

THE RELATIONSHIP BETWEEN WATER CHEMISTRY AND GOITER DEVELOPMENT
IN TWO SPECIES OF BAMBOO SHARK, *Chiloscyllium spp.*

By

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To my parents

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Abstract of Thesis Presented to the Graduate School
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Three forms of goiter have been observed in captive elasmobranchs; diffuse hyperplastic goiter, diffuse colloid goiter, and multinodular goiter. Until recently, most aquariums believed that the etiology of elasmobranch goiter was caused by insufficient iodide either in the diet or in the aquatic environment. Ozonation of aquarium water has been associated with a reduction in environmental iodide and subsequent development of goiter because it has been found to convert a majority of aquatic iodide to iodate; a form of iodine which is unusable in thyroid hormone synthesis. Histological findings in Chapter 3 support this connection between ozone and the development of goiter. We observed a female brown-banded bamboo shark (*Chiloscyllium punctatum*) develop multinodular goiter 60 days after ozone was operational on its system. In addition to dealing with reduced bioavailability of iodide in ozonated systems, increased restrictions on water use have caused many modern aquariums to operate as re-circulating system resulting in an accumulation of environmental nitrate within these systems. Recent studies have demonstrated that environmental nitrate ($\geq 55\text{mg/L NO}_3\text{-N}$) competitively inhibits the ability of the thyroid follicles to up-take iodide, resulting in over stimulation of the gland, and ultimately leading to the development of goiter. In Chapter 2, we began to evaluate the affects of elevated environmental nitrate on thyroid function in sharks. We divided ten juvenile male

white-spotted bamboo sharks (*C. plagiosum*) (80-150g) into two treatment groups (n=5 per treatment), low nitrate (<1mg/L NO₃-N) or high nitrate (70mg/L NO₃-N), for 29 days in a flow-through natural seawater system. We hypothesized that exposure to elevated nitrate would manifest via: a) alteration in growth rates; b) development of a diffuse hyperplastic goiter; and c) decreased plasma thyroxine (T₄) concentrations. Our results indicated that nitrate exposure did not affect growth rates (e.g., weight, length, and condition factor), nor did it decrease circulating plasma T₄ concentrations during the 29 day experimental period. However, histological analysis of the thyroid glands in nitrate-exposed sharks did demonstrate the development of diffuse hyperplastic goiter. The results of this study support concerns that environmental nitrate exposure, in the absence of other factors, may be goitrogenic. In summary, water chemistry plays a significant role in goiter development of captive elasmobranchs and thus health management programs need to have an increased awareness of the affects of ozone on iodide bioavailability and re-circulating filtration technology on nitrate concentrations.

CHAPTER 1
NITRATE AND ITS HEALTH IMPLICATIONS

Introduction

The Nitrogen Cycle

Today many aquarium and aquaculture facilities utilize recirculating or water reuse systems. An advantage of these systems is that there is a reduction in overall water usage, along with increased flexibility of geographic locations as facilities no longer are restricted to locations adjacent to a body of water (Spotte, 1992; Noga, 1996; Malone and De LosReyes, 1997). Successful management of these systems, and consequently of the fish, depends upon prevention of disease achieved through the proper maintenance of natural biological processes. In an aquarium, a major cause of disease is improper water quality; for example, low concentrations of dissolved oxygen can result in the death of fish within a few hours (Spotte, 1992; Noga, 1996). Other water quality parameters important to fish health are the components of the nitrogen cycle; ammonia, nitrite and nitrate (Figure 1-1).

In an aquatic system, ammonia is released from a fish's gill as the by-product of protein metabolism. At concentrations as low as 0.05mg/L NH_3 , ammonia is damaging to fish gills and other tissues increasing the susceptibility of that fish to disease (Noga, 1996; Alonso and Camargo, 2003). Nitrogen fixing bacteria convert ammonia ($\text{NH}_4^+/\text{NH}_3$) to nitrite (NO_2), also a toxic metabolite, which at concentrations of 0.1 mg/L can result in methemoglobinemia or "brown-blood disease" (Westin, 1974). NO_2 diffuses into a fish's blood stream via the gills and oxidizes hemoglobin to methemoglobins leading to hypoxia (Noga, 1996; Alonso and Camargo, 2003). In established aquariums, NO_2 is converted into nitrate (NO_3) before it can accumulate to toxic concentrations (Sharma and Ahlert, 1977; Malone and De LosReyes, 1997).

Nitrate (NO_3) is the end product of the nitrogen cycle and is a compound that commonly accumulates in aquariums, especially in marine tanks where water exchange is typically less than in fresh water systems. Traditional aerobic biofilters produce nitrate as an end-product of bacterial de-nitrification. In order for nitrate to be biologically converted back into N_2 (gas) for elimination from the system, it must be reduced through anaerobic processes by various microbes (Sharma and Ahlert, 1977; Spotte, 1992). The process by which nitrate is converted into nitrogen gas requires more technically challenging anaerobic filtration, such as sulfur-based denitrifying filters (Spotte, 1992). Historically, nitrate was removed by discharging large volumes of water and replacing that water with clean source water. Currently, in these systems (typically greater than 10,000 liters) this is no longer practical as replacing salt water is not only expensive but federal and state water restriction laws prevent “dumping” of salt water into city sewage systems. Though anaerobic filters have helped reduced nitrate levels, they are limited by the volume they can filter in a given time period and the time required for the process to occur. Because of these factors, nitrate accumulates in aquariums and can plateau at concentrations of several hundred mg/L (Spotte, 1992; Mohan and Aiken, 2004).

Nitrate – A Health Concern

In the past, nitrate has not received as much attention as other nitrogen by-products (e.g., ammonia and nitrite) as a potential water quality hazard in both natural and aquarium settings, but emerging information has implicated nitrate as a contaminant at concentrations once thought to be innocuous for aquatic vertebrates. A growing body of scientific literature suggests that nitrate can disrupt other physiological processes, especially endocrine processes that can have irreversible impacts on reproduction and development of various organisms, including humans (Guillette and Edwards, 2005). Since the 1950's, the World Health Organization has associated nitrate contaminated well water (≥ 10 mg/L $\text{NO}_3\text{-N}$) with the prevalence of methemoglobinemia

(Blue Baby syndrome) in small children and infants (WHO, 2008). This is because nitrate is converted to NO_2 in the digestive system which then interferes with the ability of oxygen to bind to hemoglobin resulting in hypoxia (WHO, 2008). The World Health Organization has recommended that nitrate concentrations in drinking water not exceed 10 mg/L $\text{NO}_3\text{-N}$, though extensive use of fertilizers in agricultural systems and increased nutrient run-off has led to an increase in nitrate concentrations in aquatic ecosystems (Rouse et al., 1999; Galloway et al., 2003).

Aquatic organisms are exposed to nitrate, primarily through epithelial absorption across gills, skin or intestines. In various aquatic species, such as amphibians and fish, nitrate has been associated with acute toxicity (Tsai and Chen, 2002; Alonso and Camargo, 2003; Hamlin, 2006), along with changes in physiology and development (Edwards et al., 2006a; Edwards et al., 2006b; Hamlin, 2007; Ortiz-Santaliestra and Sparling, 2007; Hamlin et al., 2008). Nitrate toxicity has been found to vary based on environmental conditions (e.g., salinity level, reverse-osmosis water [RO_e]) (Tsai and Chen, 2002; Edwards et al., 2006a). For example, in juvenile tiger shrimp (*Penaeus monodon*) nitrate toxicity (LC_{50} 96hr test) increased as salinity decreased from 35‰ (232 mg/L $\text{NO}_3\text{-N}$) to 15‰ (145 mg/L $\text{NO}_3\text{-N}$) (Tsai and Chen, 2002).

Edwards et al. (2006a) found that there was a significant interaction between water type and nitrate concentration on the time to metamorphosis in Southern toad (*Bufo terrestris*) tadpoles. Results showed that in a nitrate concentration of 30 mg/L $\text{NO}_3\text{-N}$, mean time to metamorphosis decreased by 5 days for tadpoles reared in RO_e when compared to tadpoles reared in natural spring water, whose metamorphosis was delayed by 7 days (Edwards et al., 2006a). Nitrate toxicity has been found to differ significantly based on age. Hamlin (2006) found that the age of sturgeon (*Acipenser baeri*) affected their sensitivity to nitrate, with older

sturgeon (673.8g fish; 397 mg/L NO₃-N) having an increased sensitivity to aquatic nitrate concentration compared to younger counterparts (6.9g fish; 1028 /L NO₃-N).

It has been demonstrated that nitrate disrupts gonadal function and sex steroid synthesis in many vertebrates. Nitrate exposure (≥ 5 mg/L NO₃-N) has resulted in the suppression of testosterone and estrogen, and reduced both sperm motility and fecundity, all indices which can have a deleterious effect on reproductive success of a population (Edwards et al., 2006b; Barbeau and Guillette, 2007; Edwards and Guillette, 2007). Additionally, nitrate laden drinking water (45.5 mg/L NO₃-N) has been found to produce histological changes, such as hepatocellular degeneration and increased oxidative stress due to alteration of erythrocyte membranes, in male Sprague-Dawley albino rats (Ogura et al., 2005). Morphologic and behavioral alterations, such as intestinal hemorrhage, bent tail, depigmentation and erratic swimming, have been observed in *Nyctibatrachus major* and *Fejervarya limnocharis* tadpoles at concentrations as low as 1 mg/L NO₃-N (Krishnamurthy et al., 2008).

The thyroid gland has been found to be influenced by nitrate in mammals, amphibians, and fish (Zaki et al., 2004; Edwards et al., 2006a; Ortiz-Santaliestra and Sparling, 2007; Radikova et al., 2008). Nitrate inhibits thyroid function by blocking the sodium-iodide symporter (NIS), an integral plasma membrane glycoprotein that transports iodide into the thyroid (Tonacchera et al., 2004). Since iodide is essential in the production of new thyroid hormones, without iodide no new hormones can be synthesized. By competing with the NIS and reducing the bioavailability of iodide, nitrate interferes with thyroid hormone synthesis resulting in changes in thyroid histopathology (Tonacchera et al., 2004). Though the thyroid gland follicles contain stores of thyroid hormone, without the production of new hormones, these fluid filled colloidal spaces become depleted, leading to a decline in thyroid hormone concentrations, overstimulation of the

gland, and ultimately the development of follicular cell hypertrophy and hyperplasia (Bloomfield et al., 1961; Tonacchera et al., 2004; Zaki et al., 2004; Hu et al., 2006).

Disruption of thyroid function is most detrimental during an organism's development. For example, thyroid hormones play a key role in amphibian metamorphosis, the process whereby a tadpole develops into an adult frog (Shi, 1999). In southern leopard frogs (*Rana sphenoccephala*), nitrate (22.7 mg/L NO₃-N or 100 mg/L NO₃⁻) was found to reduce both larval survival and development (Ortiz-Santaliestra and Sparling, 2007). Although the mechanism by which nitrate affects amphibian metamorphosis has been found to be species-specific, in general larval exposure to nitrate results in decreased body size and weight, altered time to metamorphosis whether by increasing or decreasing that time, and reduced feeding and growth rates (Edwards et al., 2006a; Ortiz-Santaliestra and Sparling, 2007). Nitrate sensitivity tends to differ depending on concentration and exposure period; studies are demonstrating that nitrate exposure can result in changes to many physiological processes including the thyroid gland, which if disrupted can impact growth and development of an organism.

The Thyroid Gland

Evolution of the Thyroid Gland

The thyroid gland, which influences many physiological processes, is structurally conserved throughout all chordates and generally found as one or two highly vascularized lobes surrounded by connective tissue or in the case of most teleosts, scattered lobes throughout the pharyngeal region (Norris, 2007). Thyroid gland development begins with ventral budding of the embryonic pharynx (endoderm) between the first and second pharyngeal pouches around week two of gestation (Norris, 2007). Initially, the gland is differentiated as cellular cords and later is separated into a single layer of epithelial cells (thyrocytes) that possess numerous microvilli. The thyrocytes are joined at their apical end to form a follicle containing a protein-

rich acidophilic colloid that serves as the storage site for thyroid hormones, a unique feature among endocrine glands (Norris, 2007). Thyroid hormones are iodinated tyrosines that are not only found in vertebrates, but also invertebrates, plants, and bacteria (Eales, 1997; Dumont et al., 2008). Though with most invertebrates (e.g., insects, sponges, and coral) and plants (e.g., marine algae), iodocompounds may result from either exogenous accumulation of organic iodine (e.g., via ingestion) or as a by-product in other signaling pathways (Eales, 1997; Dumont et al., 2008).

Thyroid Function in Fish

In teleost fishes and elasmobranchs, thyroid hormones play a role in iodide uptake, growth, development (e.g., metamorphosis), osmoregulation, and reproduction (Leatherland, 1988; Eales and Brown, 1993; Blanton and Specker, 2007; Norris, 2007). Elasmobranch studies have determined that thyroid gland activity changes seasonally due to reproductive state (Crow et al., 1999; Volkoff et al., 1999; Gash, 2000; McComb et al., 2005). Histological assessment of the thyroid gland of an ovulating viviparous female bonnethead (*Sphyrna tiburo*) shark revealed follicular hypertrophy and depleted colloidal thyroid hormones (THs) storages, indicating an increase in THs synthesis possibly caused by an elevated metabolic demand due to reproductive stage (Gash, 2000; McComb et al., 2005). Maternal thyroid condition and input of THs into yolk has been linked to success of embryonic growth and survival (Lam, 1980; Inui and Miwa, 1985; Deane and Woo, 2003). Roy et al. (2000) determined that when murrel (*Channa gachua*) and carp (*Catla catla*) are injected with gonadotropin-releasing hormone this results in the stimulation of maternal thyroid glands to increase of thyroidal iodide uptake, synthesize new THs, and incorporate the maternal THs into egg yolk (Monteverdi and Di Giulio, 2000). This incorporation of maternal THs into egg yolk has been found to significantly influence whether embryonic development and metamorphosis of many teleost species is successful (Tagawa et al., 1990; Jones et al., 2002; Deane and Woo, 2003; Einarsdottir et al., 2006; Yamano et al., 2007).

A further indication of the importance of THs in embryonic development is that growth and time to metamorphosis have been accelerated via treatment with exogenous T₄ (Lam, 1980; Inui and Miwa, 1985). In salmonid fishes, smoltification coincided with an elevation in TH concentrations (Dumont et al., 2008). During this process, a freshwater parr salmon will smolt in preparation to migrate to the ocean thereby triggering morphological, behavioral and biochemical changes (Eales and Brown, 1993). Though osmoregulation is controlled by prolactin, cortisol and arginine vasotocin (water balance), THs are important in osmotic adaption during migration from freshwater to saltwater (Dumont et al., 2008).

Thyroid Physiology

Thyroid Hormone Synthesis

The HP-thyroid axis is controlled by a negative feedback signaling from thyroid hormones (THs) on the pars distalis of the adenohypophysis. An environmental or neurological cue triggers the release of thyrotropic releasing hormone (TRH), which leads to the activation of IP₃ second messenger system and vasoactive intestinal peptide (VIP) (Norris, 2007; Zoeller et al., 2007). Through the phosphorylation of phosphokinase C, IP₃ activation caused transcription factor, Pit-1, to stimulate the synthesis of TSH β gene and increase of intracellular Ca²⁺ levels (Norris, 2007). Concurrently, VIP stimulated thyrotropic cell cAMP facilitating the release of thyroid stimulating hormone (TSH) from the pituitary (Norris, 2007; Zoeller et al., 2007). TSH binds to TSH receptor on the thyroid follicle stimulating an increase in iodide uptake by the sodium-iodide symporter (NIS) and subsequent release of THs from the thyroid gland (Norris, 2007).

Iodide Uptake

Iodide (I⁻) is transported into the thyrocytes via the sodium/iodide symporter (NIS) (Norris, 2007). Driven by Na⁺/K⁺ ATPase transport proteins, the NIS co-transporters Na⁺ and I⁻ in

a 2:1 ratio across the thyrocyte basolateral plasma membrane and then pendrin, a 86-kDa protein encoded by the Pendred syndrome gene (PDS), transports iodide across the apical membrane (Zoeller et al., 2007). Anions (thiocyanate, perchlorate, and nitrate) block the accumulation of I⁻ by follicular cells through the competitive inhibition of NIS (Tonacchera et al., 2004).

Thyroglobulin (Tgb) Synthesis

Tgb synthesis occurs at the rough endoplasmic reticulum, where it migrates to the Golgi apparatus to be folded and packaged with the addition of carbohydrate and sulfate moieties. Non-iodinated tyrosine residues are incorporated into Tgb and transported to the apical cell surface and undergo exocytosis into the follicular lumen (Hadley, 2000; Norris, 2007).

Iodination and Coupling of Tyrosine Residues in Tgb

In order for I⁻ to bind to Tgb, it must be converted from inorganic I⁻ to active I⁺ via enzymatic activity of thyroperoxidase (TPO). TPO catalyzes glucose oxidation and reduction of pyridine nucleotides to form hydrogen peroxide, which reacts with I⁻ by attaching it to a tyrosine residue on Tgb at either position 3 on the phenolic ring or position 5, forming 3-mono-iodotyrosine (MIT) and 3,5-diiodotyrosine (DIT), respectively (Hadley, 2000; Norris, 2007). The coupling of iodinated tyrosine is also enzymatically controlled by TPO. The alanine side chain on one of the iodinated tyrosines is cleaved off, and the second iodinated phenolic ring is joined to the other via the formation of an ether (-O-) linkage. Coupling of an MIT and DIT yields 3,5,3'-triiodothyronine (T₃), while coupling two DIT molecules yields 3,5,3',5'-tetraiodothyronine (thyroxine or T₄). The proportion of MIT and DIT determines the amount of T₃ and T₄ formed. Normally much more T₄ is synthesized in the thyroid than T₃, while most T₃ synthesis occurs in peripheral tissues via deiodinase activity (Hadley, 2000).

Thyroid Hormone Release and Transport

Once the thyroid gland is stimulated by TSH, Tgb is reabsorbed through endocytosis of colloid. The endosomes (colloid droplets) migrate from the apical portion to the basal portion of the cell and in the process become associated with lysosomes forming an endolysosome. Following hydrolysis of the endolysosome at the basal pole of the cell, thyroid hormones are released from Tgb and diffuse from the cell into circulation (Norris, 2007).

Due to the fact that thyroid hormones are hydrophobic, 75-85% of bound hormones are linked to α_2 -globulins called thyroid binding globulin (TBG). A small fraction (<0.1%) is transported free in blood, while the remainder is bound to prealbumin (TBPA) and albumin (TBA). T_4 is more tightly bound to serum proteins than T_3 , so T_3 is more rapidly eliminated from the blood (Norris, 2007).

Metabolism of Thyroid Hormones

Metabolism of thyroid hormones is accomplished via deiodination, resulting in an assortment of iodothyronines, including the metabolically active form T_3 and metabolically inactive form reverse T_3 . There are three types of deiodinases: type I deiodinase (D1); type II deiodinase (D2); and type III deiodinase (D3). D1 and D2 remove an iodide atom from the outer phenolic ring, while D3 (and D1) remove an iodide atom from the inner amino ring. Therefore, D1 and D2 can convert T_4 to T_3 , while D3 can convert T_4 to reverse T_3 (Norris, 2007). D1 is primarily found in the liver, kidney and thyroid, while D2 occurs primarily in the brain, pituitary, placenta, and brown adipose tissue (rodents). D3 is also found in the brain and intestine, along with placenta and fetal skin. Thus, the difference in tissue expression of these deiodinases, along with different expression patterns during development, will influence the ability of thyroid hormones to affect a particular tissue (Shi, 1999; Zoeller et al., 2007).

A second important metabolic pathway, which leads to the excretion of T_4 (convert to tetraiodothyroacetate) and T_3 (convert to triiodothyroacetic acid), included sulfonation or glucuronidation of the phenolic hydroxyl group, thus changing the solubility of iodothyronines allowing for their concentration in bile acids or hepatic excretion (Zoeller et al., 2007).

Thyroid Hormone Receptors and Mechanism of Action

Thyroid hormone receptors (TRs) belong to the super family of nuclear hormone receptors (e.g., glucocorticoid, estrogens, retinoic acid, and vitamin D) (Thorton and Kelly, 1998; Thorton, 2003). TRs have several functional domains: a N-terminal domain responsible for transcription activation; a zinc finger DNA binding domain; a hinge domain; and a C-terminal ligand binding domain (Shi, 1999; Thorton, 2003; Norris and Carr, 2006). TRs, which form a heterodimer with RXR (9-cis-retinoic acid), activation is dependent upon T_3 binding to the ligand binding domain on the thyroid response element (TRE) (Shi, 1999). In other words, without the presence of T_3 , TR activation is repressed via co-repressors (N-Cor and SMRT) that promote the binding of a cofactor (Sin3A) and histone deacetylase, thereby maintaining heterochromatin structure (Shi, 1999; Wu and Koenig, 2000). Once T_3 binds to the TR-RXR complex, it results in the dissociation of the co-repressors, a conformational change of the receptor, and the recruitment of co-activators (e.g., steroid receptor co-activator [SCR] and p300/CBP) and histone acetyltransferase (HAT) allowing for the transcription and translation of new proteins to occur (Shi, 1999; Wu and Koenig, 2000).

Diseases of the Thyroid Gland

As previously stated, the hypothalamic-pituitary-thyroid axis is controlled via negative feedback produced from thyroid hormones (THs) in the pituitary. Any alteration in this negative feedback system will change the ratio between circulating levels of THs and thyroid stimulating hormone (TSH) and consequently will result in thyroid disease. High THs and low TSH levels

result in hyperthyroidism, while low THs and high TSH produce hypothyroidism.

Hypothyroidism, which has been documented in many animals including humans, dogs, dolphins, and sharks, results from the continual release of TSH from the pituitary and a reduction in THs levels (Cotran et al., 1994).

There are three types of hypothyroidism; the first is an autoimmune disorder, the second is congenital hypothyroidism, and the third is caused by a goitrogenic compound. Disease of the thyroid gland caused by autoimmunity results when the immune system of the individual begins to attack the thyroid gland; for example, chronic thyroiditis is an autoimmune disease where an individual produces thyroglobulin (TgAA) and thyroid peroxidase (TPOAA) antibodies (Graham et al., 2007; Norris, 2007). Both Hashimoto's thyroiditis (struma lymphomatosa) and Riedel's thyroiditis (struma fibrosa) lead to the replacement of thyroid tissue with either lymphoid or fibrous connective tissue and as a result, no new THs can be synthesized. This reduction in circulating THs triggers an elevation in TSH secretion by the pituitary which ultimately results in follicular hypertrophy and hyperplasia, a reduction in colloidal THs storages, and goiter formation (Graham et al., 2007; Norris, 2007).

As stated previously, maternal thyroid condition and hormone input are important to successful embryonic growth and survival (Lam, 1980; Wilson and McNabb, 1997; Zoeller et al., 2007). Human infants are completely dependent on maternal thyroid hormone input during the first trimester, as infants do not start synthesizing thyroid hormones until the second trimester (Zoeller et al., 2007; Brown, 2009). During the first trimester, THs are critical for proper neurological development and any reduction in maternal THs can retard fetal brain development (Shi, 1999). If thyroid hormone deficiency continues, this can result in abnormal fetal pituitary development, thyroid gland development or thyroid hormone synthesis (Brown, 2009).

Congenital hypothyroidism, which occurs in 1 in 4,000 infants, results from decreased thyroid hormone synthesis in newborns (Shi, 1999; Zoeller et al., 2007; Brown, 2009). If diagnosed early, thyroid hormone replacement can ensure the normal neurological development. Untreated congenital hypothyroidism will result in severe mental and physical retardation (cretinism) due to improper cerebellum development and bone growth formation (Shi, 1999; Zoeller et al., 2007).

Iodide (I⁻) is important in thyroid hormone synthesis and a deficiency during childhood development can result in retardation of growth and intellectual development (WHO, 2004). Globally, I⁻ deficient hypothyroidism is high due to the scarcity of terrestrial I⁻ sources. According to the World Health Organization, worldwide over one-third of children between the ages of 6-12 years can be categorized as I⁻ deficient (WHO, 2004). Since 1993, global prevalence of I⁻ deficient induced hypothyroidism, or goiter, has risen by nearly 32% (WHO, 2004). Unfortunately, the global supplies of I⁻ are generally insufficient to meet the physiological demands of the thyroid gland and, accordingly, supplementation through iodized salt is necessary in order to prevent goiter.

A goitrogen is a compound that inhibits thyroid hormone synthesis through the suppression of I⁻ uptake. Examples of these compounds are flowering plants from the *Brassicae* family (e.g., cabbage, Brussels sprouts, turnips), anions (e.g., perchlorate, nitrate, thiocyanate), and pharmacological drugs (e.g., methimazole, propylthiouracil) (Norris, 2007). This reduction in I⁻ bioavailability results in development of diffuse hyperplastic goiter as the thyroid gland attempts to compensate for the iodide deficiency. As stated previously, the thyroid gland has been found to be influenced by nitrate and has been linked to goiter development in humans, rats and fish (Tonacchera et al., 2004; Zaki et al., 2004; Radikova et al., 2008). Nitrate competitively inhibits

the sodium-iodide symporter (NIS) (Tonacchera et al., 2004, Zoeller et al., 2007). By competing with the NIS, nitrate interferes with thyroid hormone synthesis, lowers circulating THs concentrations, and ultimately changes thyroid gland histopathology (Tonacchera et al., 2004; Zaki et al., 2004; Hu et al., 2006).

Thyroid Disease in Elasmobranchs

Sharks and rays have been exhibited in aquariums since the 1860's and continue to be popular exhibit animals today. The first recorded aquarium to display sharks was the Hamburg Aquarium in Germany in 1864 (Koob, 2004). Some examples of the earliest species kept on display were spiny dogfish (*Squalus acanthias*), small spotted catshark (*Scyliorhinus canicula*), nurse shark (*Ginglymostoma cirratum*), angel shark (*Squatina squatina*), spotted skate (*Raja montagui*), and cownose ray (*Rhinoptera bonasus*). With technological advances in life support systems and increased knowledge of husbandry requirements, over 150 out of the 400 known species of sharks and rays are now maintained in captivity (Koob, 2004).

Disease in captive elasmobranchs is relatively rare; however the occurrence of goiter has been well documented in captive sharks (Gridelli et al., 2003; Crow, 2004; Murray, 2009; Figure 1-2). Until recently, the aquarium industry believed that the etiology of elasmobranch goiter was largely attributed to chronic exposure to reduced bioavailability of iodide (I^-), either due to insufficient dietary access or low environmental concentrations due to the addition of the chemical filter ozone on a life support system (Sherrill et al. 2004). Ozone results in the chemical alteration of iodine species from I^- to iodate (IO_3^-), which cannot be used in THs synthesis (Pike et al., 1993; Crow et al., 1998; Crow, 2004). It not only reduces I^- bioavailability, but ozone also increases the rate of the conversion of ammonia and nitrite to nitrate contributing to nitrate accumulation in a re-circulating marine system (Spotte, 1992).

With continued nitrate accumulation in recirculating system and awareness of its goitrogenic properties, nitrate maybe an important factor in the etiology of goiter.

Bamboo sharks (genus *Chiloscyllium*) are members of the family of long-tailed carpet sharks, *Hemiscyllidae*, native to inshore Indo-West Pacific (Musick et al., 2004). These sharks are popular in aquariums and research laboratories due to their small size (maximum size 97 cm total length), sedentary and docile nature. Furthermore, bamboo sharks adapt easily to captivity and are readily available (Dehart, 2004; Koob, 2004). Due to these physical characteristics, the bamboo shark is an ideal model species for studies that begin to elucidate the affects of nitrate as a goitrogenic compound in elasmobranchs.

Objectives and Hypotheses

The thyroid gland plays a major role in growth and development and thus is very active in young animals, ranging from humans to elasmobranchs. Due to increased energetic demands, the thyroid gland is continuously up-taking iodide (I^-) and synthesizing new thyroid hormones. Sharks absorb I^- from sea water, but because ozonation reduces the bioavailability of I^- , many captive sharks are subject to chronic exposure to an I^- deficient environment ($\leq 0.15\mu M$), which if left untreated can result in the development of goiter (Sherrill et al., 2004; Crow, 2004). The multinodular goiter (chapter 3) which developed in a female brown-banded bamboo shark (*Chiloscyllium punctatum*) 60 days after ozone was operational on its system supports this connection between ozone and the development of goiter. In addition to dealing with reduced bioavailability of iodide, increased restrictions on water use have caused many modern aquariums to operate as re-circulating systems resulting in an accumulation of environmental nitrate within these aquariums. To date no studies exist which look at the affect of high environmental nitrate on thyroid function in elasmobranchs when held in an iodide rich

environment ($\geq 0.15\mu\text{M}$). Thus, the goal of this study (chapter 2) is to evaluate the affects of high environmental nitrate concentrations on thyroid function in juvenile male white-spotted bamboo sharks and to begin to elucidate its role in the development of goiter in this species. We hypothesize that exposure to elevated nitrate concentrations will manifest via, a) alteration in growth rates, b) development of a diffuse hyperplastic goiter, and c) decreased plasma thyroxine concentrations.

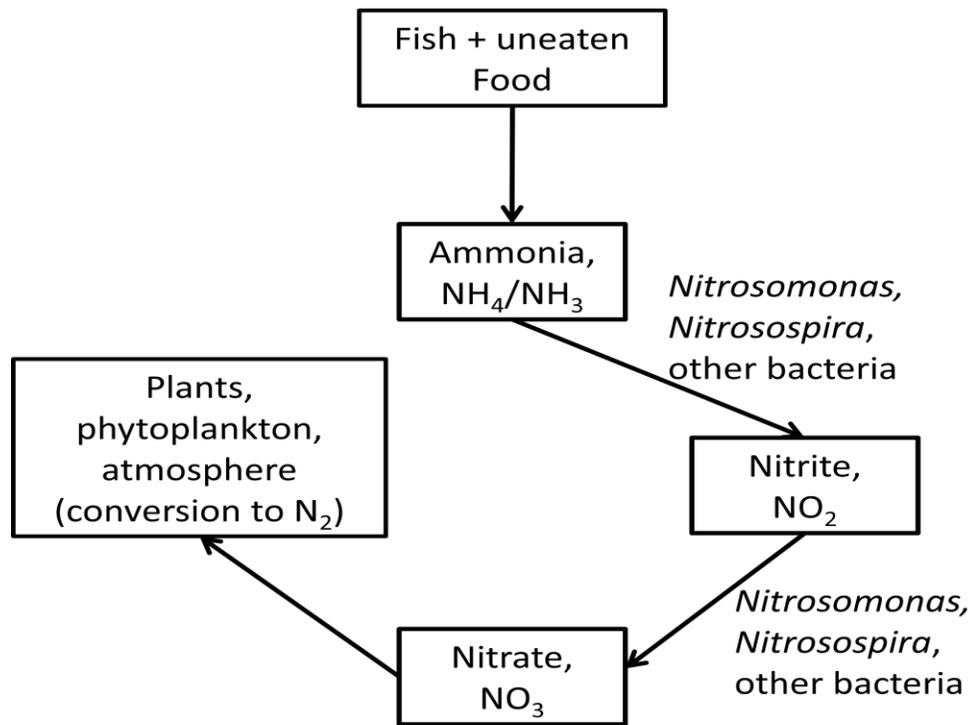


Figure 1-1. The nitrogen cycle in seawater aquariums. Adapted from Spotte, 1992.



Figure 1-2. Goiter in a female brown-banded bamboo shark, *Chiloscyllium punctatum*. Source M. Walsh (University of Florida, College of Veterinary Medicine).

CHAPTER 2
NITRATE INDUCED HYPOTHYROIDISM IN WHITE-SPOTTED BAMBOO SHARKS
(*CHILOSCYLLIUM PLAGIOSUM*)

Introduction

Elasmobranch susceptibility to goiter formation in captive environments has been documented (Crow et al., 1998). Three forms of goiter have been observed in captive sharks; diffuse hyperplastic goiter; diffuse colloid goiter; and multinodular goiter (Crow et al., 2001). Though a common, the exact etiology of this disease in captive elasmobranchs is poorly understood. Goiter can be caused by reduced iodide (I⁻) bioavailability and/or interaction with goitrogenic compounds (e.g., nitrate), which inhibit the uptake of I⁻ into the thyroid gland (Cotran et al, 1994).

In order to reduce overall water usage by public aquariums and aquaculture facilities, many of modern facilities utilize recirculating or water reuse systems. As water is “re-used”, nitrate, which is the end product of the nitrogen cycle, tends to accumulate because the biological processes required to break it down to atmospheric nitrogen gas are more complex to manage than more traditional biological filtration which breaks down ammonia to nitrite, and ultimately, to nitrate (Spotte, 1992). These anaerobic denitrification filters have limited ability to convert nitrate into nitrogen gas as they can only filter a small volume of water at one time, taking several weeks to complete the entire process. Nitrate historically was removed by discharging large volumes of water and replacing that water with source water. Currently, this practice has been restricted in aquariums due not only to local governmental water regulations but also because of the high cost associated with producing saltwater. Aquariums have decreased their water discharging practices ultimately leading to an increase concentrations of environmental nitrate which maybe several hundred mg/L in some systems (Spotte, 1992; Mohan and Aiken, 2004)

Nitrate has received less attention as a potential water quality hazard in both natural and aquarium settings, but studies have demonstrated that environmental nitrate inhibits the ability of the thyroid gland to uptake I⁻ resulting in decreased thyroid hormone synthesis (e.g., lower circulating plasma TH concentrations) and ultimately the development of goiter (Crow et al., 1998; Zaki et al., 2004; Edwards et al., 2006a). A study by Crow et al. (1998) established a preliminary relationship between high environmental nitrate and decreased serum thyroid hormone concentrations in captive male White-tip reef sharks (*Triaenodon obesus*) from two sites, Sea Life Park (SLP) and a natural seawater lagoon at the Hawaii Institute of Marine Biology, (Kaneohe, Hawaii [HIMB]). Crow et al. (1998) found that the sharks from SLP (60.5 mg/L NO₃-N or 111 μM) had lower T₄ concentrations (range of 0.93 to 0.99 ng/mL) when compared to the sharks from HIMB (T₄ range 5.61 to 7.91 ng/mL; 0.529 mg/L NO₃-N or 0.97 μM). However, chronic (4 years) exposure to low environmental iodide (<0.005 μM in SLP vs. 0.15 μM in HIMB) concentrations may have also contributed to the reduction in thyroid function and ultimately the development of goiter. Once the SLP sharks were moved from the high nitrate (60.5 mg/L NO₃-N) and low iodide (<0.005 μM) environment to an environment (HIMB) with high iodide (0.15 μM) and low nitrate (0.529 mg/L NO₃-N), goiter was abated and over the course of six months plasma T₄ concentrations returned to normal (range 3.1 to 7.9 ng/mL).

Sharks primarily absorb iodide from their aquatic environment and secondarily from their diet. A reduction in the bioavailability of I⁻, whether due to low environmental or dietary I⁻ concentrations and/or the competitive inhibition of I⁻ uptake into the thyroid due to elevated environmental nitrate, can disrupt the synthesis and release of thyroid hormones from the thyroid gland. Over time, this disruption of normal thyroid function and overstimulation of the gland

can result in a state of hypothyroidism and ultimately the development of goiter (Cotran et al., 1994).

It is possible that the aforementioned factors that influence the bioavailability of I⁻ may explain why goiter is a common health problem in captive elasmobranchs. With increased restrictions on water discharging practices coupled with the limitations of anaerobic filtration systems, many modern aquaria are faced with higher and more chronic environmental nitrate exposure to their aquatic organisms. However, nitrate is often viewed as relatively harmless and current recommendations for safe levels of nitrate in a shark exhibit are suggested to be ≤ 70 mg/L NO₃-N (Mohan and Aiken, 2004), although literature has suggested nitrate concentrations above 30 mg/L NO₃-N can disrupt normal thyroid function in (Bloomfield et al., 1961; Edwards et al., 2006a). The question remains as to the affect that this recommended nitrate concentration (70 mg/L NO₃-N) has on the elasmobranch thyroid gland. Previous research has documented the development of diffuse colloid goiter and multinodular goiter due to chronic effects of environmental iodide deficiency ($<0.005 \mu\text{M}$) and high environmental nitrate (60.5 mg/L NO₃-N) (Crow et al., 2001); however to date no studies exist which look at the effect of acute exposure (≤ 30 days) of high environmental nitrate in a relatively I⁻ rich environment on thyroid function in elasmobranchs. The objective of this study was to evaluate the effects of high environmental nitrate concentrations (70 mg/L NO₃-N) on thyroid function in juvenile white-spotted bamboo sharks (*Chiloscyllium plagiosum*) and begin to elucidate the role of nitrate in the development of goiter. We hypothesize that exposure to elevated nitrate concentrations will manifest via, a) alteration in growth rates, b) development of a diffuse hyperplastic goiter, and c) decreased plasma thyroid hormone concentrations.

Materials and Methods

Study Animals

Ten juvenile (approximately 80-150 g) captive born male bamboo sharks (*Chiloscyllium plagiosum*) were acquired from a Florida commercial source in May 2008. Animals were housed at the University of Florida's Whitney Laboratory for Marine Bioscience, St. Augustine, FL. Sharks were allowed to acclimate for a total of 3 weeks prior to start of experiment.

Tank Design

Bamboo sharks were housed in four flow-through concrete 1960 liter (518 gal) rectangular tanks located in an area with limited human traffic and minimal sound disturbances (Figure 3-1). Each tank was supplied with fresh seawater from the Atlantic Ocean, which was pumped through a series of PVC pipes that ran under the beach and was stored in a water tower. The tanks were situated outdoors (water temperature range was 23-25⁰C) but were shaded for protection from direct sunlight. Mesh net lids were placed over each tank to reduce human interaction and prevent predation. In each tank, PVC pipes (3 inch diameter) were provided for structure, hiding, and animal aggregation during the day.

Feeding Protocol

Juvenile sharks were fed six days a week (6% body weight per week) on a ration of capelin (*Mallotus villosus*), mackerel (*Scomberomorus spp.*), and silverside (*Menidia menidia*) along with a multivitamin supplement (Vita-Zu[®] Shark/Rays II Tabs; Mazuri Inc.[®]). A vitamin without iodine supplementation was prepared by Mazuri Inc.[®] and vitamins were test for iodine concentration by ABC Research Corporation (Gainesville, Fl; Hach reference method 8031). Tanks were cleaned daily to remove any fecal matter and uneaten food. Algal growth was also removed daily. Sharks were not fed for 24 hours prior to experimental blood draws.

Water Chemistry Protocol

Ammonia (ammonia salicylate method^a), nitrite (nitrite diazotization method^b), pH, and alkalinity^c were evaluated bi-weekly and on blood collection days in all tanks using a Hach DR/2700 portable spectrophotometer (Table 3-1).^d Temperature and dissolved oxygen (DO; morning only) were measured daily using a YSI 55 DO meter^e. Salinity was measured with a refractometer bi-weekly. In April 2010, total iodine was measured in the tanks using a Hach DR/4000 spectrophotometer by the ABC Research Corporation (Gainesville, FL; Hach reference method 8031).

Nitrate was tested daily with a Hach DR/2700 Spectrophotometer^d via both a chromotropic acid method^f and cadmium reduction method.^g The limit of both nitrate testing methods was 30 mg/L NO₃-N, therefore samples were diluted using a 1 to 10 dilution method. Water samples were also frozen daily during the nitrate exposure experiment from each tank. Approximately two weeks after the experiment ended, nitrate concentration (NO₃-N) in all frozen water samples were measured using ion chromatography (ICS-3000, AD25 Absorbance Detector^h).

Acclimation and Branding Protocol

Animals were distributed into four 1960 L flow-through tanks by ranking animals according to weight (largest to smallest) and dividing them among control and treatment tanks. Total biomass of each tank was within 20 g of the others. One week after arrival, all individuals were marked with silver nitrate using a dot marking scheme on their fins. Briefly, sharks were anesthetized until unresponsive to touch (minimum time 40 second, maximum time 74 seconds) in 65mg/L of buffered tricaine methanesulfonate (MS-222; Western Chemical Inc., Ferndale, WA). Sharks were removed from the anesthetic bath, measured (total length [cm]) and weighed (g). Sharks were returned to the anesthetic solution until un-responsive to touch. Then each

individual was placed on a wet ceramic tray, the fins were dried and silver nitrate q-tip was dipped in water and applied to skin for 3 second in a predetermined mark/branding pattern (Figure 3-2). Excess silver nitrate was then rinsed off with sea water and sharks were placed in pre-determined tanks and monitored for recovery from anesthesia.

Nitrate Experimental Protocol

The nitrate (NO_3) exposure experiment consisted of two treatments; a control group exposed to natural sea water and a NO_3 exposure group (Figure 3-2). The control group was exposed (N=5) to background concentrations ($<1.0 \text{ mg/L NO}_3\text{-N}$ or $< 4.4 \text{ mg/L total NO}_3^-$) of nitrate present in natural seawater for 29 days. NO_3 exposed animals (N=5) were exposed to nitrate at a concentration of $70 \text{ mg/L NO}_3\text{-N}$ ($308 \text{ mg/L total NO}_3^-$) via a continuous sodium nitrate (Jost Chemical Co; St. Louis MO) drip for 29 days. Briefly on alternating days, two tanks (4,230 liter; 1,117 gal; each) adjacent to treatment tanks were filled with sea water and 1,785 g of sodium nitrate was added to the sea water, resulting in calculated final concentration of $70 \text{ mg/L NO}_3\text{-N}$ in the treatment tanks. After 24 hours, the nitrate laden seawater from one of the reservoir tanks was pumped into both treatment tanks on a continuous basis for 24 hours. The flow rate (1.4 L per min) of water from the reservoir tank into the treatment tanks was adjusted daily to ensure a 24 hour turnover rate for each tank. Concurrently, water from the saltwater tower was pumped into the control tanks at the same flow rate ($1.4 \text{ liters per min}$) as the treatment tanks.

Plasma Sampling Protocol

During the experimental time, each shark was bled prior to nitrate exposure (day 1) and then at weekly intervals (day 8, day 15, day 22, day 29) for a total of 5 blood sampling periods. To minimize, variations due to circadian rhythm, all samples were taken 90 minutes after sunset (approximately 2300hr).

Briefly, individual sharks were netted from their tank and manually restrained. Blood (0.5 ml) was collected from the caudal vein with a 3.0-mL syringe (25-gauge needle) within 1 minute of restraint. The blood was placed into lithium heparin Vacutainer[®] tubes, and stored on ice for no more than 60 minutes before centrifugation. The plasma was separated via centrifugation (10 min at 3000 rpm), aliquated into two microvials (240 μ L each) and stored at -80°C prior to analysis.

Following venipuncture, each shark was placed into a 38 L plastic tub and anesthetized with 65mg/L buffered MS-222 solution to facilitate handling for physical examination, as well as collection of weight and length data. After health assessment, sharks were moved into a separate 30 L recovery tank (fresh seawater, no anesthesia solution and adequate aeration). When all animals from a given tank had been bled and recovered, they were returned to the original unit. Total time from net capture until animal were placed into the recovery tank was noted for each animal.

Tissue Collection

Following collection of the final blood sample (day 29), all sharks were euthanized with buffered MS-222 (1,000 mg/L). Each animal was weighed and measured and the thyroid gland was removed and placed in Bouin's fixative for histological analysis. Briefly, thyroid gland is an encapsulated organ located on top of a vascular tissue bed of loose connective tissue between the ventral side of the coracohyal and the medial side of the coracomandibular muscles (Ferguson, 1911; Honma et al., 1987; Figure 3-3). First, an incision in the skin from the anterior gill slit to the midline of the jaw was made, and then the skin was blunt dissected away from muscle layer and the thyroid gland was removed from each animal.

Condition Factor

A Fulton's condition factor (K) was calculated for each animal using the following formula $K = 100(\text{weight}/\text{length}^3)$, where weight is in g and length is in cm (Lochmann et al., 2009).

Histopathology

Thyroid gland was fixed in Bouin's fixative and transferred to 0.5 M ethylene diamine-tetracetic acid (EDTA) for clearing in August 2009. Samples were dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS). All samples were examined using Aperio Image Scope software (Aperio Technologies, Inc., Vista, CA). Histopathological findings were corroborated by an American College of Veterinary Pathologists (ACUP) Certified Pathologist in a blind reading.

Briefly, thyroid glands were examined for the presence of colloid, follicular cell hypertrophy, follicular cell hyperplasia, and glandular hypertrophy. Colloidal depletion was determined by the reduction or absence of PAS⁺ colloid in the lumen. Follicular cells were considered normal when lined with short, simple cuboidal epithelium, and hypertrophic when lined by columnar epithelium. Follicular cell hypertrophy was determined by the presence of basal nuclei and apical cytoplasm with an increase in cytoplasm to nuclear ratio. Follicular cell hyperplasia was identified by the presence of follicular cell crowding, stratification, and/or papillary infolding. Glandular hypertrophy was determined by percent of gland affected. A grading scheme for follicular hypertrophy, hyperplasia and colloidal depletion and glandular hypertrophy was assigned by methods described by Grim et al. (2009; Table 3-2).

Plasma Thyroid Hormone Analysis

A solid-phase, 96-well plate (Perkin Elmer, Boston, MA, Protein A Flash Plate Plus) radioimmunoassay (RIA) was used to determine thyroxine (T₄) concentrations. Assay buffer

was prepared from phosphate buffered saline with 0.1% gelatin (PBSG; 0.1M, pH 7.0). Prior to sample analyses, a plasma pool was serially diluted, and assayed to ensure parallelism with the standard curve. Standards, inter-assay variance controls, and samples for each assay were run in triplicate. Unknown concentrations were calculated from standard curves plotted as the % bound versus the log₁₀ concentration. Antibody specific to thyroxine (Fitzgerald Industries, Concord, MA, Cat # 20-TS40) was diluted in PBSG to a concentration of 1:100,000. Plates were coated with 100 µl of diluted antibody per well, incubated for 18 hours at 32°C, and rinsed twice with PBSG. Standards were prepared at concentrations of 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000 and 4000 pg/ml. Each well received 100 µl of standard, control, or sample in PBSG. Finally, ¹²⁵I-steroid (Perkin Elmer) was added at 12,000 cpm per 100 µl, and plates were incubated for 3 hours at 32°C. Interassay variance wells were similarly prepared from 5 pools of bamboo shark plasma. No separation of bound and free hormone is necessary with this assay, as only the bound radiolabeled steroid is capable of exciting the scintillant coating the bottom of the well. Plates were analyzed using a Microbeta 1450 Trilux counter (Perkin Elmer) in the ¹²⁵I channel with an estimated counting efficiency of 60%. Intra-assay variance averaged 2.5%, while interassay variance averaged 6.5%.

Statistical Analysis

Statistical analyses were performed using SAS for Windows (SAS Institute, Cary, NC). Proc Glimmix Repeated Measures was used to determine the effect of nitrate treatment over time on growth rate (e.g., weight, length, condition factor) and plasma T₄ concentrations. A student t-test was used to analyze changes in growth rate (e.g., weight, length, condition factor) and plasma T₄ concentrations between the start and end of the experiment. A Fisher's exact test was

used to determine the effect of treatment on the presence of diffuse hyperplastic goiter (i.e. incidence of follicular cell hyperplasia or hypertrophy).

Results

Iodine Analysis of Vitamin Supplementation

Iodine (as calcium iodate) analysis of the vitamin supplement used in this experiment was found to contain 71 µg of iodine per 0.19 g tablet compared to a normal Mazuri SharkTab vitamin which has 30 mg per 0.19 g tablet.

Water Chemistry

Water chemistry parameters were tested bi-weekly, including the day of experimentation, and were as follows: unionized ammonia (NH_3) ≤ 0.002 mg/L, nitrite ≤ 0.003 mg/L, pH 8.2, alkalinity 250 mg/L, salinity 33 ppt. Dissolved oxygen concentrations were maintained at $\geq 95\%$ through the trial and temperature was 26.1-26.5 °C. Total iodine was 0.05mg/L. Nitrate concentrations in the all tanks were tested daily and were as follows (mean \pm SE): both control tanks $\leq 1.66 \pm 0.43$ mg/L $\text{NO}_3\text{-N}$, nitrate tanks 74.95 ± 2.37 mg/L $\text{NO}_3\text{-N}$ and 77.84 ± 3.07 mg/L $\text{NO}_3\text{-N}$.

Length and Weight

Data analysis revealed no significant difference in growth rate (e.g., length) between nitrate exposed group and control sharks over the 29 day exposure ($p = 0.83$), or between groups at the start or end of experiment ($p > 0.05$; Figure 3-4). Data analysis revealed no significant difference in weight gain between nitrate exposed sharks and control sharks over 29 day exposure ($p = 0.40$), or between groups at the start or end of experiment ($p > 0.05$; Figure 3-5).

Condition Factor

The mean values obtained for condition factor of nitrate exposed sharks and control sharks over course of experiment are represented in Figure 3-6. Data analysis revealed no significant

difference in condition factor between nitrate exposed groups and control groups over the 29 day exposure ($p = 0.40$), or between groups at the start ($p = 0.2$) and end of experiment ($p = 0.4$).

Histopathology

Preservation of thyroid glands in Bouin's for one year did result in blebbing of colloid from follicular lumen, but this artifact was present in all samples. One of the control group samples was considered too small for an accurate assessment and therefore was not part of the final analysis. Thyroid glands from control group consisted of follicles in various sizes and shapes (Figure 3-7). Colloid within the lumen is uniformly PAS⁺ and abundant (Figure 3-8). Follicular epithelial cells are low cuboidal to low columnar (Figure 3-9). Overall glands were considered to be normal (Honma et al., 1987; Crow et al., 2001).

Results indicate sharks exposed to nitrate developed diffuse hyperplastic goiter (Table 3-3; Figure 3-7). Four thyroid glands from nitrate exposed sharks contained small hyperplastic follicular nests in between ectatic follicles with colloid in lumen. There was a reduction in colloid compared to control sharks (Figure 3-8). The colloid from the hyperplastic thyroid glands were considered inspissated with a cracked/solid appearance. Follicular cells were lined with tall columnar epithelium, basal nuclei and apical cytoplasm. Follicles exhibited mild to moderate hyperplasia characterized by pseudostratified and/or stratified follicular epithelium (Figure 3-9). Papillary infolding was present. The fifth thyroid gland from the nitrate exposed group exhibited mild follicular cell hypertrophy and hyperplasia but was considered "normal".

Plasma Thyroxine Concentrations

The T₄ concentrations measured in the control sharks over 29 days ranged (mean ± SE) from 9.57 to 30.50 ng/mL (14.77 ± 1.13 ; $n = 25$). The T₄ concentrations measured in the nitrate exposed sharks over 29 days ranged (mean ± SE) from 4.63 to 45.26 ng/mL (16.53 ± 1.91 ; $n = 25$; Figure 3-10). Table 3-4 shows mean weekly plasma T₄ concentrations for both treatments.

Plasma T₄ concentrations were not significantly different from the start and end of the experiment ($p > 0.05$). Nitrate exposure over the course of 29 days did not affect plasma T₄ concentrations when compared to the control group ($p = 0.3$; Figure 3-11). Table 3-5 shows individual sharks day 29 plasma T₄ concentrations along with histology grades.

Discussion

This paper describes the effects of an acute 29 day nitrate exposure on the thyroid function of juvenile male white-spotted bamboo sharks. Previous studies have described the effects of chronic (≥ 5 months) nitrate exposure on thyroid function (Crow et al., 1998; Crow et al., 2001; Zaki et al., 2004; Eskiocak et al., 2005); therefore this study provided a unique insight into the early stages of goiter development associated with nitrate exposure. In order to begin to elucidate the etiology of goiter, three indices were assessed; alteration in growth rates, histologic evidence of diffuse hyperplastic goiter, and reduction of plasma thyroxine (T₄) concentrations. Since thyroid hormones (THs) are important in growth and development of a juvenile animal, it was hypothesized that exposure to nitrate would reduce growth rates of the juvenile bamboo sharks. Results revealed that nitrate exposure did not significantly affect growth rates during the 29 day exposure period. All sharks regardless of treatment gained both weight and length over the course of the experiment. All sharks were individually hand fed, water quality was optimum and tank size was large compared to relative body size, thus providing a possible explanation for the increase in growth for all individuals. However, the short duration of this experiment may have been a contributing factor to the lack of any significant difference in growth between treatment groups.

Condition factor was used as a comparative and theoretical measure of shark physiological well-being. By calculating the relationship between weight and length gained over the 29 day experimental time period, this ratio can provide an indication as to the “condition” of the

individual. Because of THs influence on metabolism, growth and development, it was hypothesized that exposure to nitrate would reduce the condition factor of the juvenile bamboo sharks. Results revealed that nitrate exposure did not significantly affect condition factor during the 29 day exposure period. Previous studies that used condition factor as an index of health to assess the effects of goitrogenic (e.g., propylthiouracil, perchlorate) compounds on thyroid function have revealed no significant difference in condition factor of treatment animals versus control animals even when other indices (e.g., thyroid hormones, histopathology, thyroid receptor expression) to assess thyroid function were significantly affected (Mukhi et al., 2005; Morgado et al., 2009). Therefore, when interpreting an individual's condition factor between two given points in time, one must remember that condition factor is just a ratio between weight and length and consequently it may not be a good indicator as to the true overall health of the fish.

Histologic assessment of thyroid structure of nitrate exposed sharks compared to control sharks demonstrated that nitrate exposure resulted in the development of diffuse hyperplastic goiter, indicating that there was some disruption in normal thyroid function. In 4 of the 5 sharks exposed to nitrate, there was a loss of colloid, papillary infolding and follicular hypertrophy and hyperplasia throughout a 40-60% of the thyroid gland further indicating overstimulation of the gland by thyroid stimulating hormone (TSH) and a reduction in TH follicular storages. Diffuse hyperplastic goiter has been observed in individuals exposed to goitrogenic compounds, including nitrate (Crow et al., 1998; Crow et al., 2001; Hooth et al., 2001; Gridelli et al., 2003; Rivera and Lock, 2008; Grim et al., 2009). Because the control treatment sharks did not develop goiter, we believe exposure to elevated nitrate concentration (70 mg/L NO₃-N) was responsible for the pathology that was observed in our nitrate-exposed sharks. Although iodine

concentrations were measured over 1 year after the initial experiment, the total iodine concentration (0.05 mg/L) calculated was close to previous total iodine findings in natural saltwater (0.06 mg/L) (Wong, 1980). These findings support our belief that the environmental iodide concentrations were adequate for normal thyroid hormone synthesis to occur.

Diffuse hyperplastic goiter results from a disruption of the negative feedback mechanisms responsible for the synthesis and release of new THs. As circulating THs levels decrease, TSH stimulates the follicles to synthesize and release new THs from the gland. Any disruption in this process will cause TSH to continue to stimulate the thyroid follicles and overtime hormone storages decline resulting in a reduction in circulating THs (Hadley, 2000). It was hypothesized that 29 day exposure to 70 mg/L NO₃-N would reduce plasma T₄. However, results from this experiment did not support this hypothesis.

This is the first study that has measured plasma T₄ concentrations in juvenile male white-spotted bamboo sharks (*C. plagiosum*), thus there were no basis of comparison for normal plasma T₄ concentration in this species. In this experiment, our analysis revealed that normal plasma T₄ concentrations measured in the control sharks ranged from 9.57 to 30.50 ng/ml. Upon further examination, our mean plasma T₄ (14.77 ng/ml) concentrations were similar to previous studies that measured plasma T₄ concentrations in other shark species (Volkoff, 1996; Crow et al., 1998; Crow et al., 1999; Gash, 2000; McComb et al., 2005). Volkoff (1996) measured T₄ concentrations of four wild caught sharks and found the mean T₄ concentration to be as follow; blacktip reef shark (*Carcharhinus limbatus*) – 17.0 ng/ml, finetooth shark (*Carcharhinus isodon*) – 16.0 ng/ml, dusky shark (*Carcharhinus obscurus*) - 25 ng/ml, and sharpnose sharks (*Rhizoprionodon terraenovae*) - 23 ng/ml. In wild caught bonnethead shark (*Sphyrna tiburo*), T₄ concentrations have been reported to range from ≤ 1.0 to 16.95 ng/ml, while T₄ concentrations

in captive whitetip reef sharks (*Triaenodon obesus*) ranged from 1.34 - 9.24 ng/ml (Gash, 2000; Crow et al., 1999). When compared to other vertebrate species, plasma T₄ concentrations in white-spotted bamboo shark (4.63 - 45.26 ng/ml) are higher than amphibians (0.25 - 9.0mg/ml) and teleost fish (less than 1.0 ng/ml) (Brown and Eales, 1977; Volkoff, 1996; Norris, 2007). However, our small sample size likely resulted in the fairly broad range of values; additional work is needed to determine “normal” T₄ concentrations of juvenile white-spotted bamboo sharks.

A reduction in plasma THs has been found to occur after chronic exposure (≥ 5 months), where over time the thyroid follicles becomes desensitized to TSH and cease releasing THs. As a result, the thyroid follicles become enlarged due to increased colloid storages of THs, the weight of the thyroid gland increases and there is the development of diffuse colloid goiter (Cotran et al., 1994; Crow et al., 1998; Zaki et al., 2004; Eskiocak et al., 2005). A reduction in plasma T₄ has been observed with diffuse colloid goiter because the thyroid follicles become less responsive to TSH stimulation, THs are not released which results in a characteristic state of hypothyroidism (Marine and Lenhart, 1909). Because thyroid hormones are released from the thyroid gland due to TSH stimulation, plasma TSH maybe a better indicator of a diffuse hyperplastic goiter than measuring plasma T₄ concentrations directly (Kemppainen and Behrend, 2001). Due to the fact that diffuse colloid goiter was not seen in our study, that may be a possible explanation as to why our plasma T₄ concentrations from our nitrate exposed sharks were “normal” when compared to our control animals.

Previous studies have found that plasma T₄ is not a good indicator of thyroid function, as the feedback mechanisms that control TH levels can maintain normal circulating TH concentrations in spite of a depletion of colloid T₄ storages in the thyroid gland (McNabb et al.,

2004; Mukhi et al., 2005). For example, Graham et al. (2007) observed that there can be over 70% pathological changes to the thyroid gland while circulating THs concentrations remained within normal limits. Instead, it has been suggested that measuring colloidal T₄ storages may be a better indicator of disruption of thyroid function due to goitrogenic compounds. Histologic findings from this study indicate that there was a reduction in colloidal storages in nitrate exposed sharks compared to control sharks and the use of T₄ immunohistochemistry may have provided a more sensitive indicator as to the effect of nitrate exposure on synthesis of new T₄ within the thyroid gland.

Though thyroid glands from the nitrate exposed sharks exhibited histologic changes consistent with diffused hyperplastic goiter, the entire gland was not affected. This suggests that these animals still possessed some ability to synthesize new THs, providing an alternative explanation as to why no statistically significant difference in plasma T₄ concentrations was observed between the nitrate exposed sharks and the control sharks. In teleost fishes, the thyroid gland is not a discrete organ and instead can be diffuse throughout the pharyngeal region or located near the kidney, as seen in the common carp (*Cyprinus carpio*) (Geven et al., 2007). Though the thyroid gland has been described as a discrete organ in elasmobranchs (Ferguson, 1911), there is a possibility there may be functional thyroid tissue distributed in other parts of the body, and if so, those diffuse follicular cells may be able to compensate for the loss of glandular tissue, ensuring normal levels of THs are maintained.

In teleosts, control of THs concentrations occurs peripherally, mainly in the liver, rather than centrally, as in other vertebrates. This difference in thyroid hormone conversion mechanism highlights the importance of deiodinase enzyme activity in the activation and/or deactivation of THs (Eales and Brown, 1993; Blanton and Specker, 2007). For example, type I

deiodinase (D1) is responsible for the peripheral conversion of T_4 to T_3 , while Type 3 deiodinase (D3) is important in the conversion of T_4 to reverse T_3 and subsequent elimination of THs from the liver (Norris, 2007). This balance between the activation, deactivation, and elimination of THs plays an important role in how the body responds to alternation of normal thyroid function. For example, hyperthyroidism, a state of increased circulating thyroid hormones, increases D3 activity and subsequent clearance of excess THs, while the opposite is observed in hypothyroidism. Instead, hypothyroidism has been found to increase D1 activity and reduce hepatic TH clearance rates (Orozco and Valverde, 2005). This study did not measure deiodinase activity and the question remains as to how nitrate affects the conversion of T_4 to T_3 and/or reverse T_3 , and ultimately thyroid hormone clearance rates. Hamlin et al. (2008) posed the question as to whether nitrate exposure can affect sex hormone clearance rates in the liver as her results demonstrated that nitrate-exposed sturgeon had significantly higher plasma testosterone concentrations compared to controls. Therefore, possible normal T_4 concentrations observed in the nitrate-exposed sharks could have resulted from an alteration in T_4/T_3 /reverse T_3 conversion and eventual thyroid hormone clearance. Further studies are needed to understand the effects of nitrate on deiodinase activity in white-spotted bamboo sharks.

The activation of thyroid receptors and consequent control of gene transcription is controlled by T_3 and not T_4 , further supporting the role that deiodinases play in maintaining THs homeostasis. It has been suggested that measuring deiodinase activity can provide an indirect indicator of possible changes on T_3 receptor mediated effects on target cells (Blanton and Specker, 2007). Additional support of the use of deiodinase activity as an indicator of thyroid function is that in fish endogenous T_4 does not lead to an increase T_3 (Eales and Brown, 1993). Therefore, normal or elevated concentrations might produce a false positive indication of normal

thyroid function, while in reality deiodinase activity and subsequent receptor mediated effects on T_3 on target cells are altered. In order to assess thyroid function, this study only measured T_4 and did not measure any of the aforementioned indices also responsible in maintaining thyroid homeostasis. Future studies should consider using deiodinase activity or T_3 receptor mediated expression as an index in order to attempt to elucidate the effects of nitrate exposure on peripheral tissues.

Analysis of plasma T_4 concentrations observed in the nitrate exposed sharks demonstrates that a diagnosis of goiter based solely on circulating T_4 levels must be made with great caution. Though our results demonstrated that T_4 concentrations from nitrate exposed sharks were not statistically significant from the control sharks, histologic evaluation of the nitrate exposed sharks thyroid glands revealed pathologic changes. In an aquarium, it is not feasible or desirable to euthanize elasmobranchs from a collection as a means of determining thyroid gland health of the population. Therefore, alternative and minimally invasive indices need to be adopted. For example, measuring not only circulating T_4 levels but also circulating T_3 and TSH levels can provide a glimpse as to the activity of the thyroid gland. Due to the fact that THs have such a short half-life (T_4 – 5 to 7 days; T_3 – 1 day) within the blood stream, multiple sampling is needed in order to establish indices by which any future comparison can be made.

In conclusion, this study supports the concern that environmental nitrate exposure, in the absence of other factors (e.g., reduced I bioavailability), may be goitrogenic in juvenile bamboo sharks and is an important factor in the etiology of this disease in captive elasmobranchs. This experiment was an acute 29 day exposure to 70 mg/L NO_3-N , which manifested in the development of diffuse hyperplastic goiter. Previous reports on the manifestation of goiter in captive elasmobranchs have described the development of diffuse colloid goiter; which first

manifests as diffuse hyperplastic goiter, but due to chronic exposure to an I⁻ limited environment, overtime it develops into a diffuse colloid goiter (Cotran et al., 1994; Crow et al., 1998; Crow et al., 2001; Gridelli et al., 2003). Therefore, the histological finding of diffuse hyperplastic goiter from this study provides a unique contribution in our attempt to begin to elucidate the stages of goiter manifestation in captive sharks. In the nitrate exposed sharks, histological changes occurred to over 60% of the thyroid gland. For this reason, the thyroid gland may have been able to compensate during the 29 day nitrate exposure in order to maintain T₄ concentrations within “normal” ranges; providing an explanation as to why there was no significant difference in T₄ concentrations between nitrate-exposed sharks and the control sharks. Goiter is one of the most common health problems in captive elasmobranchs and this study suggests that nitrate exposure may be an important factor in the etiology of this disease.

Table 3-1. Water quality parameters from the flow-through natural saltwater system.

Parameter	Range	Units
Salinity	33.0	ppt
pH	8.2	
Dissolved O ₂	95-98	% saturation
Unionized Ammonia	<0.002	mg/L
Nitrite	<0.003	mg/L
Total Alkalinity	250	mg/L
Control Tank #1 – Nitrate	<1.66 ± 0.43	mg/L NO ₃ -N
Control Tank #2 – Nitrate	<1.66 ± 0.43	mg/L NO ₃ -N
Nitrate Exposure Tank #1	74.95 ± 2.37	mg/L NO ₃ -N
Nitrate Exposure Tank #2	77.84 ± 3.07	mg/L NO ₃ -N

Table 3-2. Severity grading scheme for follicular cell hypertrophy and hyperplasia, colloid depletion and glandular hypertrophy adapted from Grim et al. (2001).

	Grade	Descriptor	Criterion
Hypertrophy			
	0	Not	≤ 10% enlargement of gland
	1	Mild	Diffuse enlargement of gland 20-40%
	2	Moderate	Diffuse enlargement of gland 50-70%
	3	Severe	Diffuse enlargement of gland ≥70%
Hyperplasia			
	0	Not	Focal or diffuse crowding of follicular cells ≤ 10%
	1	Mild	Focal or diffuse crowding of follicular cells 20-40%
	2	Moderate	Pseudostratified or stratified follicular epithelium,
	3	Severe	Extensive hyperplasia with stratification 2-3 cells
Colloid			
	0	Not	≤ 10% colloid depletion throughout gland
	1	Mild	Colloid depletion in 20-40% of the thyroid gland
	2	Moderate	Colloid depletion in 50-70% of the thyroid gland
	3	Severe	Colloid depletion in ≥70% of the thyroid gland
Glandular			
	0	Not	≤ 10% of follicles affected throughout the thyroid
	1	Mild	20-40% of follicles affected throughout the thyroid
	2	Moderate	50-70% of follicles affected throughout the thyroid
	3	Severe	≥70% of follicles affected throughout the thyroid

Table 3-3. Incidence and severity of thyroid alterations in white-spotted bamboo sharks, *Chiloscyllium plagiosum*, after 29 days of nitrate exposure.

	Control	Nitrate sharks
Hypertrophy Severity Grade	0.25 ± 0.25	1.6 ± 0.25*
Hyperplasia Severity Grade	0.25 ± 0.25	1.8 ± 0.20*
Colloid Depletion	0.25 ± 0.25	1.2 ± 0.20*
Glandular Hypertrophy	0.25 ± 0.25	1.6 ± 0.24*

*Significantly different from corresponding grade control mean by a one-sided Fisher's exact test ($p < 0.05$).

Table 3-4. Mean weekly plasma T₄ concentrations for control sharks and nitrate exposed sharks. Data corresponds to the mean ± SE. p<0.05.

	Day 1	Day 8	Day 15	Day 22	Day 29
Control Sharks	16.98 ± 3.39	13.70 ± 2.33	16.99 ± 3.27	14.39 ± 2.19	11.79 ± 0.97
Nitrate Exposed	16.77 ± 2.76	16.01 ± 2.02	20.11 ± 5.00	17.61 ± 7.12	12.16 ± 3.78

Table 3-5. Individual shark plasma T₄ concentration and histology severity grades from day 29.

Control Sharks	T ₄ ng/ml	Hypertrophy	Hyperplasia	Colloid Depletion	Glandular Hypertrophy
T1CO1	12.84	0	0	0	0
T1CO2	15.06	n/a*	n/a*	n/a*	n/a*
T2C01	9.98	0	0	0	0
T2CO2	11.06	1	1	1	1
T2C03	10.04	0	0	0	0
Nitrate-Exposed Sharks	T ₄ ng/ml	Hypertrophy	Hyperplasia	Colloid Depletion	Glandular Hypertrophy
T3NO1	8.46	1	1	1	1
T3NO2	6.45	2	2	2	2
T4NO1	9.59	2	2	1	2
T4N02	9.14	2	2	1	2
T4NO3	27.14	1	2	1	1

*sample too small for an accurate analysis

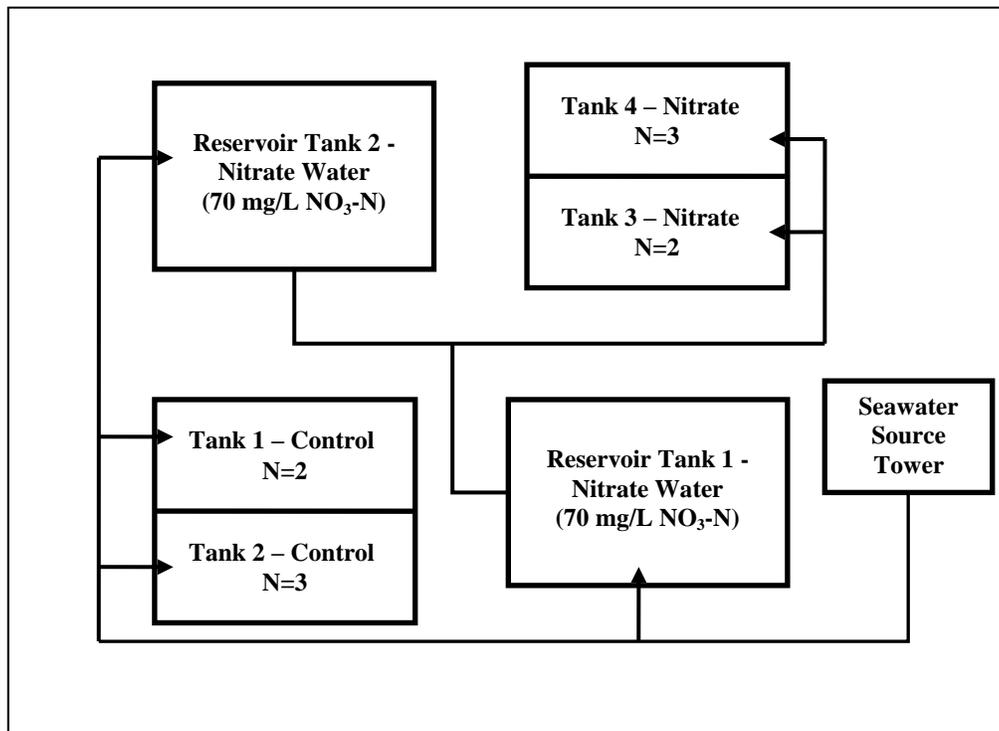


Figure 3-1. Experimental tank design. Bamboo sharks were housed in four flow-through concrete 518 gal tanks.



Figure 3-2. Individual identification marking scheme. Branding of a white-spotted bamboo shark using silver nitrate q-tip.

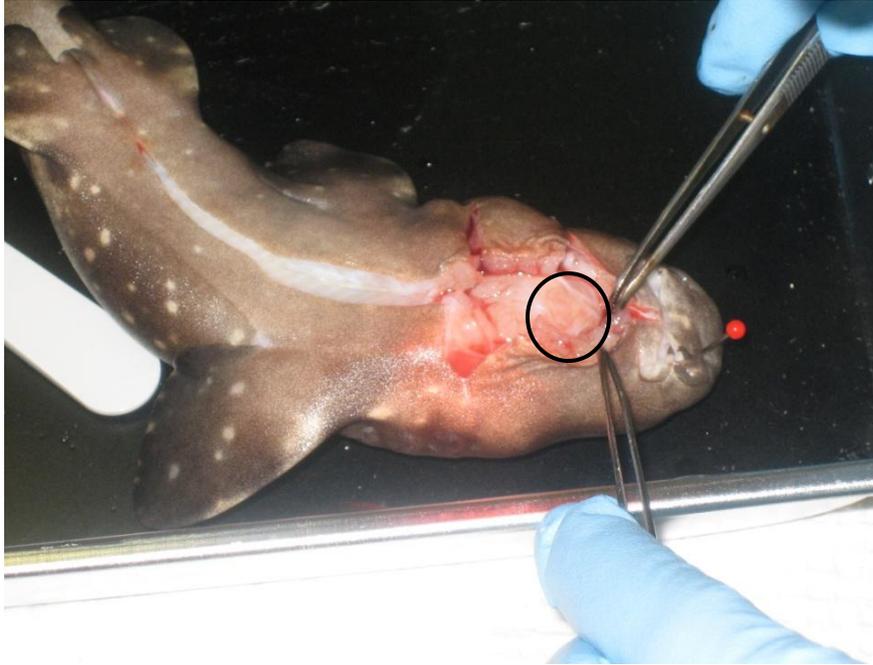


Figure 3-3. Dissection of the thyroid gland (circle) located between the ventral side of the coracohyal and the medial side of the coracomandibular muscles.

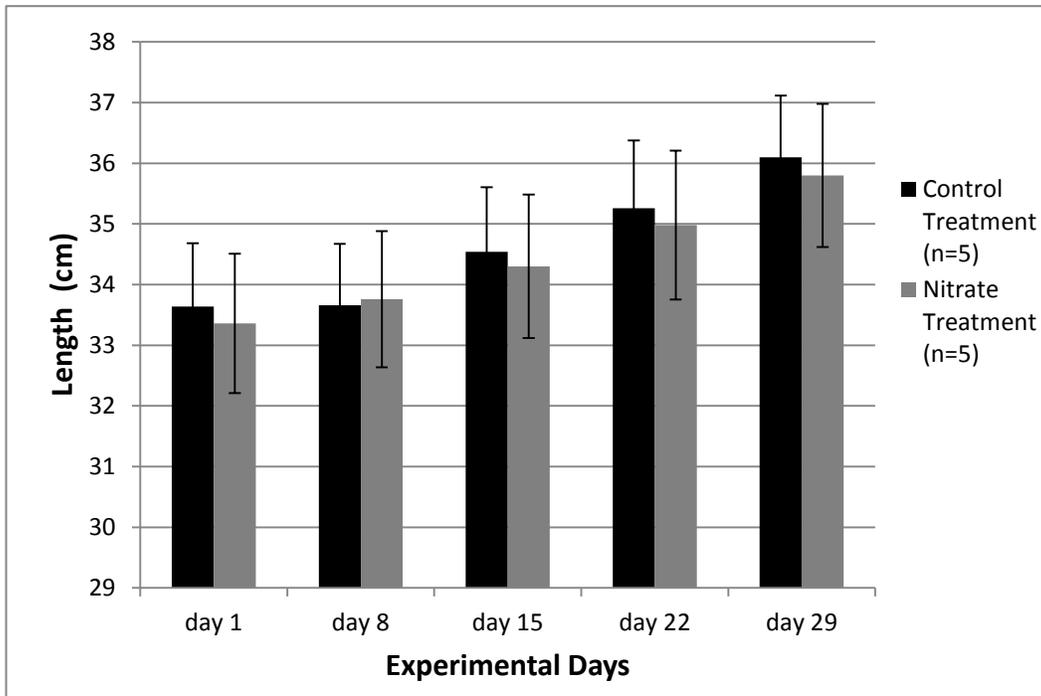


Figure 3-4. Length gain of sharks during the experimental period (29 days) for control group and nitrate exposed group. No significant difference was found in length between nitrate-exposed and control sharks at the start and end of the experiment. Data corresponds to the mean \pm SE. $p < 0.05$.

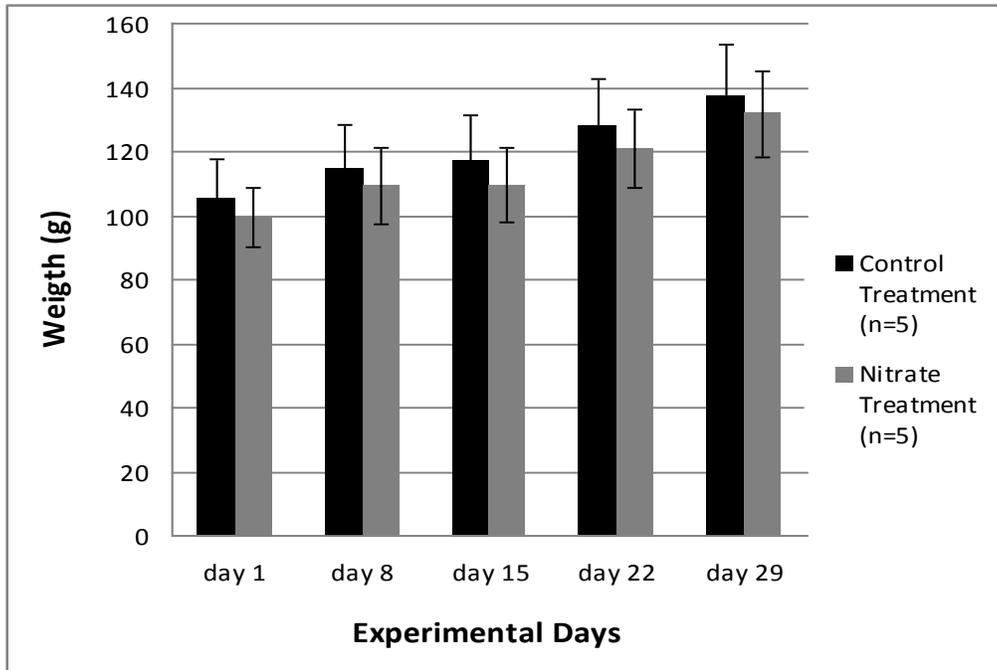


Figure 3-5. Weight gain of sharks during the experimental period (29 days) for control group and nitrate exposed group. No significant difference was found in weight between nitrate-exposed and control sharks at the start and end of the experiment. Data corresponds to the mean \pm SE. $p < 0.05$.

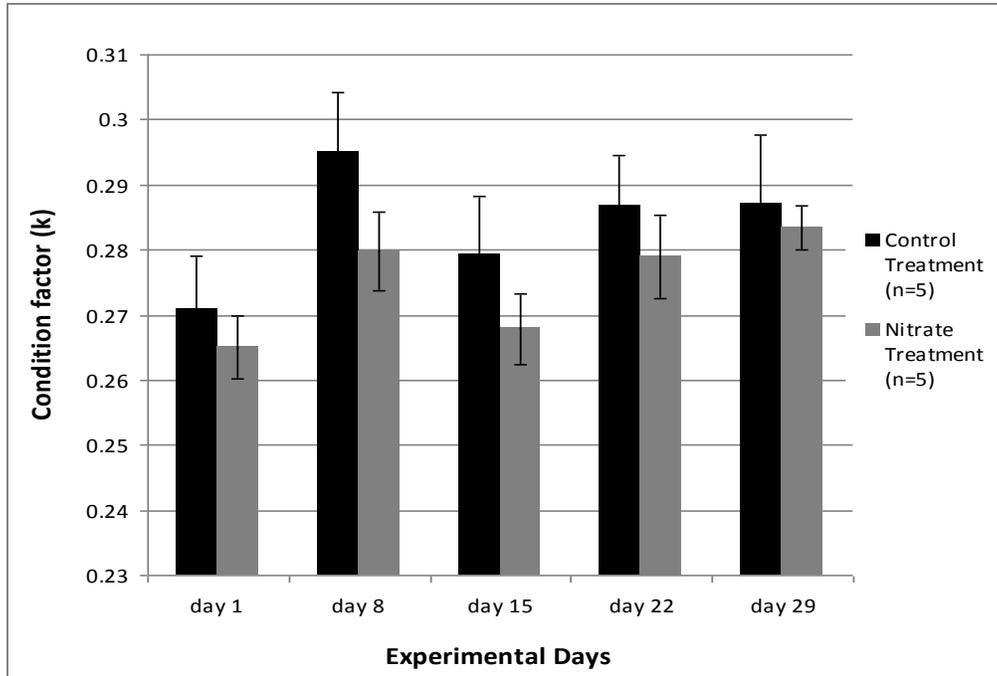


Figure 3-6. Condition factor of sharks during the experimental period (29 days) for control group and nitrate exposed group. No significant difference was found in condition factor between control sharks and nitrate-exposed sharks at the start and end of the experiment. Data corresponds to the mean \pm SE. $p < 0.05$.

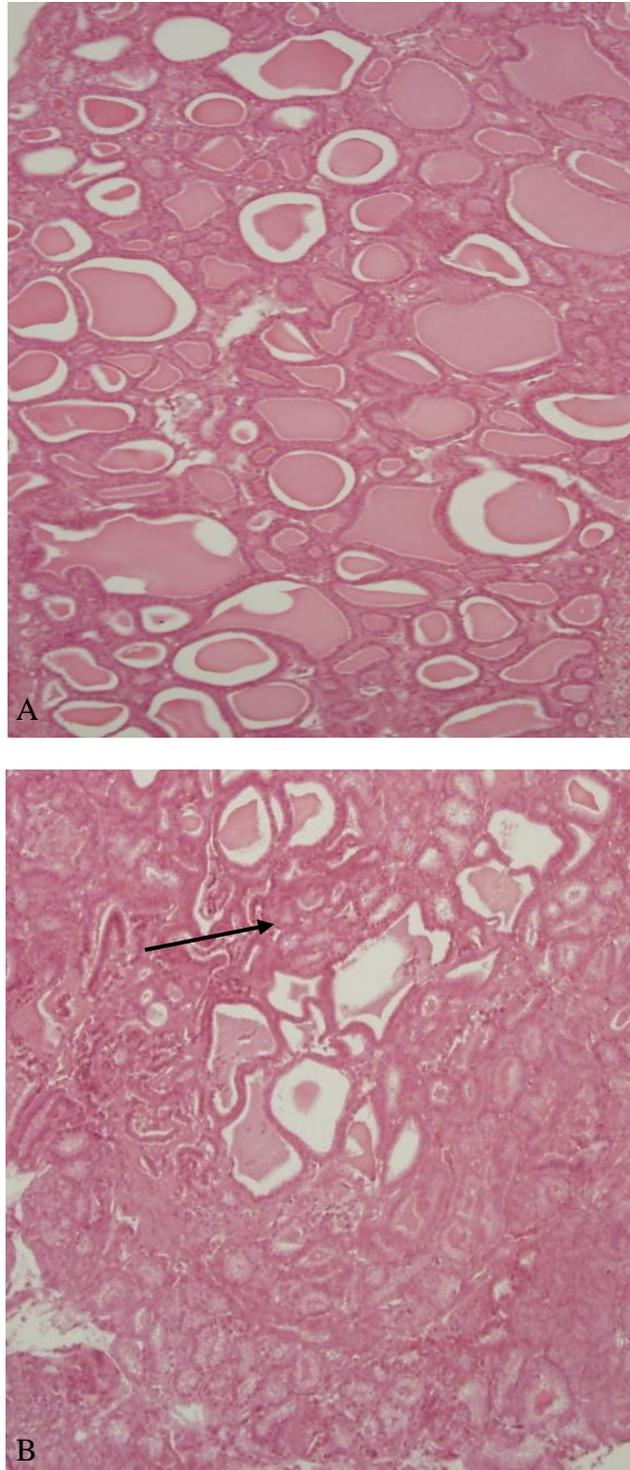


Figure 3-7. Representative images of thyroid glands from white-spotted bamboo sharks, *Chiloscyllium plagiosum*. A) Control group consist of follicles in various sizes and shapes. Colloid within lumen is uniform throughout. B) Nitrate exposed group contained small hyperplastic follicular nests (arrow) in between ectatic follicles with colloid in lumen. Stained with H&E. (10x)

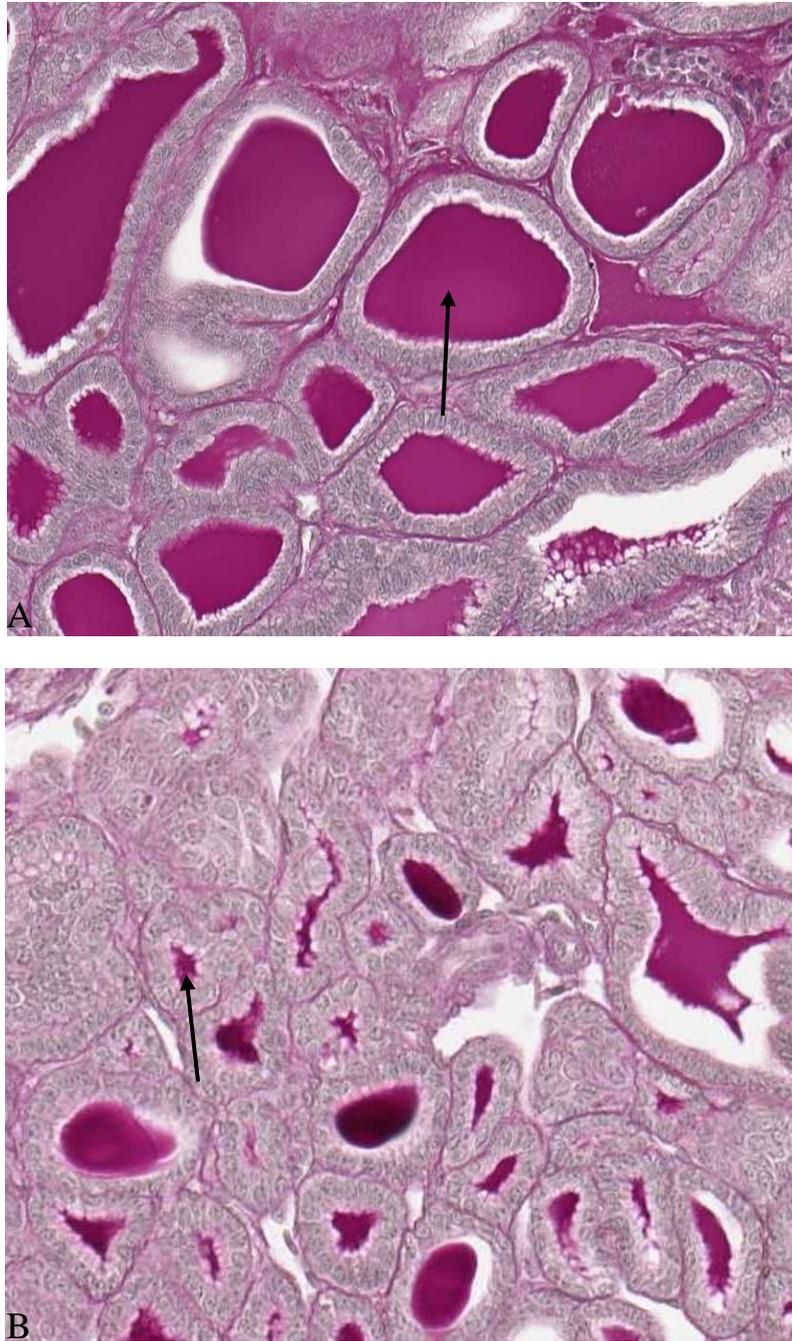


Figure 3-8. Representative images for the comparison of colloidal storage. A) Thyroid gland from control shark, colloid (arrow) within the lumen is uniformly PAS⁺ and abundant. B) Thyroid gland from nitrate exposed shark, colloid is reduced or absent from lumen (arrow). (20x)

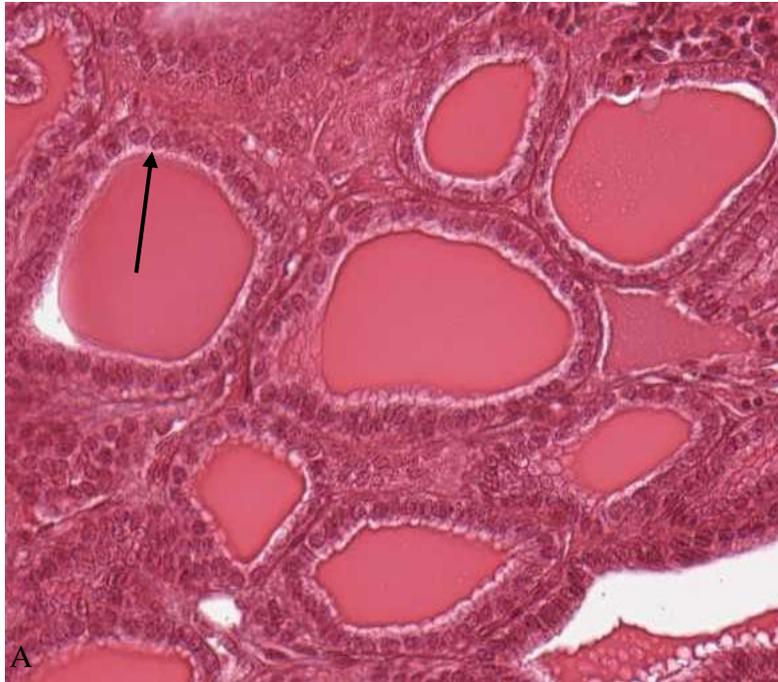


Figure 3-9. Representative images for the comparison of the follicular cell epithelium. A) Thyroid gland from control shark. Follicular cells lined by a layer of single cuboidal epithelium (arrow). No follicular cell hypertrophy or hyperplasia B) Thyroid gland from nitrate exposed shark. Follicular cells lined with tall columnar epithelium (follicular cell hypertrophy; arrow). Follicles exhibited mild to moderate hyperplasia characterized by pseudostratified and/or stratified follicular epithelium (circle). Stained H&E. (20x)

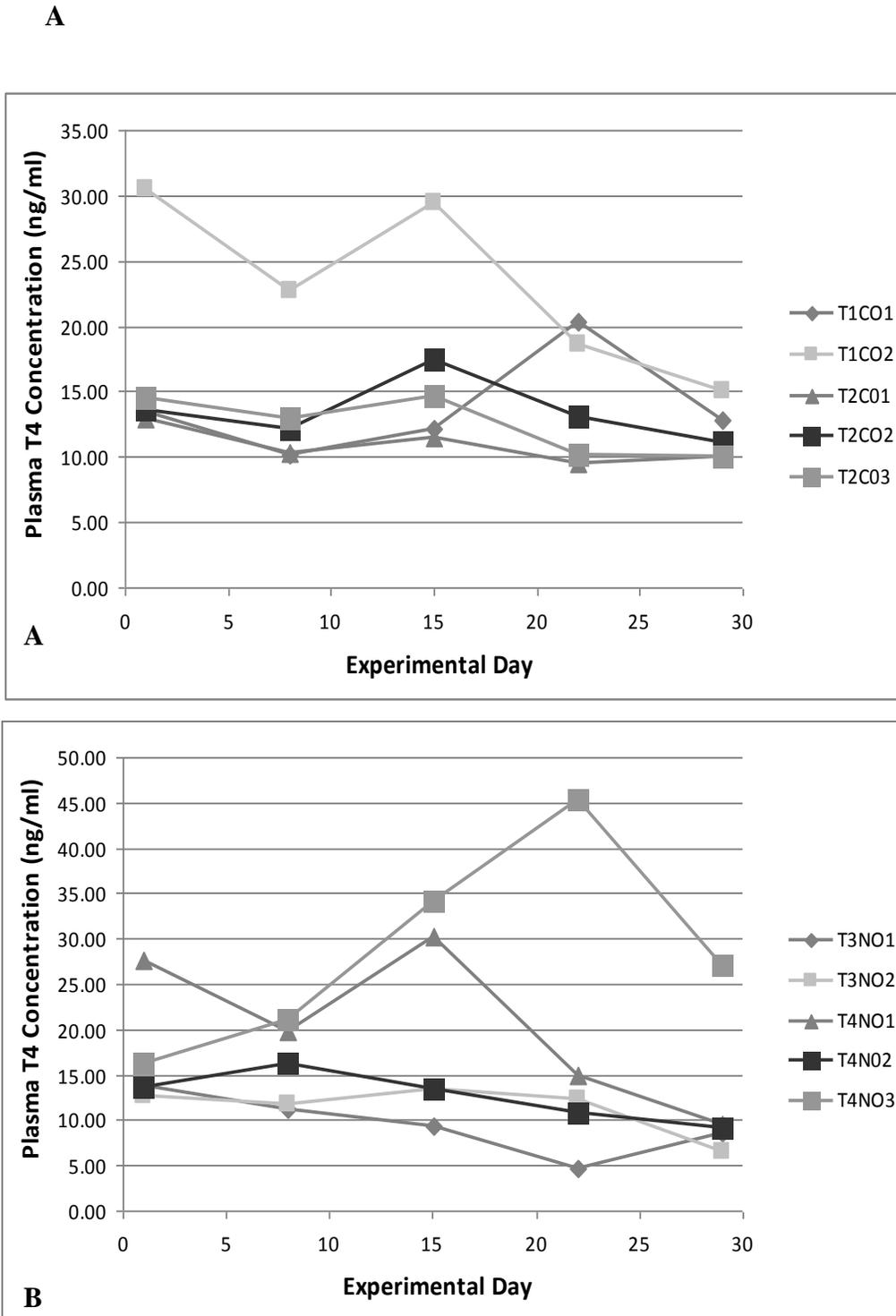


Figure 3-10. Plasma T₄ concentrations for individual white-spotted bamboo sharks, *Chiloscyllium plagiosum*, control group A) and nitrate exposed group B) over 29 days experimental period. Graph represents individual plasma T₄ concentration.

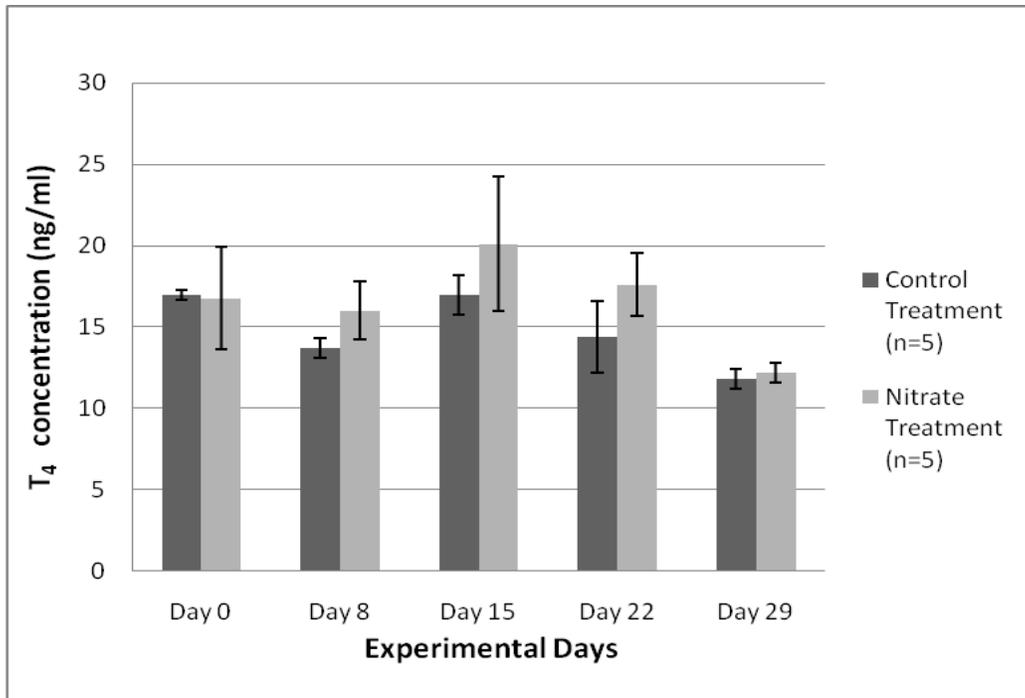


Figure 3-11. Mean plasma T₄ concentrations of sharks during the experimental period (29 days) for nitrate exposed group and control group. No significant difference was found in T₄ between nitrate-exposed and control sharks at the start and end of the experiment. Data corresponds to the mean \pm SE. $p < 0.05$.

CHAPTER 3
HYPOTHYROIDISM IN A FEMALE BROWN-BANDED BAMBOO SHARK,
CHILOSCYLLIUM PUNCTATUM

Introduction

In an aquatic environment, total dissolved iodine (~0.06 mg/L) occurs in two forms, iodide (I^-) and iodate (IO_3^-). While the exact ratio of these two forms varies with geographical location and water depth, the aquatic concentration of IO_3^- is around 0.04–0.06 mg/L and I^- is between 0.01-0.02 mg/L (Wong, 1980). Though both IO_3^- and I^- exist in an aquatic environment, only I^- can be used in thyroid hormone synthesis (Eales, 1997). Fish, including sharks, actively accumulate I^- via a gill branchial iodide pump ($\geq 80\%$), while receiving relatively little I^- through intestinal absorption ($\leq 20\%$) (Gorbman, 1955; Eales and Brown, 1993). Due the high environmental iodine concentrations in sea water (~0.06 mg/L; (Wong, 1980; Spotte, 1992)), plasma concentrations of I^- are relatively higher in fish compared to mammals. Because of the relatively stable concentrations of I^- in an aquatic environment, fish including sharks store relatively little I^- within the thyroid gland or other tissues (Gorbman, 1955; Eales and Brown, 1993). This physiological dependency on environmental bioavailability of I^- is critical to the understanding of goiter formation in captive elasmobranchs, as gradual or sudden decreases in environmental I^- concentrations can result in the development of this disease (Crow et al., 1998; Murray, 2009).

Iodide is essential for the production of new thyroid hormones and subsequent storage of thyroid hormones within the colloidal lumen. Disruption of normal thyroid hormone synthesis due to a deficiency in iodide results in the reduction of both colloid storages and circulating concentrations of thyroid hormones (Cotran et al., 1994). If limited access to dietary I^- or environmental I^- is not resolved, the excessive stimulation of the thyroid gland over time will result in histologic change of the follicles and ultimately the development of goiter. The three

forms of goiter (diffuse hyperplastic goiter, diffuse colloid goiter, and multinodular goiter) characteristic of iodide deficiency and/or exposure to goitrogenic compounds have been observed in captive elasmobranchs (Crow et al., 2001; Gridelli et al., 2003).

A diffuse hyperplastic goiter results from a reduction in plasma thyroid hormone concentrations and elevation of thyroid stimulating hormone (TSH) resulting in little to no colloid, papillary enfolding of follicular epithelium, and cellular hyperplasia and hypertrophy (Cotran et al., 1994; Crow et al., 2001). Continued exposure to a low iodide environment ($\leq 0.15\mu\text{M}$) results in the continuous attempt of the thyroid gland to stabilize hormone production. Because of the constant stimulation of the thyroid follicles by TSH over time, these follicles become desensitized to TSH stimulation and no longer recognize the TSH signal. Consequently, no hormone is released. This state of hypothyroidism is marked with a characteristic formation of a diffuse colloidal goiter (Cotran et al., 1994; Crow et al., 2001; Zaki et al., 2004; Eskiocak et al., 2005). At this stage, the thyroid gland contains enlarged thyroid follicles filled with colloid and flattened follicular epithelium. Eventually, the continued exposure to an iodide deficient environment results in the development of multinodular goiter which has flattened follicles of varying size mostly containing colloid with cuboidal to low columnar epithelial cells. Follicles are multinodular surrounded by fibrous bands, connective tissue and expanded parenchyma (Cotran et al., 1994). If left untreated, goiter can result in lethargy, anorexia, dyspnoea, and even death (Gridelli et al., 2003; Sherrill et al., 2004).

In aquaria, ozone is used to remove organic debris and to chemically filter water (Powell et al., 2004). However, this process has been shown to alter the bioavailability of iodine in saltwater converting available I^- to IO_3^- (Sherrill et al., 2004). A standard ozone setting (800mV) of an ozone contact chamber in a marine system had been found to reduce I^- concentration from

0.15 μM to 0.032 μM (Sherrill et al., 2004). Because of the potential loss of I^- from the environment due to the presence of ozone, it has been suggested that I^- levels be tested regularly (e.g., at least weekly) for all species of iodine present in the water (I^- and IO_3^-) and that a minimum I^- level of 0.10 to 0.15 μM be maintained (Sherrill et al., 2004). Due to the difficulties and time consuming nature of measuring all iodine species, many aquariums measure total iodine which does not provide adequate information on I^- bioavailability. In an attempt to circumvent the challenges associated with monitoring environmental I^- concentrations, many aquariums provide dietary I^- supplementation, especially to elasmobranchs, as a means of ensuring adequate bioavailability of I^- and thus preventing goiter formation (Stoskopf, 1993; Sherrill et al., 2004). However, as fish derive over 80% of I^- from the aquatic environment, this physiological dependency on environmental I^- emphasizes the importance of maintaining sufficient environmental I^- concentrations instead of relying solely on dietary supplementation as a means of preventing the development of goiter in captive elasmobranchs (Eales and Brown, 1993; Sherrill et al., 2004). With increased usage of ozone in many aquariums, its effects on the bioavailability of I^- for thyroid hormone systems cannot be overlooked when establishing an elasmobranch health management program. Failure to provide adequate I^- can result in the development of goiter.

Clinical History

This report describes the occurrence of goiter in a female brown- banded bamboo shark (*Chiloscyllium punctatum*), who was part of an all female population of 4 other brown-banded bamboo sharks (*C. punctatum*) (5 sharks total), 11 white-spotted bamboo sharks (*C. plagiosum*), and 2 zebra bullhead sharks (*Heterodontus zebra*) from a commercial source. All sharks were maintained in a touch tank exhibit with the following life support systems: sand filter, carbon

filter, and a protein skimmer. Initially, the system was not ozonated. When an ozone system was built, it was first turned on during the day and off at nights beginning February 25, 2009 and then was left on continuously from March 18, 2009 to April 28, 2009. In April 2009, in a period of less than 60 days, 5 brown-banded bamboo sharks and 11 white-spotted bamboo sharks developed physical evidence of goiter. The affected sharks developed lethargy and decreased appetite during this time period. The two zebra bullhead sharks were not visibly affected and showed no signs of distress.

One affected female brown-banded bamboo shark (*C. punctatum*) weighing 1369.5 g and measuring 64 cm total length was submitted to the University of Florida Veterinary Medical Center for examination on May 29, 2009 (Figure 4-1). The female *C. punctatum* presented with a subcutaneous swelling on the ventral midline one cm caudal to the mouth. Post-mortem examination revealed an accumulation of transparent, red fluid in the mandibular region.

Materials and Methods

Water Chemistry Parameters

Ammonia, nitrite, and iodine were not routinely tested, thus testing only occurred when problems arose (Hach DR/4000 spectrophotometer).^d Environmental iodine concentrations were not measured until goiter was observed on April 12, 2009 and then total iodine (Hach reference method 8031) was tested daily. Nitrate was measured monthly using the Hach DR890 calorimeter.^d Salinity was measured using a refractometer every other week. Dissolved oxygen was measured every other week using a YSI85 probe.^e

Blood Collection

Plasma (0.5 ml) was collected from the caudal vein of the shark submitted to University of Florida with a 3.0-mL syringe. The blood was placed into lithium heparin Vacutainer[®] tubes, and stored on ice for no more than 60 minutes before centrifugation. The plasma was separated

via centrifugation (10 min at 3000 rpm), aliquated into two microvials (240 μ L each) and stored at -80°C prior to analysis.

Tissue Collection

On May 29, 2009, the brown-banded bamboo shark presented to University of Florida was euthanized with buffered MS-222 (1000 mg/L) and the thyroid gland was removed by making an incision through the coracomandibular muscle and layers of connective tissue. The tissue was then placed in 10% neutral buffered formalin (NBF) for histological analysis. In August 2009, the thyroid gland sample was dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS). All samples were examined using Aperio Image Scope software (Aperio Technologies, Inc., Vista, CA).

Histopathology

Thyroid gland was examined for the presence of colloid, follicular cell hypertrophy, follicular cell hyperplasia, and glandular hypertrophy. Colloidal depletion was determined by the reduction or absence of PAS^+ colloid in the lumen. Follicular cells were considered normal when lined with short, simple cuboidal epithelium. Hypertrophy was indicated by low cuboidal epithelium lining the follicles. Follicular cell hypertrophy was identified by the presence of basal nuclei and apical cytoplasm with an increase in nuclear to cytoplasmic ratio. Follicular cell hyperplasia was identified by the presence of follicular cell crowding, stratification, and/or papillary infolding. Glandular hypertrophy was determined by percent of gland affected.

Plasma Thyroid Hormone Analysis

A solid-phase, 96-well plate (Perkin Elmer, Boston, MA, Protein A Flash Plate Plus) radioimmunoassay (RIA) was used to determine thyroxine (T_4) concentrations. Assay buffer was prepared from phosphate buffered saline with 0.1% gelatin (PBSG; 0.1M, pH 7.0). Prior to

sample analyses, a plasma pool was serially diluted, and assayed to ensure parallelism with the standard curve. Standards, inter-assay variance controls, and samples for each assay were run in triplicate. Unknown concentrations were calculated from standard curves plotted as the % bound versus the log₁₀ concentration. Antibody specific to thyroxine (Fitzgerald Industries, Concord MA, Cat # 20-TS40) was diluted in PBSG to a concentration of 1:100,000. Plates were coated with 100 µl of diluted antibody per well, incubated for 18 hours at 32°C, and rinsed twice with PBSG. Standards were prepared at concentrations of 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000 and 4000 pg/ml. Each well received 100 µl of standard, control, or sample in PBSG. Finally, ¹²⁵I-steroid (Perkin Elmer) was added at 12,000 cpm per 100 µl, and plates were incubated for 3 hours at 32°C. Interassay variance wells were similarly prepared from 5 pools of bamboo shark plasma. No separation of bound and free hormone is necessary with this assay, as only the bound radiolabeled steroid is capable of exciting the scintillant coating the bottom of the well. Plates were analyzed using a Microbeta 1450 Trilux counter (Perkin Elmer) in the ¹²⁵I channel with an estimated counting efficiency of 60%. Intra-assay variance averaged 2.5%, while interassay variance averaged 6.5%.

Results

Water Chemistry Parameters

Water chemistry parameters were kept within normal ranges within the system; pH 8.0-8.4, ammonia (unionized) ≤ 0.1mg/L; nitrite ≤ 0.1mg/L. From December 2008 to June 2009, nitrate was measured monthly with a mean (± SE) of 35.05 mg/L NO₃-N (± 5.12). In order to increase the bioavailability of I⁻, Lugol's Solution (potassium iodide; J. Crow's[®]) was added to the tank water when physical evidence of goiter developed in the bamboo sharks. From April

14th to June 9th 2009, daily testing of total iodine demonstrated a mean concentration of 0.08 ± 0.01 mg/L.

Histopathology and Plasma Thyroxine Concentration

Thyroxine concentration measured in the bamboo shark submitted to the University of Florida was 4.64 ng/ml. Histological examination of the thyroid gland demonstrated the development of multinodular goiter. Microscopic examination of the thyroid gland revealed thyroid follicles of varying size surrounded by a moderate amount of thyroid parenchyma and inflammatory aggregates (Figure 4-2). In addition, the thyroid follicles were multifocal separated by fibrous connective tissue. Follicles were large, lined by cuboidal to low columnar epithelium, and colloid was present in only thyroid follicles which retained their shape. Moderate follicular hyperplasia was also present (Figure 4-3). There were moderate numbers of collapsed follicles which not did contain colloid within the lumen. PAS⁺ stain of the thyroid gland revealed basement membrane of these collapsed follicular cells but no lumen (Figure 4-4).

Discussion

Five female brown-banded bamboo sharks and eleven female white-spotted bamboo sharks developed physical evidence of goiter two months after ozone was added to their life support system. Upon identification of goiter in the bamboo sharks, ozone was turned off and iodine (potassium iodide) was added to the water daily as a means of increasing the concentration of total environmental iodine to 0.1 mg/L (mean $0.08 \text{ mg/L} \pm 0.01$). By June, the 4 remaining brown-banded bamboo sharks and 2 white-spotted bamboo sharks died. Clinical signs of goiter resolved in the 9 remaining white-spotted bamboo sharks by June. Interestingly, the two female zebra bullhead sharks inhabiting the same tank did not develop physical evidence of goiter, possibly indicating a potential species-specific difference in susceptibility to the condition.

Upon microscopic examination, thyroid follicles were multifocal within a central solid parenchyma. Thyroid follicles were small with colloid present in the lumen and hyperplasia of the follicular epithelium was observed. Surrounding the parenchyma, large amounts of eosinophilic material consistent with focal hemorrhage and inflammatory aggregates were present. Pathological assessment of the thyroid gland demonstrated the development of multinodular goiter, which is characteristic of chronic iodide deficiency (Cotran et al., 1994). Furthermore, the plasma T₄ concentration (4.64 ng/ml) measured in the female brown-banded bamboo shark was compared to the mean plasma T₄ concentrations measured from the control juvenile male white-spotted bamboo sharks (14.77 ng/ml; range 9.57 to 30.50 ng/ml; n=25) from Chapter 3 and was found to be considerably lower. This low plasma T₄ concentration suggests a more severe case of hypothyroidism than was observed in the white-spotted bamboo sharks after a 29 day exposure to nitrate exposure as previously described.

Although goiter was not observed until after almost to two months of ozone exposure, the histologic identification of multinodular goiter observed in this female bamboo shark suggests that the sharks were subject to a chronic reduction in the bioavailability of I⁻ (Cotran et al., 1994). Though environmental I⁻ concentrations may have been within normal ranges prior to the addition of ozone, environmental nitrate concentrations in the system were elevated (mean 35.05 mg/L NO₃-N). Analysis from Chapter 2 demonstrated the exposure to elevated nitrate (70 mg/L NO₃-N) for 29 days resulted in the development of diffuse hyperplastic goiter. In addition, other studies have demonstrated that chronic exposure (≥ 5 months) to elevated nitrate (≥ 30 mg/L NO₃-N) can result in the development of diffuse colloid goiter (Crow et al., 1998; Zaki et al., 2004; Eskiocak et al., 2005). The possibility exists that the elevated environmental nitrate concentrations (mean 35.05 mg/L NO₃-N) observed in this system competitively inhibited the

uptake of I into the thyroid gland and reduced thyroid hormone synthesis prior to the addition of ozone to the system. Further studies are needed to elucidate the transition from diffuse hyperplastic goiter and the development of multinodular goiter in elasmobranchs and how alternations in I bioavailability affects the progression of this disease.

Goiter is a common health problem in captive elasmobranchs and this case report further supports the recommendation of Sherrill et al. (2004) that regular monitoring of environmental I, as well as with ensuring adequate dietary iodine, is important in the prevention of this disease. Because of the potential loss of I from the environment in ozonated systems, weekly testing of I levels is recommended to ensure that I level remain at a minimum concentration of 0.15 μM (0.01-0.02 mg/L) (Sherrill et al., 2004). However, many aquariums only have the technical capabilities of measuring total iodine, which does not provided adequate information as to I concentrations. Because of the difficulties of monitoring I concentrations and the expense that is involved in adding iodine solutions, such as Lugol's solution, to a system, ensuring proper iodine supplementation is critical to preventing goiter. The current recommended iodine dietary requirement for captive sharks is 100-300 mg/kg body weight of fish (Janse et al., 2004). In conclusion, the addition of iodine to both the aquatic environment and diet can help prevent and treat goiter captive elasmobranchs.



Figure 4-1. Goiter in a female brown-banded bamboo shark, *Chiloscyllium punctatum*. Source M. Walsh (University of Florida, College of Veterinary Medicine).

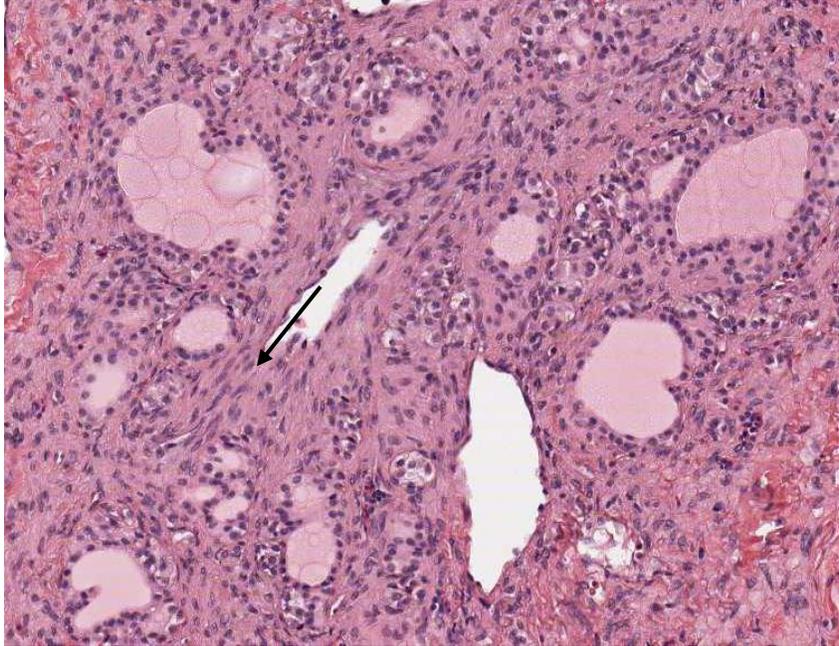


Figure 4-2. A representative image of a multinodular goiter from brown-banded bamboo shark, *Chiloscyllium punctatum*. Colloid filled thyroid follicles of varying size are surrounded by solid parenchyma and connective tissue (arrow). Stain H&E. (10x)

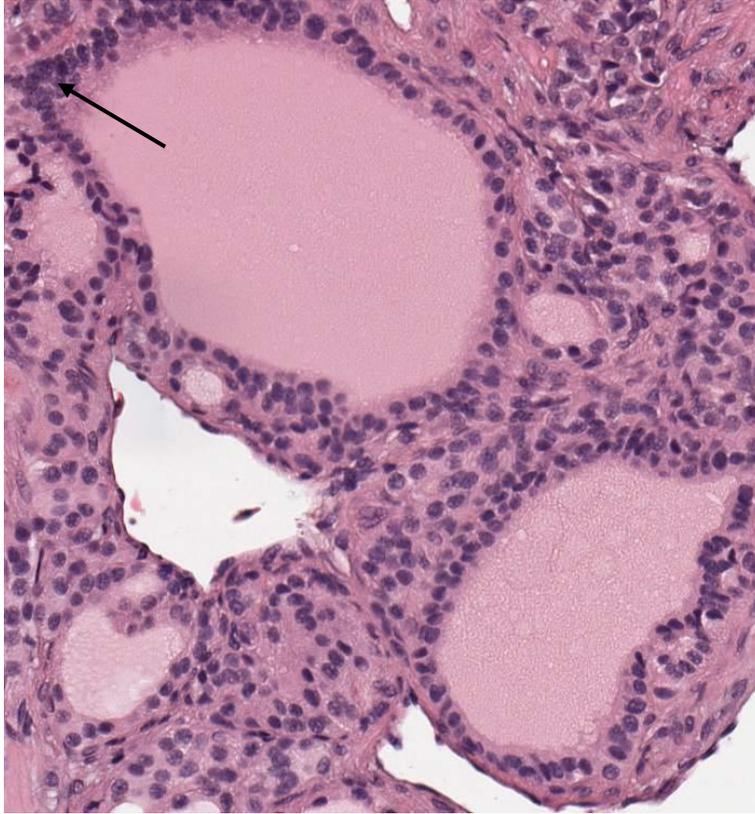


Figure 4-3. A representative image of multinodular goiter with variable size thyroid follicles surrounded by parenchyma. Note flattened epithelial lining, low columnar epithelium and follicular cell hyperplasia (arrow). Stain H&E. (20x)

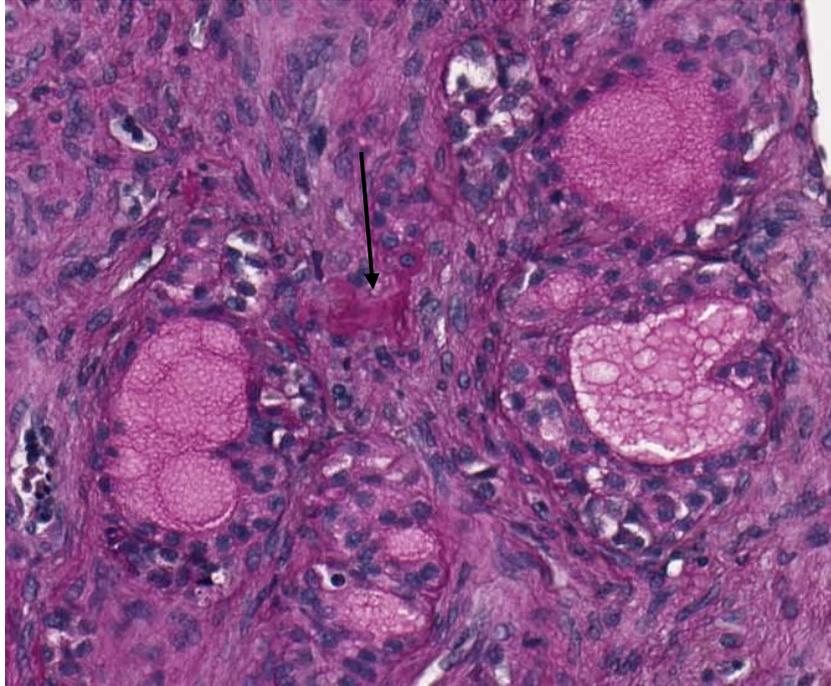


Figure 4-4. A representative image of a PAS stained section of the thyroid gland from a female *Chiloscyllium punctatum*. Note ruptured follicle (arrow) with basement membrane but no follicular lumen. Stained PAS. (20x)

CHAPTER 4 CONCLUSION

Elasmobranchs susceptibility to goiter formation in captive environments has been well documented (Crow et al., 1998). Three forms of goiter have been observed in captive sharks. These are diffuse hyperplastic goiter; diffuse colloid goiter; and multinodular goiter (Crow et al., 2001). Though a common disease, the exact etiology of this disease in elasmobranchs is poorly understood. Goiter can be caused by reduced iodide (I⁻) bioavailability and/or caused by exposure to goitrogenic compounds, including nitrate, which inhibit the uptake of I⁻ into the thyroid gland (Cotran et al., 1994).

In the past, nitrate has received less attention as a potential water quality hazard in both natural and aquarium settings compared to other parameters, but studies have demonstrated that environmental nitrate inhibits the ability of the thyroid gland to uptake I⁻ resulting in decreased thyroid hormone synthesis (e.g., lower plasma thyroid hormone concentrations) and ultimately the development of goiter (Crow et al., 1998; Zaki et al., 2004; Eskiocak et al., 2005). Chapter 2 describes the effects of a 29 day high environmental nitrate exposure (approximately 70 mg/L NO₃-N) on thyroid function of juvenile white-spotted bamboo sharks (*Chiloscyllium plagiosum*). Results demonstrated that 29 day high environmental nitrate concentrations did not affect growth rate, condition factor or decrease circulating plasma T₄ concentrations. However, sharks exposed to elevated nitrate concentrations did develop histologic changes consistent with diffuse hyperplastic goiter. This study supports concern that environmental nitrate exposure, in the absence of other factors, may be goitrogenic and may be an important factor in the etiology of this disease in captive elasmobranchs.

Caution must be used in diagnosing goiter when based solely upon circulating thyroid hormones concentrations. In our analysis, we found that mean plasma T₄ concentrations (16.53

± 1.91 ng/ml) in our nitrate-exposed shark were higher when compared to data from a single female brown banded shark with a clinical case of goiter (4.64 ng/ml). Results from this study indicate that though thyroid hormone concentrations may appear normal, pathological changes to the thyroid gland may occur as a result of continual stimulation by thyroid stimulating hormone on the thyroid follicles in a physiological effort to synthesize new thyroid hormone. Thyroid hormone concentrations measured in nitrate-exposed sharks in this study were not considered to be low despite histologic evidence of disease in the thyroid gland. In contrast, plasma T₄ concentrations were markedly lower in a brown-banded bamboo shark with gross evidence of goiter. Measuring thyroid stimulating hormone, deiodinase enzyme activity or T₄ colloidal storage may be better indicators as to whether or not the thyroid gland is being over stimulated (Mukhi et al., 2005; Blanton and Specker, 2007; Graham et al., 2007).

It was been suggested that chronic exposure to elevated nitrate (≥ 56.8 mg/L NO₃-N) results in the thyroid attempting to normalize circulating thyroid hormone concentrations, resulting in increased uptake of I⁻ by thyroid follicles (Eskiocak et al., 2005). It is with this increase in thyroidal I concentrations that the thyroid gland will synthesize new thyroid hormones. Overstimulation of the thyroid follicles by thyroid stimulating hormone (TSH) results in the follicles becoming desensitized to this stimulus and consequently they lose the ability to release new thyroid hormones into the blood stream. Thyroid follicles begin to swell because of increased colloidal storage and the follicular epithelium becomes flattened (Cotran et al., 1994; Crow et al., 2001). Continued stimulation from TSH results in rupturing of follicles and development of multinodular goiter as characterized by small follicles surrounded by fibrous tissues (Cotran et al., 1994). Results from Chapter 3 support previous findings that

elasmobranchs subjected to a chronic iodide deficient environment can develop multinodular goiter.

Health management programs for captive elasmobranchs should include consideration of goiter and its prevention by increasing the awareness of management practices needed for aquarium life support systems, especially for systems using re-circulating technology or ozonation. In these systems, nitrate accumulation should be anticipated with the understanding that iodide bioavailability may be reduced. Monitoring I^- and IO_3^- , rather than total iodine, is necessary to ensure that I^- remains at a minimum concentration of approximately $0.15\mu M$ (Sherrill et al., 2004). The current recommended iodine dietary (as $CaIO_3$) requirement for captive elasmobranchs is 100-300 mg/kg body weight of fish (Janse et al., 2004). In order to combat the possibility of reduced iodide bioavailability due to ozonation of water and competitive inhibition of iodide uptake into the thyroid due to nitrate, it may be necessary to increase nutritional iodine supplementation.

The form of iodine which is generally used for captive sharks is normally in the form of calcium iodate ($CaIO_3$), because potassium iodide is known to have a short shelf-life compared to the calcium form (Dr. Liz Koutsos, Mazuri Inc., personal communication). Recently, questions and concerns have been raised as to the bioavailability of iodide when administered orally in the form of $CaIO_3$. The reason for this concern is because in an aquatic environment IO_3^- cannot easily diffuse through the gills for use in thyroid hormone synthesis. Therefore, questions have arisen about the best form of iodine used for supplementation. The majority of iodine metabolism studies have been conducted using ruminants as the experimental animal (Miller and Ammerman, 1995). Because of the concern regarding the bioavailability of I^- and the void that exists in the literature on bioavailability data for sharks, there is a need for nutritional

studies aimed at determining the form of iodine that is most available to sharks in order to prevent goiter.

In conclusion, this study provides histological data showing that sharks subjected to an environment containing approximately 70 mg/L $\text{NO}_3\text{-N}$ for 29 days resulted in the development of diffuse hyperplastic goiter. Results from this study support concerns that environmental nitrate exposure, in the absence of other factors, may be goitrogenic. With increasing restrictions on water use, most modern aquaria operate as re-circulating systems, resulting in higher and more chronic nitrate exposure to the aquatic organisms within such systems. Goiter is one of the most common health concerns in captive elasmobranchs and the data collected in this study suggests that elevated environmental nitrate is an important factor in the development of this disease.

APPENDIX
WATER CHEMISTRY TEST AND MANUFACTURERS

- a. AmVer™ Diluent Reagent LR, Hach Company, Loveland CO, Cat #2602200.
- b. NitriVer®3 Nitrite Test ‘N Tube, Hach Company, Loveland CO, Cat #1406500.
- c. Buret titration Method, Hach Company, Loveland CO, Cat #8221.
- d. Hach Company, Loveland CO.
- e. Yellow Springs Instruments, Yellow Springs OH.
- f. NitraVer® X Reagent HR method 10020, Hach Company, Loveland CO, Cat #2605345
- g. NitraVer®5 Nitrate Reagent; Hach Company, Loveland CO, Cat #1403599.
- h. Dionex Co™; Sunnyvale CA.

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BIOGRAPHICAL SKETCH

Alexis L. Morris spent much of her childhood getting her toes wet in the ocean, digging in the sand searching for shark teeth, scuba diving in the ocean searching for sharks. When she was unable to go to the ocean, she spent much of her time wondering through aquariums sneaking glances at the sharks on exhibit. Alexis graduated from The Chapin School in 2001, and then began studying for her Bachelor of Science degree in environmental studies from Emory University in Atlanta, GA. After graduating in May 2005, she worked as a research associate in a zooplankton behavior laboratory at Georgia Institute of Technology.