al. (1989). Briefly, yolk was thawed and 200 mg was placed in a glass vial with 2.5 ml of extraction solution (1 mM of 6-*N*-propylthiouracil in methanol and 10 N NaOH) and homogenized using a tissue homogenizer (Omni International, Warrenton, VA). The homogenizer tip was rinsed with an additional 1.0 ml of extraction solution which was retained. A trace amount of ^{125}I T_3 (ICN Pharmaceuticals) was added to each sample tube (~1000 cpm) for recovery quantification. Vials were vortexed, centrifuged, decanted, and the remaining pellets were resuspended in 2 ml of methanol, vortexed, and centrifuged again. The pooled supernatants were dried for 12 h under vacuum at 37 °C and reconstituted with 250 µl ANS (8-anilino-1-naphthalenesulfonic acid)/barbital buffer, 250 µl methanol, and 2.5 ml chloroform, vortexed, and centrifuged. The aqueous top layer was collected, and the remaining chloroform was extracted again by additions of 250 µl ANS/barbital buffer and 250 µl methanol. The extract was pooled and dried for 12 h under vacuum at 37 °C, and reconstituted with 400 µl of hormone-free human serum. Assay methods were subjected to validation tests including parallelism, recovery, intra- and interassay variation. No significant non-parallelism was found, intra- and interassay variability (calculated as the coefficient of variation, CV, for at least eight samples in three assays) for both assays was less than 6%. All samples were run in duplicate and the extraction recovery efficiency averaged 77.2%. All data were individually corrected to account for recovery.

2.3. Maternal serum radioimmunoassay

Concentration of thyroxine (T_4) and triiodothyronine (T_3) in maternal serum was determined using a competitive binding solid phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles). All samples were run simultaneously and in duplicate to reduce intra-assay variability. The concentration of T_4 and T_3 was determined by extrapolation from the standard curve. Validation tests including parallelism, and intra- and interassay variation were run. No significant non-parallelism was found, and intra- and interassay CV for both assays was less than 8%.

2.4. Embryonic thyroid gland histology

Ten embryos from Florida Bay were collected during several developmental stages (1.4–5.6 cm total length) and the whole body or entire jaw region was trimmed and fixed in a 10% neutral buffered formalin solution for 24 h. Tissues were then transferred in a graded series to 70% ethanol for storage until processing. The tissues were dehydrated to 100% absolute alcohol, cleared in xylene, infiltrated with Paraffin wax, and embedded into molds. Sagittal sections were cut at 10 µm thick and stained with periodic acid Schiff (PAS) reaction counterstained with Orange G. All slides were examined under a light microscope (400×) for the presence or absence of thyroid follicles (the functional unit of the thyroid gland) as described by Honma et al. (1987).

2.5. Statistics

All data were grouped by reproductive stage based on female condition; the presence and location of ova, and the presence of embryos. Differences in serum and yolk TH concentrations associated with geographic region and reproductive stage were examined using two-way ANOVA. Tukey's test was used for all pairwise multiple comparisons. Hormone concentrations in yolk versus serum were compared by Mann–Whitney rank sum tests for each reproductive stage within each location. Comparisons of length and weight of maternal and embryonic sharks were made using *t* tests. Results were considered significant at $P \leqslant 0.05$.

3. Results

3.1. Maternal length and weight

Maternal sharks collected adjacent to Tampa Bay from each reproductive period were greater in stretch total length than those from Florida Bay ($P = 0.001$). The weight of Tampa Bay sharks was significantly greater than those from Florida Bay ($P = 0.001$).

3.2. Yolk thyroid hormone concentrations

The T_4 concentration in yolk samples from reproductive periods in Tampa Bay was significantly higher than those from Florida Bay ($P = 0.002$). Specifically, the concentration of T_4 in Tampa Bay samples was significantly higher than Florida Bay during the pre-ovulatory stage ($P = 0.030$) and the pregnancy stage ($P = 0.001$; Fig. 1A). The analysis of yolk T_3 revealed Tampa Bay samples were significantly higher than those from Florida Bay ($P = 0.043$). Specifically, the concentration of T_3 was higher in Tampa Bay during the pregnancy stage ($P = 0.045$; Fig. 1B) than in Florida Bay. In Tampa Bay, the pregnancy stage was higher than the pre-ovulatory period ($P = 0.041$). There was a stepwise increase in T_3 as development proceeded in both sites.

3.3. Maternal serum thyroid hormone concentrations

Analysis of serum revealed that Florida Bay T_4 concentrations were significantly higher ($P = 0.028$) than Tampa Bay. In Florida Bay samples, T_4 in the pregnancy stage was significantly higher than the pre-ovulatory stage ($P = 0.048$; Fig. 2A). In Tampa Bay serum samples, the concentration of T_3 was significantly higher ($P = 0.046$) during the pregnancy stage than the pre-ovulatory stage (Fig. 2B).

3.4. T_3/T_4 ratios

The T_3/T_4 ratio in yolk tended to increase as development progressed and the highest ratio occurred during the latest stages of pregnancy sampled (Fig. 1C). In Tampa Bay, the ratio during pregnancy was significantly

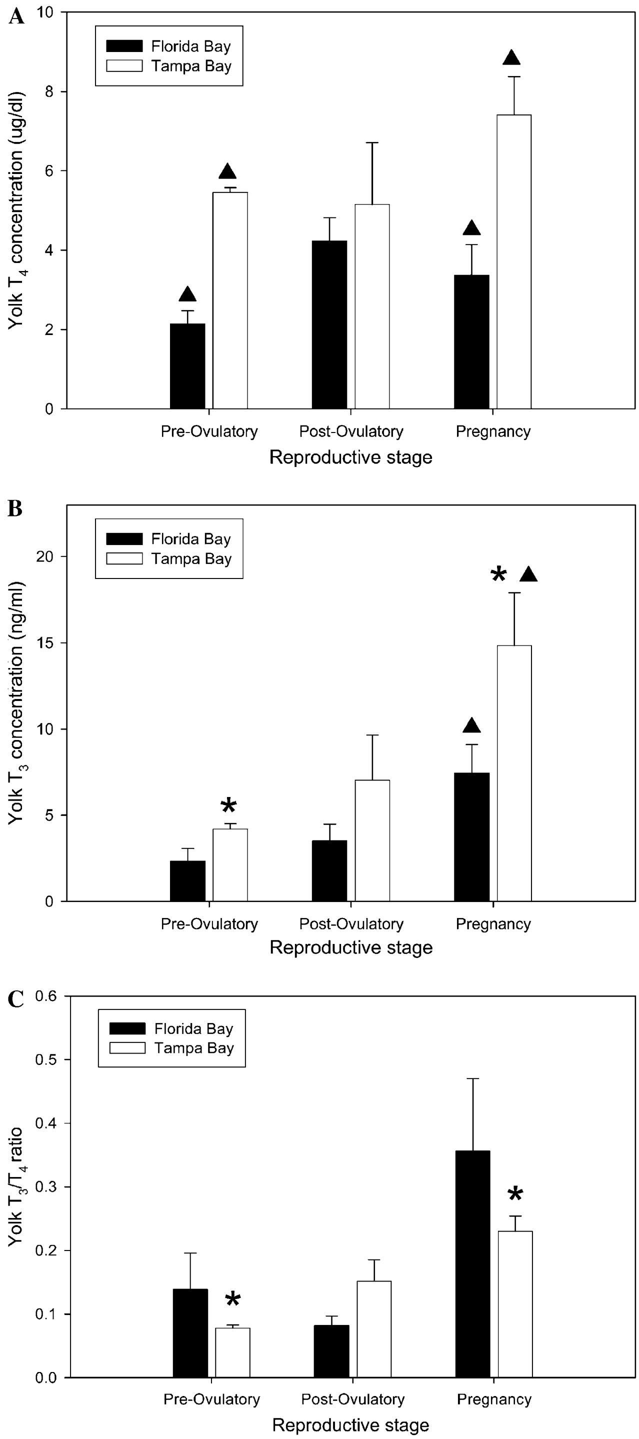

Fig. 1. Yolk TH concentrations during reproductive stages (pre-ovulatory through pregnancy) of the bonnethead shark *(S. tiburo)* from Florida Bay and Tampa Bay. (A) T_4, (B) T_3, and (C) T_3/T_4 ratio. Significance within yolk T_4 samples (A): Tampa Bay pre-ovulatory > Florida Bay pre-ovulatory, Tampa Bay pregnancy > Florida Bay pregnancy. Significance within yolk T_3 samples (B): Tampa Bay pregnancy > Tampa Bay pre-ovulatory, Tampa Bay pregnancy > Florida Bay pregnancy. Significance within the T_3/T_4 ratio in yolk (C): Tampa Bay pregnancy > Tampa Bay pre-ovulatory. The (▲) indicates significant differences between sites during a specific reproductive stage and * indicates a significance between reproductive stages within only one site.

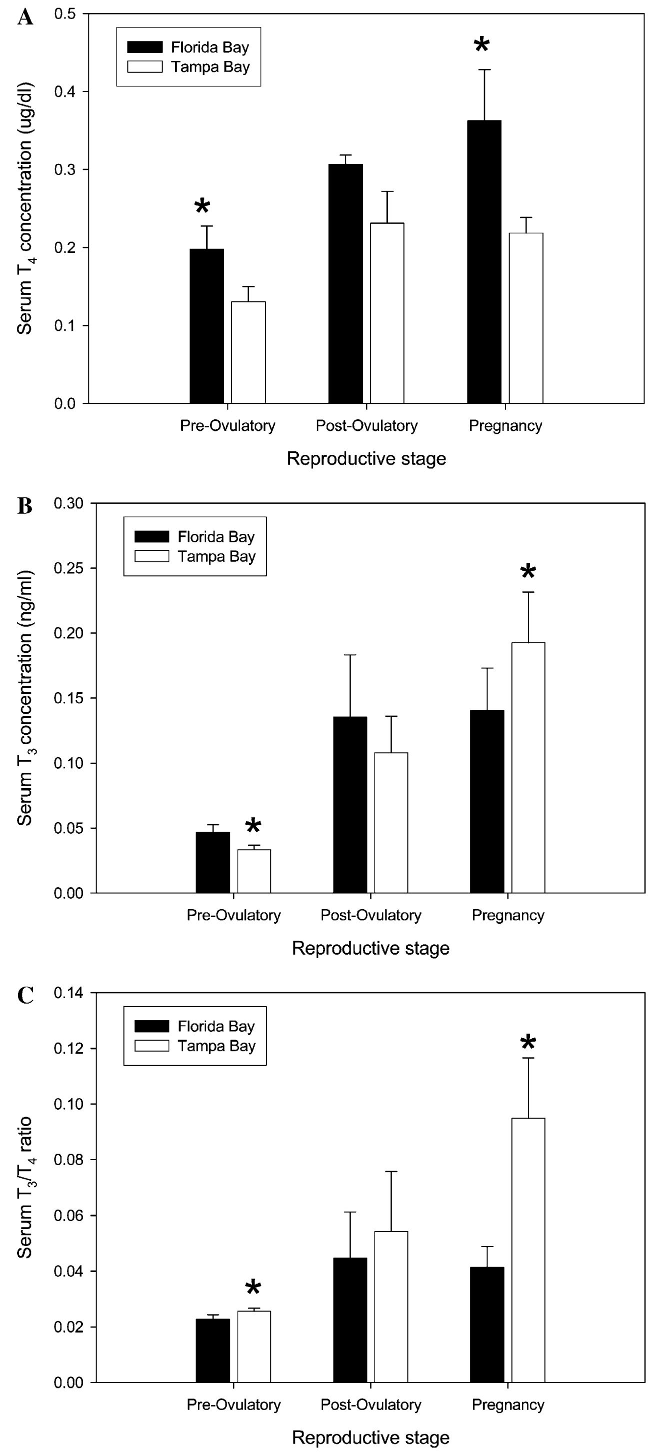

Fig. 2. Maternal serum TH concentrations during reproductive stages (pre-ovulatory through pregnancy) of the bonnethead shark *(S. tiburo)* from Florida Bay and Tampa Bay. (A) T_4, (B) T_3, and (C) T_3/T_4 ratio. Significance within serum T_4 samples (A): Florida Bay pregnancy > Florida Bay pre-ovulatory. Significance within serum T_3 samples (B): Tampa Bay pregnancy > Tampa Bay pre-ovulatory. Significance within the T_3/T_4 ratio in serum (C): Tampa Bay pregnancy > Tampa Bay pre-ovulatory. The * indicates a significance between reproductive stages within only one site.

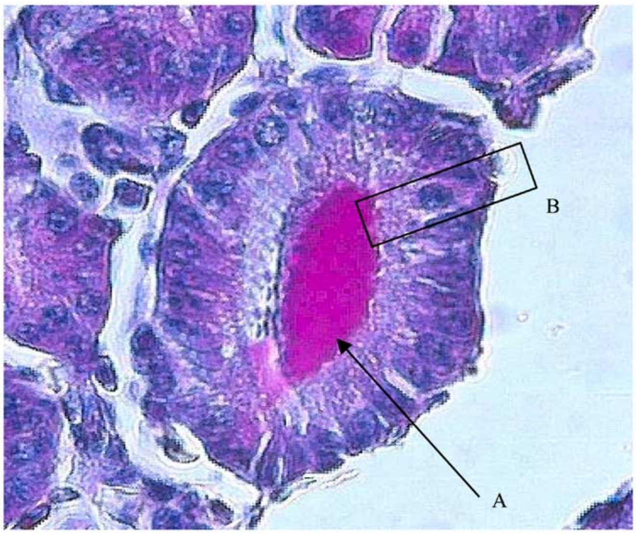

Fig. 3. The thyroid follicle of a female bonnethead embryo (15.6 cm TL) with PAS+ staining (pink) within the follicle lumen (A) indicative of the presence of thyroglobulin. Epithelial cells (B) surround the follicular lumen and are columnar in shape which suggests active secretion of TH. PAS+ stains were found in animals 5.9 cm and greater.

higher than pre-ovulatory ($P = 0.014$). The T_3/T_4 ratio in serum showed a general increase in both sites as development progressed with Tampa Bay pregnancy being significantly higher than Florida Bay pregnancy ($P = 0.027$; Fig. 2C).

3.5. Comparison of yolk and serum

When yolk and serum TH were compared by stage, the T_4 and T_3 concentration in yolk was significantly higher than that found in maternal serum ($P < 0.05$ for both sites in all stages).

3.6. Embryonic thyroid gland histology

From the 10 embryos assessed for thyroid follicles, no follicles were observed in embryos 1.4–1.9 cm total length. Thyroid follicles were observed in embryos 5.9–15.6 cm total length (Fig. 3). Embryos less than 10 cm total length are yolk dependent and have not established placental associations. The purpose of the histological assessment was not comparative but rather an attempt to pinpoint at which developmental stage (in utero), a functional thyroid gland was present (thereby potentially increasing the TH content in yolk by embryonic endogenous production).

4. Discussion

Thyroid hormones are known to influence the development and growth of vertebrates, and manipulations of TH in egg yolk have been used successfully to increase the size and survivorship of aquacultured teleost species (Brown et al., 1988; Power et al., 2001). However, fewer studies have examined the role of TH in cartilaginous fish (Crow et al., 1999). Volkoff et al. (1999) reported T_4 and T_3 concentrations in serum of several elasmobranch species and concluded there were correlations to reproductive status. Gash (2000) measured circulating T_4 in the serum of mature *S. tiburo* females from Florida Bay and Tampa Bay, and found concentrations increased from the pre-ovulatory to pregnancy periods with the highest peak occurring during the implantation stage. This study found similar TH concentrations in the maternal serum, and a general increasing trend in both T_4 and T_3 from the pre-ovulatory to pregnancy stages.

From the analyses of TH concentration in serum, an increase in T_3 was observed in both sites as development proceeded. This trend was not unexpected as significantly elevated serum T_3 levels have been reported during ovulation and through gestation in the Atlantic stingray, *Dasyatis sabina* (Volkoff et al., 1999). However, the fact that the TH concentration in maternal serum from Florida Bay was higher than that of Tampa Bay was unexpected. Due to the size, growth and temperature differences reported between the two sites, it was expected that the TH concentration in the Tampa Bay samples would be higher, potentially reflecting this additional energetic investment. There are a few plausible explanations that can be considered: (i) the thyroid gland is more active at higher temperatures and (ii) less T_4 is deiodinated at higher temperatures, (iii) the higher T_4 concentration in the serum is a result of unused T_4.

This study is the first to demonstrate that TH is found within the egg yolk of *S. tiburo* and the concentration of each hormone is subject to considerable change as embryonic development proceeds. Patterns of increase in T_3 content of yolk, and consequently in the T_3/T_4 ratio, may have developmental significance. A distinct geographic variation in TH concentration of yolk between developing sharks within Florida Bay and Tampa Bay was also detected. A steady pattern of increase in T_3 was observed as development proceeded, but no clear pattern of change in T_4 concentration emerged. This is consistent with the view that T_3 is important in the regulation of development, and is the metabolically active hormone that exerts cellular action (Eales et al., 1992). The lowest T_3 level was found during the pre-ovulatory period, suggesting that provisioning may be a cumulative process. During pregnancy the concentration was even higher; throughout this stage embryos are present and actively utilizing yolk reserves. The increase in T_3 corresponds to embryonic somatogenesis, definition of the nervous and sensory system, and chondrogenesis. These are periods of elevated energetic demand by the embryo and rapid yolk depletion. This finding suggests that T_3 is available for uptake and utilization by the embryos as yolk consumption proceeds.

The progressive increase in the T_3/T_4 ratio over development suggests generation of T_3 by monodeiodination of T_4 in yolk and potentially within the embryo itself. The histological assessment of embryos from Florida Bay revealed functional thyroid follicles in embryos 5.9 cm and greater. Embryos of this size do not have placental associations. It is possible that the embryonic thyroid gland is functional, and that exogenous T_4 is being produced and converted to T_3 within the embryo and its yolk sac. Additionally, the increase of T_3 during pregnancy may be a result of passive uptake of T_3 within uterine fluids. Tagawa and Brown (2001) demonstrated that yolk of tilapia (*Oreochromis mossambicus*) eggs immersed in fluids acquired T_3 by way of passive diffusion. Similarly, Raine and Leatherland (2003) showed that trout (*Oncorhynchus mykiss*) egg T_3 content fluctuated depending on concentrations in the surrounding ovarian fluid.

The study sites are separated by nearly three latitudinal degrees so it is reasonable to expect differences in environmental cues, and in physiological responses to them. The higher temperature within Florida Bay may cause *S. tiburo* to have a higher metabolic rate, possibly contributing to differences in growth and development, and an overall smaller body size. It is also likely that some environmental signals activate endocrine transducers, such as TH, which are known to have involvement in the mediation of growth, development and reproductive processes. The fact that serum TH concentrations are higher in Florida Bay than in Tampa Bay may be a consequence of different environmental conditions. These two sites are reported (Gelsleichter et al., 2005) to differ in the concentration of organochlorine contaminants (Tampa Bay is higher) which have been hypothesized to be linked with differences in reproductive success between the two populations (Parsons, 1993). The present study suggests a possible maternal physiological response to environmental factors.

Determination of the exact timing of embryonic endogenous TH production may improve our understanding of maternal provisioning strategies, and studies that address this are underway. On a larger scale, a transplant or "common garden" study may reveal influential environmental factors on TH levels and potential plasticity with respect to maternal provisioning. It is possible that changes in the maternal endocrine profile are reflected in yolk content, and may in turn physiologically "preadapt" offspring to one set of environmental conditions or another (Dufty et al., 2002).

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