SODIUM IODIDE SYMPORTER LOCATION, EXPRESSION AND REGULATION IN RED DRUM, *SCIAENOPS OCELLATUS*

A Dissertation

by

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ABSTRACT

Iodine, a non-metallic trace element, is one of the heaviest elements essential for normal biological function. Vertebrate animals must obtain iodine from the environment to manufacture thyroid hormones and sufficient iodine supply is thus critical for normal endocrine function. In mammals, iodine uptake from the environment is achieved by the sodium iodide symporter (NIS), but little is known outside a few mammalian orders about how animals obtain iodine. Because the basic biochemical pathways for thyroid hormone synthesis and receptor activation appear conserved in vertebrates, the mechanisms through which non-mammalian species accumulate iodine for thyroid hormone manufacture have also been assumed to be similar. However, few studies have identified the NIS gene or characterized the regulation of its activity in fish. The perciform teleost fish, red drum (*Sciaenops ocellatus*) is easily obtained from hatcheries and has been used extensively for nutritional and thyroid hormone research, therefore providing an informative species in which to study the mechanisms of iodide uptake and utilization in a nonmammalian species.

I developed a fast, non-lethal methodology using Positron Emission Tomography and Computed Tomography PET/CT imaging of 124I injected fish to produce three dimensional images identifying areas of active radioiodide uptake in the subpharyngeal region of red drum, thus allowing me to establish a methodology for consistently obtaining thyroid tissue. I then confirmed that an NIS homolog is expressed in red drum thyroid, stomach and intestine, but found little evidence for its expression in gills.

Utilizing quantitative RT-PCR, I demonstrated that thyroid stimulating hormone stimulated *nis* expression in red drum subpharyngeal thyroid tissue but not any other *nis* expressing tissue, similar to mammalian thyroid NIS. *nis* expression in red drum subpharyngeal, stomach, intestine, and gill tissues was relatively unchanged during feeding, salinity transfer, and environmental iodide exposure, suggesting that these fish have physiological mechanisms of dietary iodine uptake, thyroid hormone synthesis, and iodide recycling similar to those described in mammals. Minimal *nis* expression in gills across a variety of osmotic environments and nutritional conditions suggests that this transporter does not serve as a mechanism for direct environmental iodide uptake via gills.

DEDICATION

For my friends, family and all women in science.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Iodine, a non-metallic trace element, is one of the heaviest elements essential for normal biological function. Vertebrate animals must obtain iodine from the environment to manufacture thyroid hormones. Remarkably, while researchers have spent years studying the physiological regulation of uptake of other essential ions such as sodium and chloride (Evans et al 1999), very little is known outside a few mammalian orders about how animals obtain iodine. Because iodide transport proteins have been demonstrated in the digestive tract of mammals (Nicola et al 2009) most fish research has assumed that iodine pumps are present in similar gastrointestinal locations (Gregory & Eales 1975). However, animals living in aquatic environments have an alternate possibility: the direct uptake of iodide from the water to the circulatory system across the integument or respiratory organs. Although it has been suggested that teleost fish are able to accumulate iodine directly across their gills (Fromm 1965), no iodine transport protein has been described in detail. A better understanding of the mechanisms of iodine uptake in teleost fish could help to identify novel mechanisms of iodine transport in aquatic vertebrates that are important for maintaining endocrine homeostasis in aquaculture or in the face of a changing environment.

Iodine is a naturally occurring element that is found in concentrations ranging from 0.5mg/kg in soil to 10-20ng/m^3 in the atmosphere and 46-60 ug/L in the oceans (Moreda-Piñeiro et al 2011). The primary natural source of iodine for biological

systems is seawater which contains 450nM dissolved iodine (Küpper et al 2011, Truesdale & Spencer 1974). In an oxygenated marine environment at 100% salinity, iodine exists primarily in two forms: iodate (IO_3^-) and iodide (I^-) (Zhang et al 2010). Iodide (I^-) is the biologically available form of iodine because it can be transported across biological membranes (Dai et al 1996, Nicola et al 2009). It has been hypothesized that iodide first acquired a role in biological systems as an antioxidant and a catalyst for biochemical pathways involved in salt balance, energy production and tyrosine synthesis (Crockford 2009). Iodotyrosine may also have served as an early paracrine communicator as it diffuses easily between cells. As iodotyrosine acquired more important regulatory roles, animals developed the ability to synthesize it endogenously in the thyroid gland and thus required mechanisms for environmental iodide uptake to guarantee a sufficient supply (Crockford 2009). Since iodine is important for maintaining thyroid hormone production in all vertebrates, it has been proposed that iodide transport mechanisms have been conserved throughout vertebrates.

Thyroid epithelial cells utilize iodide to synthesize the hormone thyroxine (T_4) which is subsequently secreted to the circulation (Denver et al 2009). T_4 is converted in peripheral tissues to 3,5,3'-triodothyronine (T_3) by the cleavage of iodine from the outer phenolic ring via outer ring deiodinase enzymes (Sutija & Joss 2006). Peripheral tissue deiodinases can remove iodide from T_3 and T_4, thus releasing iodide back into circulation for either excretion or transport back to the thyroid (Fujimoto et al 2012, Kopp 2008). Once secreted into circulation, thyroid hormones are transported to target tissues where they enter the cell, translocate to the nucleus, and bind to thyroid hormone

receptors which regulate fundamental physiological processes including metabolism, endocrine function, reproduction and development (Moreno et al 2008, Yen 2001). Therefore, sufficient iodine supply is critical for normal endocrine function. Conversely, iodide deficiency results in an impaired ability to synthesize T_4 (Goldsmith 1949, Leblond 1951, Zoeller et al 2007) leading to hypothyroidism which can result in serious developmental, metabolic, and reproductive disorders in humans (Pearce et al 2013).

In mammals, iodine uptake from the environment is achieved by the sodium iodide symporter (NIS), a membrane protein that belongs to the family of SLC5 ion transporters (Dohan et al 2003, Nicola et al 2009, Portulano et al 2014). NIS is synthesized in the enterocytes of the mammalian small intestine and stomach where it is responsible for dietary iodide absorption (Carrasco 1993, Kotani et al 1998, Nicola et al 2009). Iodide is then transported via the circulation to mammalian thyroid cells where is it transported into the thyroid via the same NIS. Located in the basolateral membrane of thyroid epithelial cells, NIS concentrates iodide within cells by simultaneously transporting two Na^+ and one I^- through the cell membrane (Dohan et al 2003, Portulano et al 2014). NIS activity has also been identified in several other secretory tissues, including the mammary gland, placenta, kidney, choroid plexus, and salivary gland (Dohan et al., 2003). While it been studied in many mammalian species, NIS has only been investigated in a small number of non-mammalian species. Whereas mammalian NIS generally exhibits greater than 75% sequence identity (Dohan et al 2003), human NIS also retains 69% identify with *Xenopus laevis* NIS, indicating that, at least in tetrapods, its structure has been highly conserved (Carr et al 2008). Therefore, while

mammals are the common NIS model, it is believed that both mammalian and non-mammalian species have similar mechanisms for accumulating environmental iodide and concentrating it in the thyroid.

Mammalian NIS has been extensively characterized because of this critical role in providing iodide needed for thyroid hormone synthesis (Dohan et al 2003). The NIS nucleotide sequence codes for an intrinsic membrane protein consisting of approximately 618 amino acids with 13 transmembrane segments (Darrouzet et al 2014). Recent molecular studies have provided support for a close relationship between NIS expression and iodide transport activity in cells. Increases in NIS RNA expression accompany an increase in sodium and iodide cotransport into cells (Dai et al 1996), while decreased NIS mRNA expression is associated with reduced iodide uptake (Chung 2002). Additionally, induced expression of NIS cDNA in cancer cells activates NIS protein synthesis and iodide uptake (Pan et al 2013, Zhang et al 2014). Thyroid function is diminished in response to high iodide, a process known as the Wolff-Chaikoff effect (Roti et al 1997). High iodide causes plasma membrane NIS to move to intracellular compartments, NIS mRNA expression to decrease and a reduction of iodide uptake (Nicola et al 2012). In contrast, NIS synthesis and activity are stimulated in the thyroid by thyroid stimulating hormone (TSH) (Carrasco 1993, Dohan et al 2003, Kogai et al 1997, Nicola et al 2009). Recently, increased NIS expression has been effectively used in rats as an index of iodide transport (Gonçalves et al 2013). This correlation of iodide uptake and NIS expression in mammals suggests that characterization of tissue NIS

expression may serve in nonmammalian species as a useful means for identifying iodide transporting tissues, as well as an index of transport activity.

Because the basic biochemical pathways for thyroid hormone synthesis and receptor activation appear conserved in vertebrates (Eales 1979), the mechanisms through which non-mammalian species accumulate iodine for thyroid hormone manufacture have also been assumed to be similar. However, few studies have identified the NIS gene and characterized the regulation of its activity in fish. It is known that fish obtain environmental iodide and deliver it to thyroid tissue for thyroid hormone synthesis (Eales & Brown 1993, Maqsood et al 1961). Dietary supplementation studies have suggested fish accumulate iodide via the gut (Gregory & Eales 1975, Ribeiro et al 2011, Robertson & Chaney 1953) and thus diet, as in mammals, appears to be the primary source of iodide. Once iodide enters circulation it is concentrated in thyroid tissue in the lower jaw (Hunn 1963, Tarrant Jr 1971). Even though gut and thyroid have been identified in fish as iodide transporting tissues, the specific iodide transport mechanisms in these tissues have not been characterized. However, evidence does exist for conservation of the NIS gene in fish. The zebrafish (*Danio rerio*) NIS gene has been sequenced and NIS expression was used to confirm thyroid follicles were present in the lower jaw (Alt et al 2006, Porazzi et al 2009). Thus, evidence from a single teleost species has confirmed that an NIS homolog is expressed in fish thyroid tissue, although these studies did not examine NIS expression at extra-thyroidal locations, nor did they investigate how NIS expression might be regulated.

Whereas mammalian models suggest the gut is the primary location for environmental iodide uptake, there is considerable evidence for an alternative uptake location in fish. Early studies in which brook trout goiter was treated by supplementing pond water with iodide suggested that fish can also obtain iodide via direct absorption from the environment (Marine & Lenhart 1910). Later studies immersing fish in radioiodide suggested that iodide enters fish through the gills to be transported via circulation to iodide-concentrating organs, including the thyroid, head kidney, kidney, and ovary (Baker 1958, Geven et al 2007, Gregory & Eales 1975, Hunt & Eales 1979, Tarrant Jr 1971). Halibut larvae iodine uptake decreased after exposure to perchlorate, thus supporting the argument that a transport protein is responsible for environmental iodide uptake (Moren et al 2008). While it can be hypothesized that NIS is the transport protein functioning in fish gills, no one has identified NIS presence in the gills or confirmed that it is the same NIS present in fish thyroid. Since environmental iodide is only absorbed via the gut in mammalian models, demonstration of NIS expression in gills would suggest a novel pathway for environmental iodide uptake in fish. This knowledge could then be used to examine how gill or gut NIS expression change based in response to alterations in environmental or dietary iodide availability. Since iodide uptake is essential for thyroid hormone production, this information would help us understand how these distinct iodide transport systems are used in aquatic environments to maintain thyroid homeostasis in fish.

A first step in establishing the physiological significance of fish thyroid, gut, and gill NIS would be to characterize the presence of NIS gene expression in these tissues.

Once techniques have been developed to quantify NIS expression it would then be possible to determine how this expression is regulated in response to physiological or environmental stimuli. For example, TSH rapidly activates mammalian thyroid NIS expression (Kogai et al 1997). Since TSH functions appear to be highly conserved across vertebrates (Sherwood et al 2012), including fish (MacKenzie et al 2009), it is expected that increases in circulating TSH would similarly stimulate NIS expression in fish thyroid tissue. Conversely, it is known that high iodide levels inhibit NIS expression in both mammalian thyroid and extrathyroidal tissues including the intestine (Nicola et al 2012), therefore it can be hypothesized that low levels of iodide should activate NIS expression. If alterations in ambient iodide similarly regulate NIS expression in gills or intestine, fish would possess a unique mechanism to compensate for decreases in environmental iodide levels by up-regulating branchial and gastrointestinal transport. Because the ratio of total dissolved iodine to salinity is generally held at a constant value of 12.8nM/ppt (Küpper et al 2011) ambient iodide concentrations should change with changes in salinity. Euryhaline fish are therefore likely to encounter variable levels of iodide and thus represent a good experimental model for the investigation of environmental regulation of gill and gut NIS expression. In freshwater fish, ionocytes in gills maintain ion concentrations by active uptake of environmental sodium and chloride. Conversely, saltwater fish excrete sodium and chloride via the same gill ionocytes (Dymowska et al 2012, Evans et al 2005). Assuming gill iodide transport responds to changes in ion concentrations in a similar way as gill sodium and chloride transport, it

can be hypothesized that a decrease in environmental iodide would cause an increase in NIS mRNA expression in the gills.

Red drum, Sciaenops ocellatus, are a euryhaline perciform fish common along the Gulf and east coasts of the United States. Due to their popularity as a game fish red drum are cultured as a food source and to supplement wild populations. Because red drum are easily obtained from hatcheries and have been used extensively for nutritional and thyroid hormone research (MacKenzie et al 2009, Pohlenz & Gatlin III 2014) they serve as an informative species in which to study the regulation of NIS. Their euryhaline capabilities mean red drum can be effectively utilized in studies of the impact of alterations in environmental salinity on physiological function (Gatlin III et al 1992, Gullian-Klanian 2013), thereby providing an excellent model to examine if NIS expression increases as iodide availability decreases. Additionally, Miller (Miller 2011) noted that red drum held for more than six weeks in a low salinity recirculating system became progressively insensitive to thyroid stimulation by TSH, whereas fish held in other systems remained responsive to TSH stimulation over the same time. Subsequent water measurements (Wilkes, unpublished data) revealed that iodide content of the systems containing the insensitive fish progressively decreased over time, suggesting that hypothyroidism may have been induced in response to insufficient ambient iodide. Since NIS is hypothesized to be the iodide transport protein in fish gills and intestine, it is possible that low environmental iodide induces a compensatory increase in NIS expression in the gill and gut in an effort to avoid hypothyroidism. I therefore propose to

use red drum to identify locations of NIS expression in a euryhaline teleost fish and to examine the effects of alterations in environmental iodide on NIS expression.

Objectives

I propose to determine if a homolog to mammalian NIS is present in red drum thyroid tissue, intestine, and gills. Additionally, I will determine whether red drum NIS expression in each of these locations is regulated in a manner that is similar to that in mammalian thyroid and extrathyroidal tissues.

Specific objectives are:

1) To clone and sequence the sodium-iodide symporter (NIS) gene in red drum.

2) To develop PCR-based techniques suitable for quantifying and identifying anatomical locations of expression of NIS mRNA.

3) To determine whether NIS expression is regulated in a tissue specific manner with stimulation by decreased iodide in all locations but by TSH in the thyroid.

This research should provide a better understanding of how euryhaline fish obtain iodide and regulate iodine uptake. Unlike the mammalian model of iodide uptake via the gut, fish may possess a unique ability to obtain environmental iodide via the gills, which may have important implications for maintenance of thyroid homeostasis in euryhaline and aquacultured species.

CHAPTER II

VISUALIZATION OF THYROID TISSUE BY ^{124}I UPTAKE IN RED DRUM, *SCIAENOPS OCELLATUS*, AND TILAPIA, *OREOCHROMIS AUREUS*

Introduction

The thyroid gland plays a central role in fish metabolism (Chavin & Bouwman 1965), reproduction (Goto-Kazeto et al 2009, Kumar et al 2000, Vischer & Bogerd 2003), migration (Rankin 1991), and metamorphosis (Einarsdóttir et al 2006, Power et al 2001). For over one hundred years, fish thyroid gland morphology has been studied as a means for understanding environmental and physiological regulation of fish thyroid function. Studies of endocrine disruption (Liu et al 2011, Mukhi et al 2005, Patiño et al 2003, Zhang et al 2013), development (Liu et al 2013, Schnitzler et al 2016), acclimation to osmotic stress (Arjona et al 2008, Arjona et al 2010) and ecology have all benefitted from assessment of thyroid gross or microscopic morphology. Fish thyroid glands are typically found in subpharyngeal areas along the ventral aorta and lower branchial arteries, and non-encapsulated thyroid follicles can be found in multiple locations adjacent to the heart or within the head kidney and the kidney itself. Parrotfishes (Labroidei), mudskippers (Gobiidae), elasmobranchs (Chondrichthyes), lungfish (Neoceratdontidae) and swordfish (Xiphiidae) all have compact subpharyngeal thyroid glands (Barton 2007, Eales 1979, Eales & Brown 1993). However, other bony fishes such as goldfish (Cyprinidae) (Barton 2007, Eales 1979), platyfish (Poeciliidae) (Baker 1958), trout (Salmonidae) (Eales, 1964) and tilapia (Cichlidae) (Geven et al 2007) have thyroid follicles dispersed around both the head kidney and subpharyngeal regions.

Research on the environmental and physiological regulation of fish thyroid function would benefit from a technique that allowed for faster and easier visualization of difficult to locate fish thyroid follicles.

Research comparing the relative contributions of subpharyngeal and heterotopic thyroid tissue to overall thyroid function has produced conflicting results. Although little functional significance was traditionally assigned to the heterotopic thyroid tissue located outside the subpharyngeal region, in goldfish (Barton 2007) and carp (Geven et al 2007), histological examination suggests that heterotopic tissues make a significant contribution to fish thyroid function. In carp (Cyprinidae), which have thyroid follicles in both subpharyngeal and renal tissues, the kidney thyroid follicles appear responsible for most of the thyroid function (Geven et al 2007). However, in snakeheads (Channidae) heterotopic thyroid follicle epithelial cell height indicated that heterotopic follicles were less active than subpharyngeal thyroid tissue (Bhattacharya et al 1976, Geven et al 2007). Heterotopic follicles may secrete significant amounts of thyroid hormones but quantifying thyroid follicle activity can be challenging using established techniques.

Thyroid activity is usually characterized via histology or radiology. Both of these techniques are able to establish the location of subpharyngeal and heterotopic thyroid tissue, but these methodologies have limitations. For example, histology can be used to locate unknown areas of dispersed thyroid tissue but requires sectioning through large areas or entire organs. Once sectioned, tissues must be stained and visually observed, taking considerable effort and resources. Additionally, histology only provides

an indirect index of thyroid activity by characterizing the structure of thyroid epithelial cells. In contrast, radiological techniques can evaluate thyroid tissue activity via isotope uptake, but the images produced are two dimensional and involve lethal dissection and organ removal to quantify isotope uptake in specific tissues (Martinez et al 2014). While newer imaging techniques have been widely used in clinical medicine as an effective means to evaluate thyroid function, little work has been done with radiologically imaging thyroid tissue in non-mammalian species.

The ability of tissues to concentrate radioiodide has been used extensively as a method for imaging thyroid function for diagnosis or treatment of thyroid disease (Di Cosmo et al 2010). In clinical medicine, activity of thyroid tissue can be assessed relatively easily through noninvasive imaging of radioiodine uptake (DeGroot & Jameson 2010). While traditional imaging procedures such as neck ultrasonography, MRI and scintigraphy laid the foundations of thyroid location, structure, activity and disease (Elisei & Pinchera 2012), modern nuclear imaging techniques have revolutionized clinical medicine in the identification, treatment and understanding of human thyroid cancer (Bertagna et al 2009, Freudenberg et al 2004, Grewal et al 2016, Mirallié et al 2007). In particular, whole-body PET/CT is being widely used in the detection and treatment of thyroid, lymph, ovarian and other types of cancer in humans (Kwon et al 2017). PET/CT combines positron emission tomography scanning with x-ray imaging to reveal the distribution of tissues with isotope uptake overlaid on CT anatomical imaging. This precise anatomical localization is used widely in human clinical diagnostics for oncology, surgical planning, and radiation therapy (Freudenberg

et al 2004, Glaser et al 2003) (Freudenberg et al 2004, Glaser et al 2003, Jakoby et al 2011) by using isotope uptake as a measure of tissue activity for comparison across different tissue types. This technique was once limited to larger (>10kg) animals but advancements in technology allow for smaller animals less than 1kg (Green et al 2001, Martinez et al 2014) to be successfully scanned, thus opening up PET/CT as a technique that can be applied to non-mammalian species. PET/CT of mammalian species such as dogs, rats and mice is becoming a popular way to track tumor development in order to better understand human disease (Wong & Kim 2009). Despite the increased use of tissue imaging in mammalian research organisms, PET has not yet been applied to fish thyroid imaging although F-18-fluorodeoxyglucose (FDG) PET/CT has been used successfully in fish to show that standard uptake values for glucose in select organs were more similar to humans than to mice or dogs (Browning et al 2013). A more accurate methodology to locate and quantify active thyroid tissue in fish would be beneficial to fish thyroid physiological and eco-toxicological research. Our goal was to develop a fast, non-lethal methodology that combined the benefits of histology and radiology to produce images that can be used to identify tissues with active radioiodide uptake.

In this study, we describe a new technique for evaluation of fish thyroid activity using high resolution, three-dimensional imaging of radioiodide uptake. As in all vertebrates, teleost fish obtain iodine from their environment and deliver it to their thyroid gland to manufacture the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) (Blanton & Specker 2007, Eales & Brown 1993, Maqsood et al 1961). Once in circulation, iodine is imported into thyroid epithelial cells via the sodium iodide

symporter (Alt et al 2006, Porazzi et al 2009). PET/CT of fish injected with radioiodide should therefore identify the anatomical locations of active thyroid follicles. If feasible for nondestructive imaging of thyroid activity, PET/CT should provide a novel, rapid quantitative method for simultaneous assessment of location and activity of thyroid tissue in intact fish.

To demonstrate that this technique can be used as a rapid, non-destructive, and accurate method to locate fish thyroid tissue with active radioiodide uptake we chose to examine two fish species. Red drum (*Sciaenops ocellatus*) and tilapia (*Tilapia mossambicus*) are common aquaculture species which have been used extensively for nutritional and endocrine research (Geven et al 2007, MacKenzie et al 2009, Pohlenz & Gatlin III 2014, Sukumar et al 1997). In addition, thyroid function has been extensively studied in red drum and tilapia (Cohn et al 2010, Geven et al 2007, Jones 2012, Jones et al 2013, Leiner & MacKenzie 2001, Leiner & MacKenzie 2003, Oba et al 2001, Sukumar et al 1997) providing a broader context for interpretation of our imaging results.

Materials and Methods

Animal procedures

Eight tilapia (*Oreochromis aureus*) weighing an average of 448.25 ± 114.6 with a standard length of 29.13 ± 3.56 cm (Table 1) were obtained from the Aquaculture Research and Teaching Facility (ARTF) at Texas A&M University. Eight red drum (*Sciaenops ocellatus*) weighing an average of 911.8 ± 288.8 grams with a standard length of 43.1 ± 8.74 cm were obtained from Texas Parks and Wildlife's Sea Center in Lake Jackson, TX. To keep red drum and tilapia in the same holding tanks, red drum were acclimated from 30ppt to 15ppt and tilapia were acclimated from 3ppt to 15ppt. All fish were maintained in recirculating 1900 L tanks at 8ppt, 25 ± 2°C, 12L:12D photoperiod, and fed Rangen© commercial fish diet to satiation for two weeks prior to use. Fish were fasted for 24 hours prior to the experiment.

Table 1. Subpharyngeal standard uptake values (SUV) for red drum (*Sciaenops ocellatus*) and tilapia (*Oreochromis aureus*). Fish were injected with 124I and imaged using PET/CT. Subpharyngeal SUV was calculated as decay corrected activity (Bq) located in the designated ROI divided by injected dose (Bq) per gram of body weight and expressed in millicuries (mCi).

					Mean +/- s.e.	
Species	**Time Imaged**	**Subpharyngeal SUV**	**GI Tract SUV**	**Treatment**	**Subpharyngeal SUV**	**GI Tract SUV**
Tilapia	6 hr	4.81×10^{-4}	3.00×10^{-7}	PBS		
Tilapia	6 hr	4.21×10^{-4}	1.58×10^{-7}	bTSH		
Tilapia	6 hr	4.08×10^{-4}	6.52×10^{-7}	bTSH		
Tilapia	6 hr	3.44×10^{-4}	1.73×10^{-7}	bTSH	**0.413 ±0.0056**	**1.74×10^{-4} ±9.65. x 10^{-5}**
Tilapia	24 hr	7.45×10^{-4}	1.05×10^{-7}	PBS		
Tilapia	24 hr	1.19×10^{-4}	1.55×10^{-7}	bTSH		
Tilapia	24 hr	1.19×10^{-4}	3.47×10^{-7}	bTSH		
Tilapia	24 hr	1.53×10^{-4}	1.02×10^{-7}	bTSH	**0.284 ±0.0308**	**6.43×10^{-4} ±4.58 x 10^{-4}**
Red Drum	6 hr	4.26×10^{-4}	4.18×10^{-7}	PBS		
Red Drum	6 hr	3.45×10^{-4}	3.62×10^{-7}	bTSH		
Red Drum	6 hr	8.14×10^{-4}	1.08×10^{-7}	bTSH		
Red Drum	6 hr	4.35×10^{-4}	3.31×10^{-7}	bTSH	**0.505 ± 0.021**	**3.05×10^{-4} ± 1.36 x 10^{-4}**
Red Drum	24 hr	1.64×10^{-4}	1.14×10^{-7}	PBS		
Red Drum	24 hr	4.51×10^{-4}	8.67×10^{-7}	bTSH		
Red Drum	24 hr	9.60×10^{-4}	1.94×10^{-7}	bTSH		
Red Drum	24 hr	5.58×10^{-4}	5.58×10^{-7}	bTSH	**0.533 ± 0.033**	**6.91×10^{-4} ± 4.08 x 10^{-4}**

Injection protocol

For isotope injection, fish were netted from the holding aquaria, sedated with 100 mg/ml buffered Finquel (Tricaine Methanesulfonate, Western Chemical), injected with 135.2 millicuries (mCi) 124I (3DImaging, University of Arkansas for Medical Sciences, Maumelle, AR) in 0.5 mL of phosphate buffered saline (PBS, Gibco, Invitrogen), and placed in one of two 800 L static holding aquaria. 124I was injected intravenously as a bolus in the caudal vein as described in Browning et al. (Browning et al 2013). At 6 and 24 hours post injection, eight fish from each species were euthanized by overdose of MS-222® (Finquel, Argent Laboratories, Redmond, Washington) and placed in plastic bags for transport to the Texas A&M Institute for Preclinical Studies (TIPS) imaging facility where they were imaged with combined PET/CT as described below. Samples of the 124I injection solution were also transported to TIPS and measured using a Capintec® R25 Well Counter to confirm radioiodine dose. The injected 124I dose was calculated by measuring the amount of radioactivity in a full syringe and subtracting the measured amount remaining in each injection syringe after injection. Radioactivity in millicuries (mCi) at time of injection was corrected using known decay rates and then converted to becquerels (Bq).

To promote radioiodine uptake by thyroid tissue, six fish from each species received two injections of bovine thyroid stimulating hormone (bTSH, Sigma Chemical Co.) prior to imaging. Lyophilized bTSH (1.8 IU/mg) was dissolved and diluted in PBS to a concentration of 2.5 IU/mL. Six fish from each species were injected intraperitoneally with 10 mIU/g body weight bTSH using a two injection protocol

(MacKenzie 1982); the first half of the dose was given 12 hours prior to radioisotope injection and the second half was given at time of isotope injection. The remaining 2 fish of each species served as controls, receiving similar volumes of PBS. Three TSH-injected and one control fish were taken for imaging at each sampling time. Following injection, residual TSH injection solutions were assayed for immunoreactive TSH to confirm TSH injection dose using an Immulite 200XPi (Siemens) at the Endocrine Diagnostic Laboratory of the Texas A&M Veterinary Medical Diagnostic Laboratory.

Imaging

Whole body PET/CT was performed using a 128 slice Siemen's Biograph mCT scanner as previously described (Browning et al 2013, Jakoby et al 2011). The CT parameters were a slice thickness of 0.6 mm, 120 kv, 340 mas, pitch 0.6, H31s medium smooth filter kernel. PET scans were acquired using 3D acquisition mode and PET data were attenuation corrected based on CT data (Kinahan et al 1998). Following scans, 6 fish (2 tilapia and 2 red drum from the 6 hour experiment and 1 tilapia and 1 red drum from the 24 hour experiment) were preserved in 10% buffered formalin (EMD Chemicals) for histological analysis following radioactive decay of carcasses.

Acquired PET images were reconstructed using a 3D OSEM reconstruction with point spread function modeling and time of flight compensation (3 iterations, 21 subsets). Reconstructed images were smoothed using a Gaussian filter setting for FWHM of 3.0 mm in all directions with a matrix size of 400 x 400 and a physical pixel size of 0.3571 mm. All images were viewed using TrueD® visualization presets: CT

W/L presents—Baby Extremity, PET LUT—PET Rainbow (16 Bit), and Fused LUT—PET Rainbow (16 Bit). Measured 124I activity in all field of view positions was attenuation corrected to the start time of the whole body PET scan.

Images were transferred for post-processing to the Siemens workstation. Images were subjectively filtered to optimize viewing of PET and CT. To maintain anatomical landmarks and more easily visualize the regions of interest, the images were best viewed at a masking setting of 50% PET and 50% CT. Filtering of PET and CT was only for visualization purposes and did not affect isotope uptake calculations. Specific regions of interest (ROIs) for analysis were subjectively drawn within these areas on the sagittal, coronal and transverse planes to ensure three-dimensional positioning was correct. All ROIs were drawn to encompass the region that displaced localized radioiodide uptake. The software automatically calculated the mean, minimum and maximum 124I activity per volume within ROIs on the images. 124I standard uptake values (SUV) for each ROI were calculated by dividing the mean attenuation-corrected ROI activity (Bq/cm^3) by the injected activity (Bq)/body weight (g). The injected activity was corrected for radioactive decay by using the following equation:

$$dose_{corrected} = dose_{initial} * 2^{\left(\frac{T_{scan} - T_{assay}}{T_{HL}}\right)}$$

T_{scan} is the time the fish was imaged. T_{assay} is the time the radiation level of the 124I was tested. The half-life of 124I (T_{HL}) is 100.32 hours. 124I SUV for each ROI was calculated for each fish to obtain 124I SUV per volume of tissue. Mean SUV and standard error for the ROI was calculated for all fish at each time point.

Histology

To confirm that the anatomical locations identified by PET-CT as concentrating radioiodide were truly thyroid tissue we utilized the high resolution imaging capabilities of the CT scan to record anatomical coordinates for the most active locations. The distance in centimeters on the digital images was measured in three dimensions: from the anteriormost point of the head to the subpharyngeal ROI and the posteriormost point of the tail fork to the ROI along the anteroposterior axis and from the ventral subpharyngeal margin and the exterior dorsal surface along the dorsoventral axis to the ROI (Figure 1). Measurements were collected from four individuals from each species. These measurements were then applied to the preserved fish to triangulate the exact location of radioiodide concentration and dissect these regions for histological examination. Digital measurements from CT scans were transferred to preserved specimens using a scale and the ROI tissue was collected by dissection. Tissues were decalcified with Cal-Ex© (Fisher Scientific) for at least 12 hours or until tissue was pliable. Tissues were rinsed with running water for 3-4 hours, trimmed and placed in cassettes for histologic processing and infiltration. After dehydration and embedding in paraffin, tissues were sectioned to 4um, stained with hematoxylin and eosin (Sigma), examined under a light microscope (Olympus BX 52), and photographed with an Olympus (Santa Valley, PN) DP 72 microscope digital camera system. Examination confirmed that this region contained thyroid follicles (Figure 2).

Figure 1. Midsagittal section of a red drum with triangulation measurements used to locate the subpharyngeal region of interest (ROI) with high radioiodide uptake. Reference 1 on the image provides the length, width, volume and average iodide uptake of the subpharyngeal ROI. References 2-6 provide a length and average uptake values along the colored lines. All lengths are in cm.
2: length from most anterior point on the head to most posterior point on the tail of the fish
3: length from ROI center to the posterior margin of the tail
4: length from ROI center to the dorsal margin of the head
5: length from ROI center to the ventral margin of the lower jaw along the dorsoventral axis and 6: length from the ROI center to the anterior margin of the head along the anteroposterior axis.

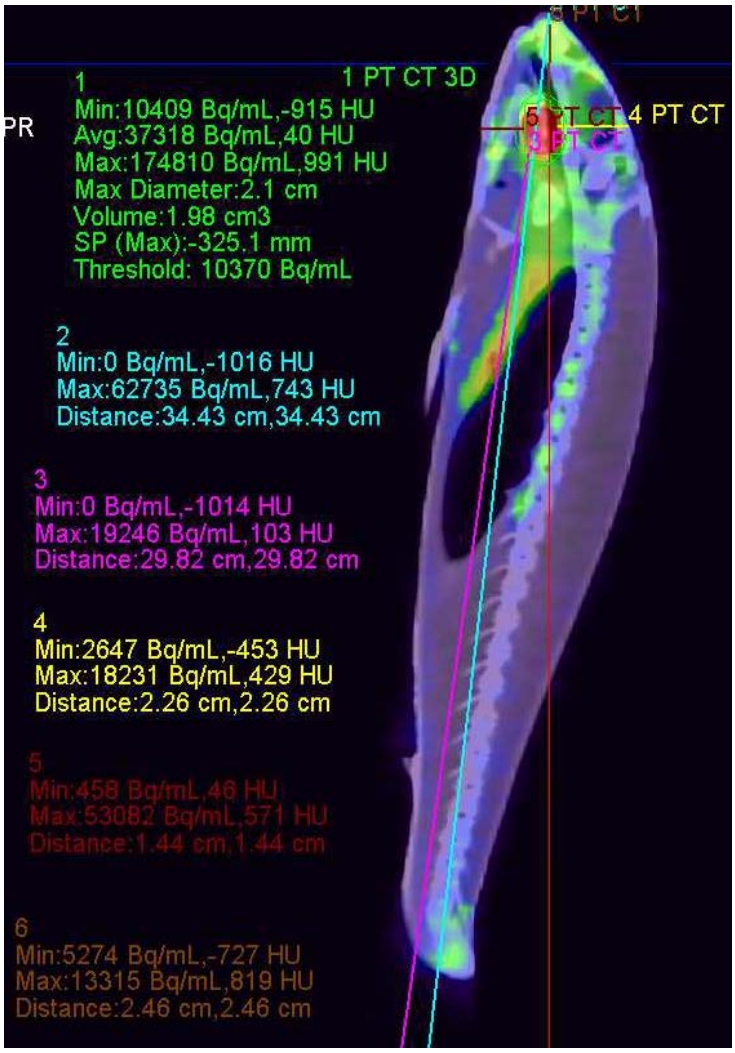
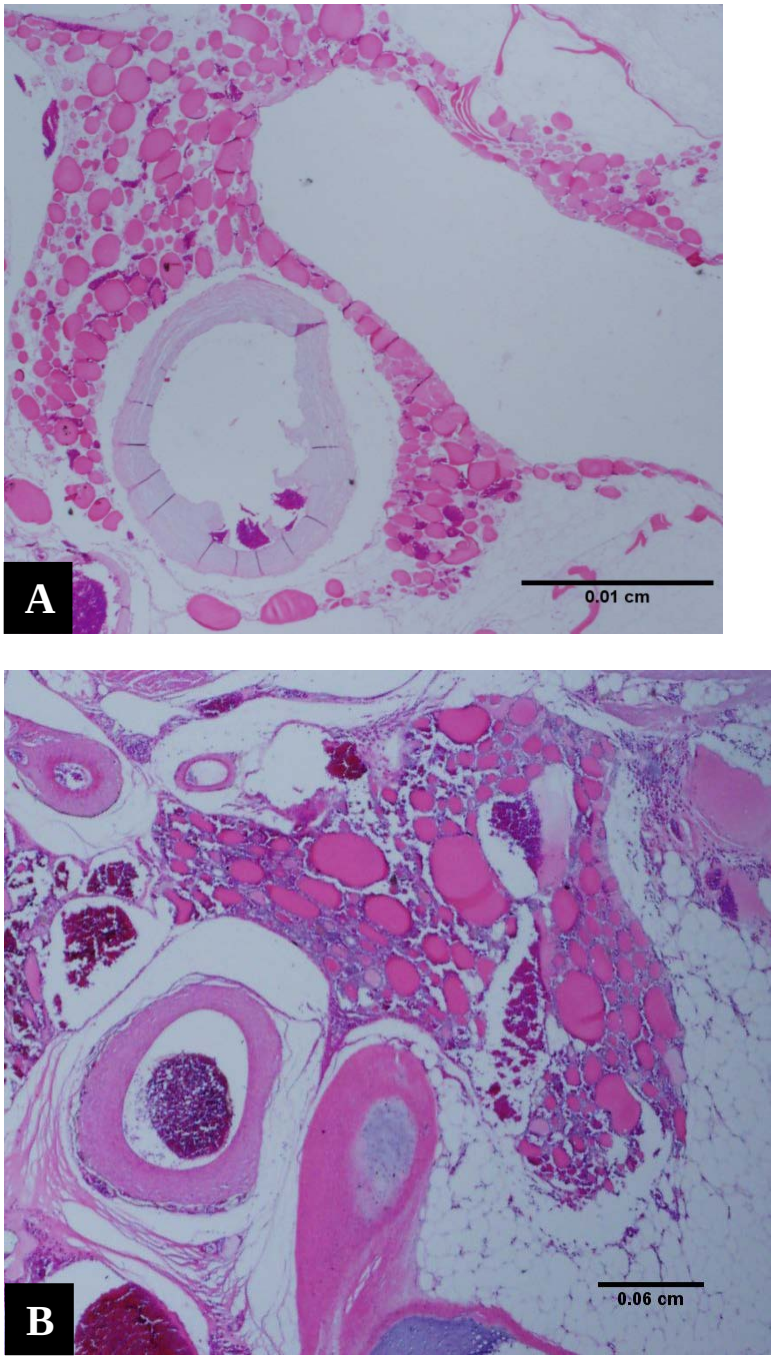


Figure 2. Histological section of tilapia (A) and red drum (B) subpharyngeal region showing greatest 124I uptake. Thyroid follicles of various sizes are visible around the ventral aorta ventral to the pharynx.



Water sampling

Water samples were collected and 124I measured throughout the experiment to determine isotope loss to tank water. Duplicate 1ml water samples were collected 1 hour prior to injection and 3, 4, 8, 22 and 24 hours post-injection from both the 6 hour and 24 hour treatment tanks. Samples were transported to TIPS and measured using a Capintec® R25 Well Counter. Each sample was measured three times and then averaged to obtain a mean counts per minute (CPM). CPM was corrected for background by subtracting CPM detected in an empty vial and converted to mCi.

Data analysis

Data were checked for homogeneity of variance. Student's t-tests were performed to compare SUV for subpharyngeal regions (thyroid) and skeletal muscle (control tissue) between the two time points and fish species. To compare fish injected with TSH to fish injected with PBS, mean SUV for the control fish and the stimulated fish were analysed using the z-test for two sample means (Excel) and resulting p-values were compared to $\alpha = 0.05$.

Results

All eight fish, at each time point, were scanned simultaneously (Figures 3 and 4) in less than 10 minutes. Greater than 90% of isotope uptake was localized in the subpharyngeal and gut regions. Computer image reconstruction took another 20 minutes to complete. Unprocessed PET/CT images (Figures 3A, 3A) had equal ratios of PET and

CT (100% PET and 100% CT). Masking filters were then set to show 0% PET: 100% CT (Figures 3B, 3B) and 100% PET: 0% CT (Figures 3C, 3C). To more easily visualize regions of interest while maintaining anatomical landmarks images were viewed at masking filters of 50% PET and 50% CT (Figure 5).

Figure 3. PET/CT of tilapia and red drum simultaneously imaged 6 hours after I124 injection. Images are displayed to show equal amounts (100% and 100%) of PET and CT (A) then filtered to show 0% PET:100% CT (B) and 100% PET:0% CT (C).

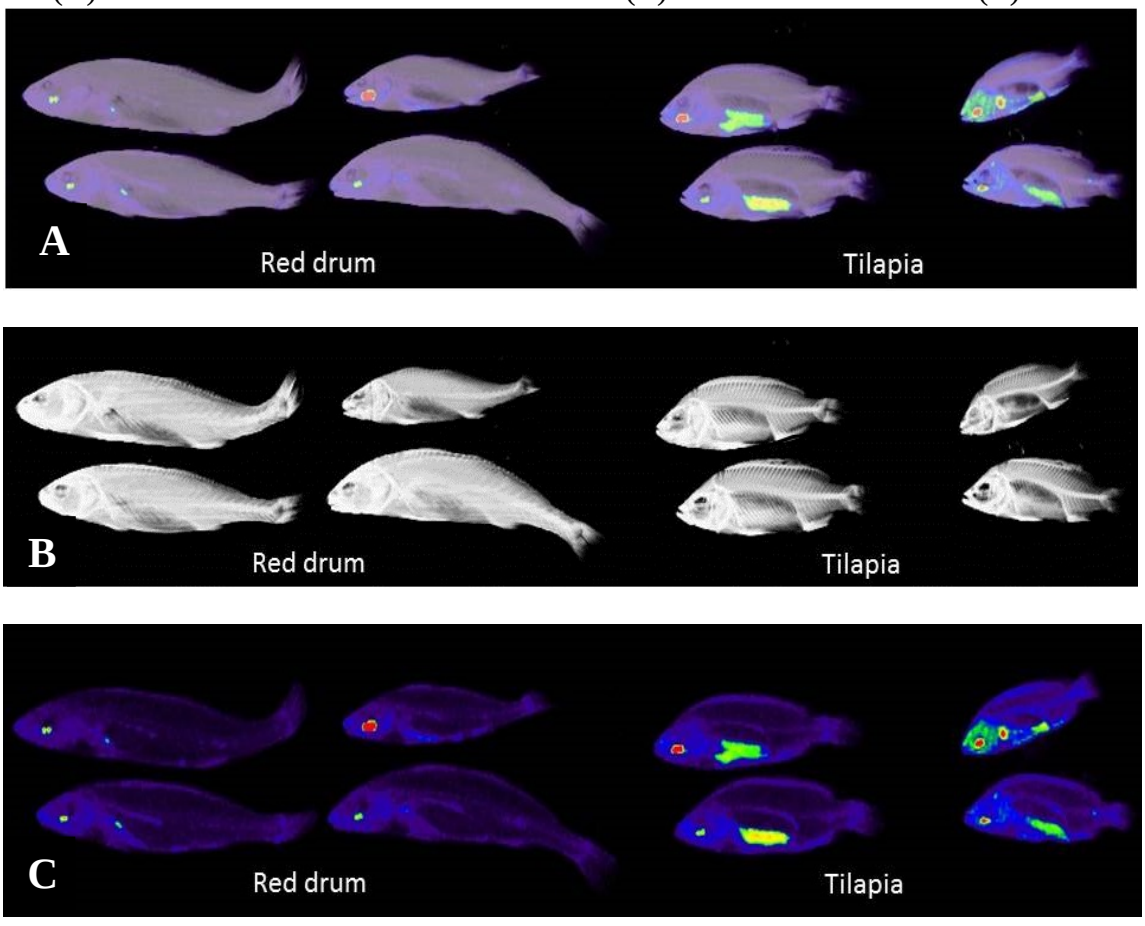


Figure 4. PET/CT of tilapia and red drum simultaneously imaged 24 hours after 124I injection. Images are displayed to show equal amounts (100% and 100%) of PET and CT (A) then filtered to show 0% PET:100% CT (B) and 100% PET:0% CT (C).

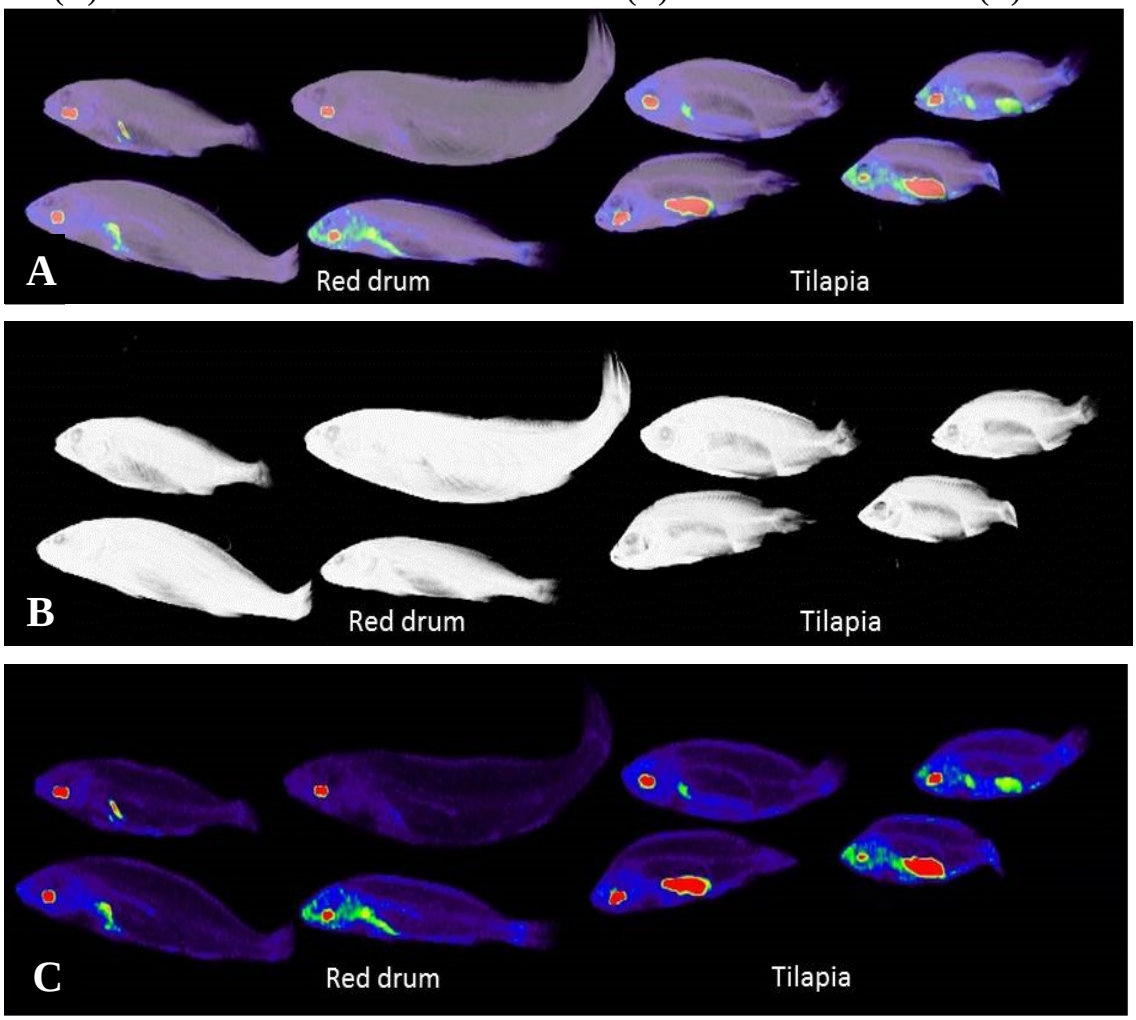


Figure 5. Lateral view of tilapia (A) and red drum (B) imaged 6 hours after 124I injection and tilapia (C) and red drum (D) 24 hours after injection. Images were filtered to 50% PET and 50% CT. All fish showed radioiodine uptake in the subpharyngeal regions. Tilapia showed radioiodine in the gastrointestinal tract 6 and 24 hours post injection (A). Red drum scans show radioiodide uptake in the anterior region of the gastrointestinal tract (B, D).

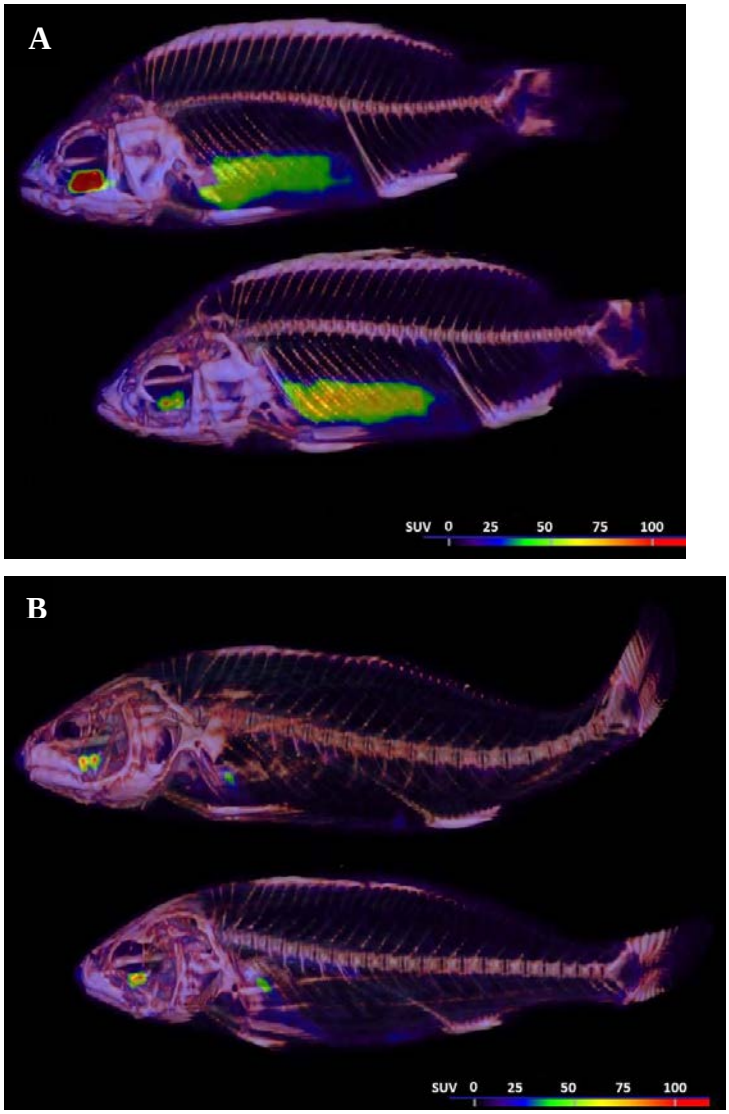
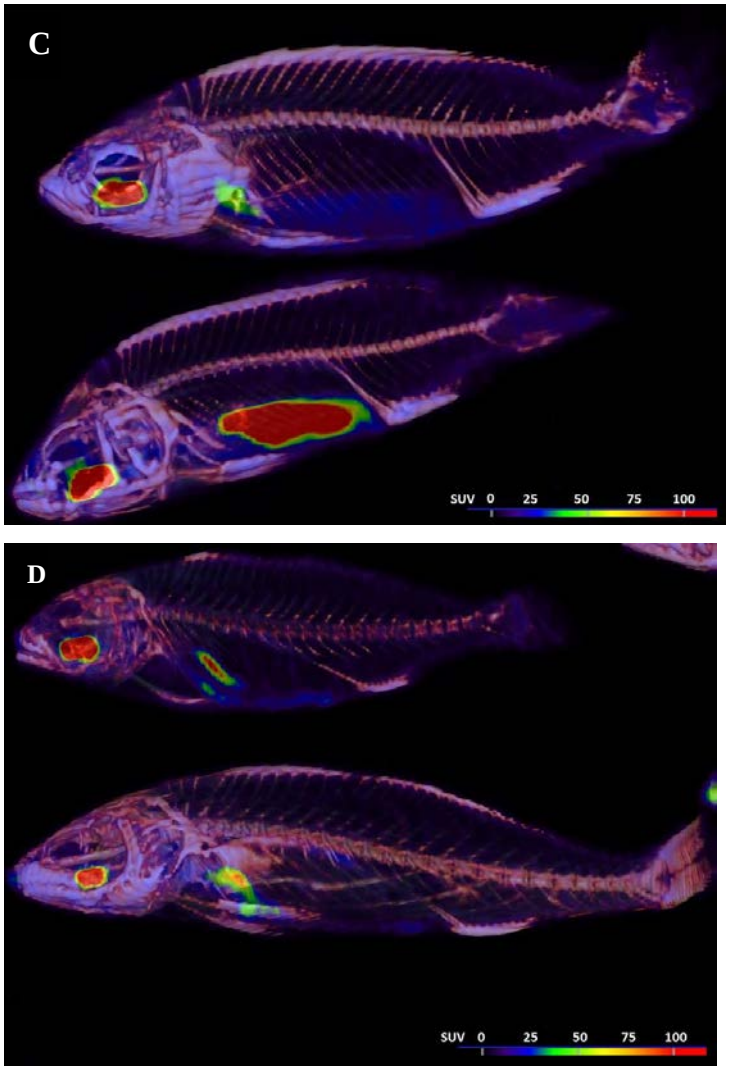
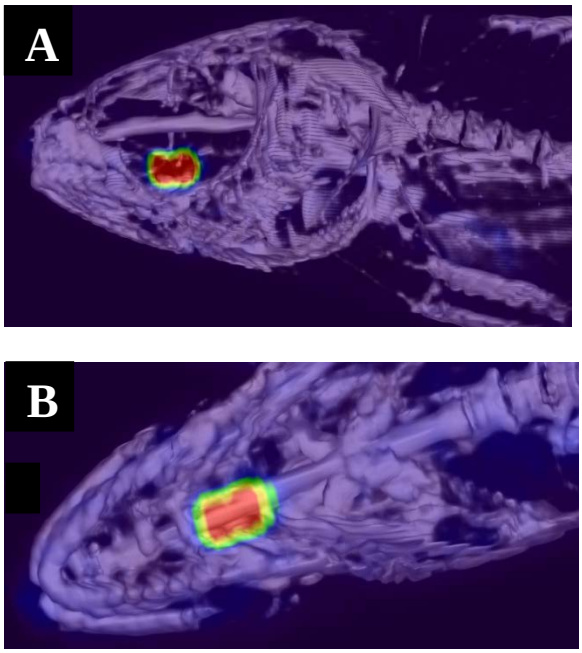


Figure 5 continued



All fish exhibited focal radioiodine uptake in the subpharyngeal region (Figure 5). Images were rotated and subpharyngeal ROI were verified to be along the midline, ventral to the parasphenoid bone (Figure 6). Histological examination of tissues collected at the subpharyngeal ROI in both species confirmed the presence of characteristic thyroid follicles in these locations (Figures 2A and B). Additionally, both species showed radioiodide uptake localized in the gastrointestinal tract (GI) at 6 and 24 hours post 124I injection (Figures 5A-D).

Figure 6. Lateral (A) and ventral views (B) views of red drum injected with 124I and imaged via PET/CT six hours after injection.

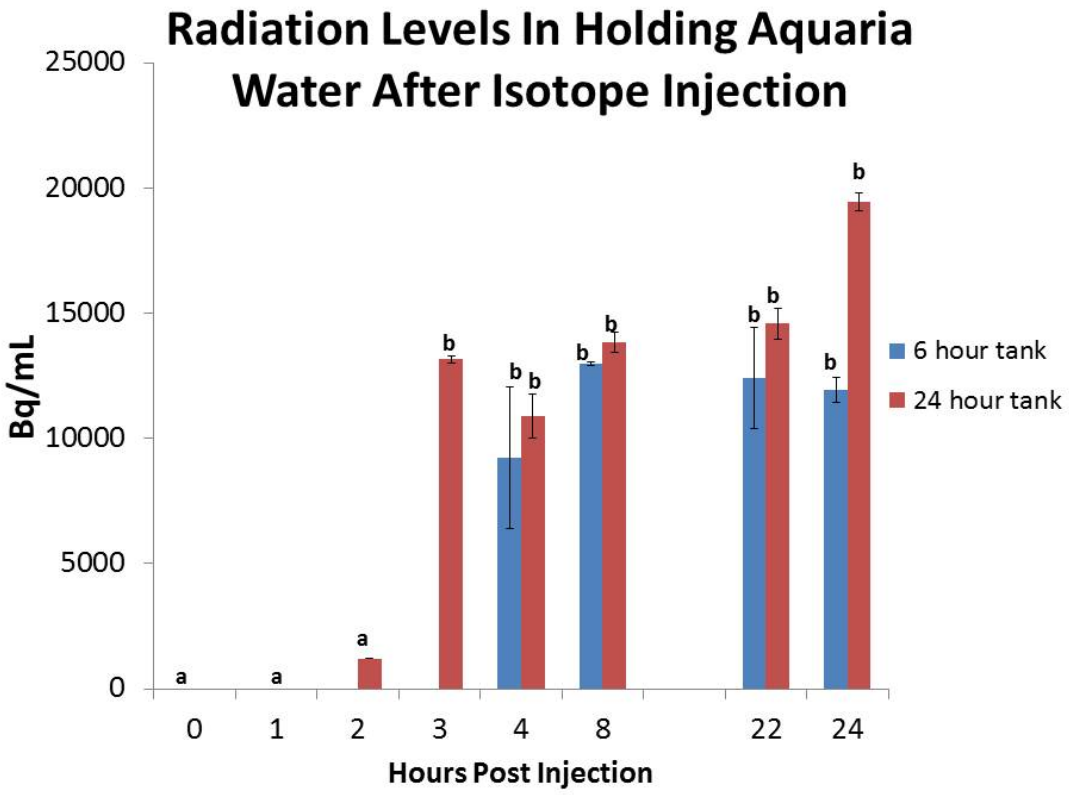


There was no significant difference in either species (red drum p = 0.86; tilapia p = 0.08) in subpharyngeal SUV between fish injected with bTSH (N=6/species) or PBS

(N = 2/species). Therefore, bTSH- and PBS-treated fish data were combined to increase statistical power. There was also no significant difference between subpharyngeal SUV (Table 1) between 6hr vs 24hr red drum ($p = 0.0764$) nor 6hr vs 24hr tilapia ($p = 0.0623$). Radioiodide uptake in the gut of red drum and tilapia combined was significantly higher in the 24hr vs the 6hr fish ($p = 0.012$). Gut SUV in red drum imaged 24hrs post injection was significantly higher the gut SUV imaged 6hrs post injection ($p = 0.04$). There was no significant difference in 6hr vs 24hr gut SUV in tilapia ($p = 0.05$).

Prior to isotope injection, water radioiodine was undetectable but was detectable at 3 hours post injection (Figure 7). In the 6 hour tank, levels continued to rise until 6 hours post injection; once fish were removed radioiodine levels began to decrease. Radioiodine levels in the 24 hour tank continued to increase until the fish were removed 24. The maximum level of 124I measured in the 24 hour holding aquaria water was 0.99Bq. Total amount of iodine in the water represented less than 0.02% of the total isotope injected into the fish.

Figure 7. Radioactivity (Bq/ml) of tank water holding fish injected with 124I. Water radioactivity was undetectable prior to isotope injection but increased beginning at 3 hours post injection. Radioactivity at and beyond 3 hours post injection was significantly higher than at 0, 1 and 2 hours post injection, as indicated by letters ($p<0.03$).



Discussion

Histological observation of changes in colloid diameter, thyroid vascularization and thyroid epithelial cell height have traditionally been used as a means for understanding environmental and physiological regulation of fish thyroid function (Eales & Brown 1993, Raine & Leatherland 2000), endocrine disruption (Liu et al 2011, Mukhi et al 2005, Patiño et al 2003, Zhang et al 2013), development (Einarsdóttir et al 2006,

Liu et al 2013, Schnitzler et al 2016) and acclimation to osmotic stress (Arjona et al 2008, Arjona et al 2010). For example, Patino et al. determined that zebrafish reared in water containing ammonium perchlorate, a known endocrine disruptor, experienced diminished thyroid function as indicated by thyroid hypertrophy, angiogenesis, hyperplasia and colloid depletion (Patiño et al 2003). Similarly, histological examination of thyroid follicle number, area and width along with thyrocyte number, height and width were used to determine that 10ppm perchlorate decreased thyroid controlled health and slowed development (Petersen et al 2015). Thyroid follicle area and volume of sole larvae was used to conclude that iodine supplementation of live feed is crucial for early life development when fish are reared in recirculating systems (Ribeiro et al 2011). While histological sectioning through large areas has been the accepted method for finding active thyroid tissue, this technique is laborious since teleost fish have thyroid follicles in widely distributed locations (Barton 2007, Eales 1979). In an effort to simplify simultaneous assessment of thyroid follicle location and activity, we explored the use of PET/CT as a new method to identify tissues with active radioiodide uptake.

In the present study, we injected tilapia and red drum with radioiodide and successfully used PET/CT to locate active thyroid follicles in the subpharyngeal region of all fish at both six and twenty four hours after injection. Scanning took less than 10 minutes and all fish were able to be imaged simultaneously. Additionally, we used the imaging software to adjust ratios of PET and CT to allow for visualization of PET with CT anatomical landmarks. Image rotation allowed for clear identification of radioiodide uptake regions in three dimensions and fast quantification of radioiodide uptake by

tissues of interest. These three dimensional images could then be used to triangulate the anatomical location of the tissues with active radioiodide uptake. All fish in our study showed most active 124I uptake in the subpharyngeal region, between the second and fourth gill arches, confirming previous research identifying iodide concentrating thyroid tissue in tilapia subpharyngeal region (Geven et al 2007). Previous histological research indicated that red drum have discrete clusters of thyroid follicles distributed along the ventral aorta between the third and fifth gill arches (MacKenzie 1988). A similar location of PET-identified maximal radioiodine uptake between the second and fourth gill arches in red drum was confirmed histologically to be thyroid tissue. As has been seen for tilapia, active thyroid follicles were only found in the subpharyngeal region of both red drum and tilapia at both time points.

Since the gastrointestinal tract is not known as a location for active thyroid follicles, radioiodide observed in the GI tract of all tilapia at both times, and in one 24 hour red drum, may have arisen from two potential sources. Thyroid hormone and thyroid hormone conjugates are stored as glucuronide or sulphate conjugates and excreted via the bile in carp (Geven et al 2007), rainbow trout (Finnson & Eales 1996) and brook trout (Sinclair & Eales 1972). As soon as two hours, and up to 96 hours after, radioiodide injection circulating radioiodide is used for the manufacture of thyroid hormone that is then released into circulation (Leloup & Fontaine 1960, Smith & Eales 1971). Radioiodinated thyroxine is stored in the gallbladder and excreted from plasma directly into the gut lumen (Collicutt & Eales 1974). Additionally, circulating radioiodine not used to manufacture thyroid hormones or enzymatically removed from

thyroid hormones is excreted via kidney filtration and urine excretion, or across gut, branchial and integumentary surfaces (Eales & Brown 1993, Geven et al 2007, Leloup & Fontaine 1960). Both red drum and tilapia at both time points displayed radioiodide uptake in the anterior region of the peritoneal cavity consistent with radioiodide accumulation in bile. Radioiodine uptake in the anterior region of the gut was seen in previous work that detected radioiodide in the gall bladder of rainbow trout (Maqsood et al 1961), channel catfish, (Collicutt & Eales 1974) and tilapia (Geven et al 2007). However, since our fish had been fasted for 48 hours prior to imaging and retained radioiodide in the gallbladder region, their digestive tracts were presumably quiescent. If these fish had not released bile for digestive purposes and thus retained radioiodinated thyroid hormones in the gallbladder, radioiodine in the gut may have come from an alternative source.

Detectable levels of radioiodide were present in the water within two hours after injection. This environmental radioiodide most likely represented excreted radioiodide since there had been insufficient time for radioiodinated thyroid hormones to have been manufactured and excreted (Eales & Brown 1993, Leloup & Fontaine 1960, MacKenzie 1982, Miller et al 2010). While we did not evaluate the chemical nature of radioiodine in the water, excretion of radioiodinated hormones may have contributed to water radioiodine within 24 hours (Eales & Brown 1993, Geven et al 2007). Our images indicate that tilapia exhibited radioiodide in the GI tract at both 6 and 24 hours with no significant difference in GI SUV between those time points. This may be explained by the fact that euryhaline teleost fish drink water when acclimated to higher salinities

(Gonzalez 2012, Miyazaki et al 1998). For example, Mozambique tilapia can drink up to 30% of their body weight after transition from fresh water to salt water (Johnson 1973). Increased drinking rates after acclimation to a higher salinity would cause a greater volume of radioactive tank water to enter the tilapia GI tract. In contrast, red drum had low levels of radioiodide in the GI tract at 6 hours but had a significant increase in GI uptake at 24 hours. Saltwater fish that move into freshwater stop drinking and instead filter out large volumes of water through the kidney (Barton 2007). Red drum acclimated to the lower holding salinity likely decreased their drinking rates and thus had smaller volumes of water enter into the GI tract during initial acclimation to higher salinities while increasing radioiodide excretion due to an increase in urine production. Meanwhile, tilapia increased drinking rates to acclimate to the higher salinity. Differences in the presence of radioiodide in the gastrointestinal tract of red drum and tilapia may thus be due to differences in ingestion of environmental radioiodine while fish worked to maintain osmoregulatory balance. Adding radioiodide to the water instead of injecting into fish and imaging fish to look for radioiodide uptake would help determine if GI radioiodine is due to water ingestion.

We found radioiodide uptake in expected areas such as the GI tract, gallbladder and subpharyngeal thyroid tissue and we found trace levels of radioiodide uptake in the muscle, brain and liver. Radioiodide is not known to penetrate muscle (Geven et al 2007, Hunn 1963, Maqsood et al 1961, Tarrant Jr 1971, Wiggs 1971, Witt et al 2009), with any radioiodide detected in muscle most likely due to accumulation of radioiodinated thyroid hormone (McNabb & Pickford 1970). Enzymatic removal of iodide from thyroid

hormones has been described in fish liver and brain but the free and conjugated thyroid hormone metabolites are then excreted via urine and bile rather than retained in deiodinating tissue (Eales & Brown 1993, MacLatchy & Eales 1992). Absence of radioiodide accumulation in tilapia kidney and gills was surprising, however. Sea bream and trout are reported to excrete 70% of radioiodide from gills and the rest via the gut and kidney (Fromm 1965, Hunn & Fromm 1964), yet our images showed no traces of radioiodide in the gill or kidney of any of our fish. This suggests that tilapia and red drum may not use gills or kidney to excrete excess iodide or, more likely, that excretion is rapid with no residual radioiodide accumulating in excretory tissues. Since radioiodide was found in the water two hours after injection and thus before radioiodinated thyroid hormones could have been manufactured or excreted, it is likely that the radioiodide was being excreted quickly.

In all jawed vertebrates (Gnathostomata), TSH binds to receptors on thyroid epithelial cells to stimulate iodide uptake from circulation for the manufacture of thyroid hormones suggesting thyroid function is highly conserved (MacKenzie 1982, Miller et al 2012, Miller et al 2010). Consequently, we injected fish with bovine TSH in an attempt to increase radioiodide uptake by thyroid tissue. While subpharyngeal radioiodide uptake was seen in all fish, no significant effects of TSH injection on radioiodide uptake were observed. Although our lab has successfully used similar doses of bTSH to stimulate T_4 release in previous experiments with red drum and goldfish, we did not confirm that the doses used in the present study were effective since we did not measure resulting circulating T_4 levels. Additionally, the small sample size (three TSH and one control fish

per species per time point) and high variability most likely limited the ability to observe significant bTSH stimulation effects. This experiment should be repeated with a broader range of doses and an increased sample size to determine how TSH stimulation affects radioiodide uptake in fish.

With PET/CT imaging of 124I injected fish we have developed a fast, non-lethal methodology that combines the benefits of histology and radiology and produces high resolution three-dimensional images that can be used to identify fish tissues with active radioiodide uptake. Histological observation of changes in colloid size, thyroid tissue vascularization and thyroid epithelial cell height have been used extensively as a means for understanding normal and pathological fish thyroid function (Eales & Brown 1993, Patiño et al 2003, Raine & Leatherland 2000) but PET/CT provides a new approach to quantify how thyroid status in fish responds to ecological, chemical and developmental stimuli. For example, studies of environmental and physiological regulation of fish thyroid function would benefit from PET/CT by providing fast and simultaneous quantification of subpharyngeal and heterotopic thyroid follicle activity. Rather than laborious histological sectioning through large sections of fish, PET/CT imaging would have quickly identified active thyroid follicles in carp renal tissue (Geven et al 2007). Additionally, comparing SUVs of heterotopic and renal thyroid tissue would provide quantifiable data on thyroid tissue activity beyond indirect histological measures. Endocrine disruption studies that are forced to use histopathology to determine the effects of endocrine disruptors on thyroid function could use PET/CT to track thyroid disruption during chemical exposure. PET/CT imaging could track the location and

activity levels of thyroid tissue throughout development and metamorphosis of flatfish (Einarsdóttir et al 2006) with iodide uptake values instead of visual classification of thyroid number and shape. We can assume that tissues that concentrate radioiodide will express the sodium iodide symporter (NIS). PET/CT images and triangulation software can be used to identify the most likely locations for NIS expression in order to collect tissues for quantitative analysis of how environmental conditions affect NIS expression.

CHAPTER III

IDENTIFICATION AND REGULATION OF THE SODIUM IODIDE SYMPORTER IN RED DRUM, *SCIAENOPS OCELLATUS*

Introduction

Iodine, a non-metallic trace element, is one of the heaviest elements essential for normal biological function. Vertebrate animals must obtain iodine from the environment to manufacture thyroid hormones. Thyroid epithelial cells utilize iodide, the biologically functional form of iodine, to synthesize the hormone thyroxine (T_4) which is subsequently secreted into circulation (Denver, et al. 2009). T_4 is converted in peripheral tissues to 3, 5, 3'-triodothyronine (T_3) by the cleavage of iodine from the outer phenolic ring via outer ring deiodinase enzymes (Sutija and Joss 2006). Peripheral tissue deiodinases can remove iodide from T_3 and T_4, thus releasing iodide back into circulation for either excretion or transport back to the thyroid (Fujimoto, et al. 2012; Kopp 2008). Once secreted into circulation, thyroid hormones are transported to target tissues where they enter the cell, translocate to the nucleus, and bind to thyroid hormone receptors which regulate fundamental physiological processes including metabolism, endocrine function, reproduction and development (Moreno, et al. 2008; Yen 2001).

Iodide uptake in mammals is achieved by the sodium iodide symporter (NIS), a membrane protein that belongs to the family of SLC5 ion transporters (Dohan, et al. 2003; Nicola, et al. 2009; Portulano, et al. 2014). Located in the basolateral membrane of thyroid epithelial cells, NIS concentrates iodide within cells by simultaneously

transporting two Na^+ and one I^- through the cell membrane (Dohan et al. 2003; Portulano et al. 2014). NIS is also synthesized in the enterocytes of the mammalian small intestine and stomach where it is responsible for dietary iodide absorption (Carrasco 1993; Kotani, et al. 1998; Nicola et al. 2009). NIS is vital to providing sufficient iodide from the environment and into the thyroid gland. In fact, malfunction of NIS and subsequent insufficient dietary intake of iodide can result in hypothyroidism (Nicola et al. 2009; Organization 2007).

Because the basic biochemical pathways for thyroid hormone synthesis and receptor activation appear conserved in vertebrates (Eales 1979), the mechanisms through which non-mammalian species accumulate iodine for thyroid hormone manufacture are also assumed to be similar. NIS presence, function and regulation are well characterized in mammals and it is believed that both mammalian and non-mammalian species have similar mechanisms for accumulating environmental iodide and concentrating it in the thyroid. The presence of thyroid NIS has been confirmed in two species of frog, *Xenopus laevis* and *Xenopus tropicalis* (Carr, et al. 2008), and one species of fish, *Danio rerio* (Porazzi, et al. 2009). *X. laevis* NIS protein sequences show a 99% homology to the *X. tropicalis* NIS protein and the *Xenopus* NIS sequences show 69% homology to the human NIS protein amino acid sequence (Carr et al. 2008) indicating that, at least in tetrapods, NIS structure has been highly conserved.

A better understanding of the mechanisms of iodine uptake in teleost fish could help to identify novel mechanisms of iodine transport in species that are important in aquaculture or in the face of a changing environment. Whereas researchers have spent

years studying the physiological regulation of transport of other essential ions such as sodium and chloride (Evans, et al. 1999) few studies have identified the NIS gene and characterized the regulation of its activity in fish. As in mammals, fish obtain iodide from their environment and deliver it to thyroid tissue for thyroid hormone synthesis (Eales and Brown 1993; Maqsood, et al. 1961) and there is evidence that NIS may be responsible. Halibut (*Hippoglossus hippoglossus*) larval iodine uptake decreased after exposure to perchlorate, a known NIS blocker, thus supporting the argument that an NIS-like transport protein exists in fish (Moren, et al. 2008). Additional evidence that NIS is involved in this process comes from studies in zebrafish, in which the NIS gene was sequenced, confirmed by BLAST for amino acid comparison and NIS expression was then used to identify the location of thyroid follicles during development (Alt, et al. 2006; Porazzi et al. 2009). Although these studies confirmed that an NIS homolog is expressed in fish thyroid tissue, they did not examine NIS expression at extra-thyroidal locations, nor did they investigate how NIS expression might be regulated. Therefore, our first goal of this study was to confirm the expression of NIS in thyroid tissue of a second species of fish in which physiological and environmental regulation of NIS could be examined. Since pituitary thyrotropin (TSH) is known to be a powerful activator of expression of mammalian NIS mRNA (Kogai, et al. 1997) and TSH functions appear to be highly conserved across vertebrates (Sherwood, et al. 2012) including fish (MacKenzie, et al. 2009), an important confirmation of the conserved function of NIS in fish is the demonstration that TSH activates thyroid *nis* expression. Therefore, our

second goal was to confirm that increases in circulating TSH would similarly stimulate *nis* expression in fish thyroid tissue.

Dietary supplementation studies have suggested fish, like mammals, accumulate iodide via the gastrointestinal tract (Gregory and Eales 1975; Ribeiro, et al. 2011; Robertson and Chaney 1953) and thus diet appears to be a primary source of iodide. Even though the gastrointestinal tract has been identified in fish as a primary iodide transporting tissue, its specific iodide transport mechanism has not been identified. Additionally, while the mammalian literature has focused on elevated iodide effects on NIS, no one has investigated if intestinal NIS mRNA expression changes in response to dietary iodide. In mammals, high iodide levels inhibit *nis* expression in intestinal tissues (Dohan et al. 2003; Nicola, et al. 2012; Uyttersprot, et al. 1997). Therefore, our third goal was to test for the presence and regulation of NIS in fish gastrointestinal tract. We hypothesized that fish would utilize NIS in the stomach and intestine for dietary iodide uptake. If NIS is present in fish gastrointestinal tract and regulated by dietary iodine, it would provide further evidence for the conservation of the physiological function of NIS throughout vertebrates. If NIS is not present in the intestine it would indicate that fish have developed alternative physiological mechanisms of iodide uptake.

Whereas the gastrointestinal tract is the primary location for environmental iodide uptake in mammals and the proposed primary location for fish, there is evidence for alternative iodide uptake locations in fish. Studies of radioiodide immersion suggest that iodide enters fish through the gills to be transported via circulation to iodide-concentrating organs, including the thyroid, head kidney, kidney, and ovary (Baker

1958; Geven, et al. 2007; Gregory and Eales 1975; Hunn and Reineke 1964; Hunt and Eales 1979; Tarrant Jr 1971). It has been assumed that fish utlizie NIS to obtain iodine via direct uptake from the water (Geven et al. 2007), but no one has yet identified NIS presence in the gills. Confirmation of *nis* expression in fish thyroid and gastrointestinal tract would confirm that NIS is present in fish in similar locations to mammals while identification of NIS in gills would suggest a novel pathway for environmental iodine uptake in fish. Since NIS is hypothesized to be the iodide transport protein in fish gills and intestine, it is possible that low environmental iodide induces a compensatory increase in NIS expression in the gill and gut in an effort to avoid hypothyroidism. If alterations in ambient iodide similarly regulate NIS expression in gills or intestine, fish would possess a unique mechanism to compensate for decreases in environmental iodide levels by up-regulating branchial and gastrointestinal transport. Therefore our fourth goal was to determine if NIS is expressed in fish gills where it could potentially serve to absorb iodide directly from the water. We hypothesized that a decrease in salinity would cause an increase in *nis* mRNA expression in the gills.

If fish are dependent on non-dietary acquisition of environmental iodide to maintain thyroid homeostasis, fish in variable salinity environments may be subject to fluctuations in iodide availability that would require a physiological response. The ratio of total dissolved iodine to salinity is generally held at a constant value of 12.8nM/ppt (Küpper, et al. 2011); therefore ambient iodide concentrations should decrease with decreasing salinity. To respond to these changes, euryhaline fish are able to adjust branchial populations of freshwater absorptive ionocytes and saltwater excretory

ionocytes (Takei and McCormick 2013) thus regulating ion transfer in response to changes in ambient ion levels. Assuming gill iodide transport mechanisms respond to changes in iodide concentrations similar way to gill sodium and chloride transport, we hypothesized that a decrease in environmental iodide would cause an increase in *nis* mRNA expression in the gills. Therefore our final goal was to determine if ambient iodine levels influence *nis* expression in fish gill. Since iodide uptake is essential for thyroid hormone production, this information would help us understand how distinct iodide transport systems are used in aquatic environments to maintain thyroid homeostasis in fish.

To address these objectives we developed techniques to identify and quantify *nis* expression in red drum, *Sciaenops ocellatus*. Red drum is a euryhaline perciform fish common along the Gulf and east coasts of the United States, easily obtained from hatcheries, which have been used in nutritional, osmoregulatory (Watson, et al. 2014), and thyroid hormone research (Esbaugh and Cutler 2016; Gatlin III, et al. 1992; Jones 2012; Jones, et al. 2013; Leiner and MacKenzie 2001; MacKenzie et al. 2009; Miller 2011; Pohlenz and Gatlin III 2014; VanPutte, et al. 2001) and can thus serve as an informative species in which to study the regulation of NIS. Euryhaline fish are likely to encounter variable levels of environmental iodide and the euryhaline capabilities of red drum mean they can be effectively utilized in studies of the impact of alterations in environmental salinity on physiological function (Gatlin III et al. 1992; Gullian-Klanian 2013), thereby providing an excellent model to examine *nis* expression in response to alterations in ionic environment.

Materials and methods

Animals

Red drum fingerlings were obtained from The Texas Parks and Wildlife Department's hatchery at Sea Center Texas (Lake Jackson, TX) and transported to the Sea Life Center (SLC) at Texas A&M in Galveston (Galveston, TX). Upon arrival, 10% water changes were performed every 20 minutes until temperature and salinity in transportation tanks matched temperature and salinity in the holding aquaria and fish were moved into the holding tanks. SLC fish were held in a 1000L recirculating system of natural seawater held at 32ppt salinity, 26^{o}C and 12L: 12D photoperiod. Fish were fed Aquamax (PMI Nutrition, Brentwood, MO) to apparent satiation twice daily. Once fish reached an average weight of >20g they were transported to the Department of Biology's BioAquatics Facility in Biological Sciences Building East (BSBE) at Texas A&M in College Station and acclimated to holding tank salinity and temperature. BSBE fish were housed in one of two 2000L recirculating artificial seawater (SuperSalt, Fritz Industries, Mesquite, Texas) systems at 9ppt salinity, 26^{o}C and 12L: 12D photoperiod.

nis identification

nis sequences for teleost fish (zebrafish, medaka, gobi, stickleback, and tilapia) were obtained from published literature and Gen Bank and used to generate PCR primers (Alt et al. 2006; Darrouzet, et al. 2014) using Clustal X2 software for Multiple Sequence Alignment and FastPCR software for primers. The forward NIS primer was chosen to be

ATT$^{C}_{T}$T$^{G}_{C}$AA$^{C}_{T}$CAAG$^{C}_{T}$NACNGG and the reverse NIS primer was GCCATNGC$^{G}_{A}$TT$^{G}_{A}$AT$^{G}_{A}$CTNGT$^{G}_{A}$GA.

RNA was isolated from red drum thyroid tissue using a ZR RNA MiniPrep kit (Zymo Research, Irvine, CA). All RNA samples were treated with DNAse I (Zymo Research) according to the manufacturer's protocol prior to PCR. PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI) according to the manufacturer's protocol. PCR reactions were run on a DNA gel and visualized under UV light (Cohn, et al. 2010). PCR products were sequenced to confirm identity using BigDye reagent (Applied Biosystems/Invitrogen) according to the manufacturer's protocol at the Gene Technologies Laboratory in the Department of Biology at Texas A&M University. Sequences were confirmed to be *nis* by BLAST analysis. Clustal X2 and FastPCR were used on the red drum sequences to create red drum specific consensus alignments and *nis* primers (rdNIS). The forward primer was GCTGTGATCTGGACTGATGTGTTCCA and the reverse primer was TCCCGCTGTATGCACAGGCAAGAA.

bTSH injection studies

To determine whether TSH activated red drum *nis* expression we performed a TSH challenge test. Four groups of 7 fish were removed from holding tanks, anesthetized by immersion in tricaine methanesulfonate (MS-222) (200mg/L) and weighed. Fish were injected intraperitoneally (IP) with 0.157, 0.625, or 2.5 mU/g body weight of bovine TSH (bTSH, Sigma, 2.2 IU/mg) in BSA-saline. Control fish were

injected with BSA-saline. Fish were returned to holding tanks, recaptured five hours later and euthanized for tissue collection. Heparinized syringes were used to collect 250-750µl of blood from the caudal vasculature. Blood was centrifuged to separate plasma which was frozen for hormone determination. To confirm successful injection an IMMULITE 2000 canine TSH immunoassay (Siemens) was used to measure circulating bTSH as previously described (Miller, et al. 2012). Following blood collection subpharyngeal tissues, intestine, and gills were collected and placed in RNAlater (Ambion), stored for 24 hours at 4^{o}C and then moved to -80^{o}C for storage until RNA isolation. For statistical analysis, homogeneity of variance was tested with Levene's test and normality with the Kolmogorov-Smimov test using SPSS version 24.0. Significance between treatment groups was determined by one way ANOVA. Significance was determined if $p > 0.5$.

Postprandial nis expression

To examine the effects of feeding on *nis* expression red drum were entrained to a set daily feeding schedule for six weeks. Fish were fed to satiation a diet of Aquamax (PMI Nutrition, Brentwood, MO) every morning between 8:30-9:00am. All noises and potential feeding disruptions were minimized for 20 minutes to allow fish to feed with no distractions. Any uneaten food was removed immediately afterward. On the day of the experiments, five fish were euthanized for tissue collection at 1 and 0.5 hours before, and 0.5, 1, 1.5, 2.5, 3, and 3.5 hours post feeding. Intestine, gills, stomach and subpharyngeal tissues were collected and placed in RNAlater (Ambion) and stored for

24 hours at 4^{o}C then moved to -80^{o}C for storage until RNA isolation. At each time point three water samples were collected in syringes, filtered through a 45μ syringe filter and stored at -20^{o}C. Samples were shipped on ice to the Laboratory for Oceanographic and Environmental Research at Texas A&M University in Galveston, TX for iodide determination (Zhang, et al. 2010). We used a one way ANOVA among time points within each tissue to look for statistical significance in *nis* expression in a tissue over time. Significance was determined if $p > 0.5$.

Salinity effects on nis expression

To examine the effects of ambient salinity on *nis* expression, a total of 60 fish, with an average mass of 35g, were held for one month at 35ppt, 25^{o}C and a 12:12 light cycle. On the day of the experiment, water changes were performed to bring 20 fish to each treatment salinity using the following protocol: A single water change using 4ppt artificial seawater was performed to decrease the salinity from 35ppt to 20ppt. Two water changes using 4ppt artificial seawater were performed 24 hours apart to decrease the salinity from 35ppt to 5ppt. A single water change with 35ppt artificial seawater was performed on the 35ppt control tank. Five fish per salinity treatment were euthanized 1, 5, 7 and 14 days after the final water change. Intestine, gills and subpharyngeal tissues were collected, placed in RNAlater (Ambion), stored for 24 hours at 4^{o}C, and then moved to -80^{o}C for storage until RNA isolation. Water samples were collected at each time point and salinity treatment, stored at -20^{o}C, and shipped on ice to Laboratory for

Oceanographic and Environmental Research (LOER) at Texas A&M University at Galveston for iodide determination described below (Zhang et al. 2010). We used a general linear model including all interactions comparing among salinity and tissues for each time point. Post-hoc Tukey's tests were performed to determine differences within groups with statistically significant differences. Significance was determined if $p > 0.5$.

Iodide supplementation

To examine directly the effects of ambient iodide on *nis* expression, 5ppt water was supplemented with potassium iodide (KI) to increase iodide concentrations to levels found in 35ppt. Iodide quantification indicated low levels of iodide and iodate in our artificial seawater at this salinity. Preliminary studies found that daily addition of 0.65g KI/1000L to our recirculating systems increased iodide levels by approximately 159 ug/L/day (Julie Butler, Texas A&M University, unpublished research). Based on these results, a single dose of 0.3g KI was added to the charcoal free recirculating systems to increase ambient iodide levels. Five fish were euthanized 0, 1, 5, 7 and 14 days after the iodide supplementation. Intestine, gills and subpharyngeal tissues were collected, placed in RNAlater (Ambion) and stored for 24 hours at 4^{o}C and then moved to -80^{o}C for storage until RNA isolation. At each time point and salinity three water samples were collected in syringes, filtered through a 45µ syringe filter and stored at -20^{o}C. Samples were shipped on ice to LOER (Texas A&M University at Galveston) for iodide determination (Zhang et al. 2010). We performed a general linear model including all

interactions comparing among tissues for each time point. Post-hoc Tukey's tests were performed to determine differences within groups with statistical significant differences.

Iodide quantification

Iodide concentrations were measured in our artificial seawater to assess the effects of carbon, biological filtration, and fish presence on environmental iodide exposure of red drum. Fritz Super Salt Concentrate© was mixed with Morton's Salt© and filtered reverse osmosis (RO) water was used to obtain full strength artificial seawater at 35ppt. Seawater was allowed to mix for 24 hours and then added to two empty 2,000L aquatic raceway systems. After 17 days of recirculation 10L of activated carbon was added to the filter. Live Nitrifying Bacteria© (Drs. Foster & Smith, Rhinelander, WI) was added 12 days later. Fish were added 16 days later. Water samples were collected in triplicate, filtered through a 45µ syringe filter and stored at -20^{o}C until iodide quantification.

Iodide and iodate were quantified by LOER using previously described techniques (Santschi, et al. 2017; Zhang et al. 2010). For iodide quantification a 5 ml aliquot of sample water was mixed with 0.5 mL of 1% acetic acid and 1 mL of phosphate buffer in a culture tube (16 × 150 mm). Internal standard (50 µL), N, N-dimethylaniline solution (50 µL), and 2-iodosobenzoate solution (0.4mL) were then added to each tube and shaken on a Touch Mixer for 1 min. Next, cyclohexane (0.5 mL) was added to the tubes and shaken on a Touch Mixer for 20 s. The top cyclohexane layer

was removed and placed into an auto sampler vial for GC-MS analysis (Zhang et al. 2010).

For iodate quantification, a 5 mL aliquot of sample or standard was mixed with 50 μL of 1 M HCl and 100 μL of 0.01M of sodium meta-bisulfite in a culture tube (16 × 150 mm). This solution was reacted at room temperature for 30 min. Next, 1 mL of phosphate buffer was added and mixed. Internal standard (50 μL), N, N-dimethylaniline solution (50 μL), and 2-iodosobenzoate solution (1.0 mL) were added, and the solution was shaken on a Touch Mixer for 1 min. This solution was then extracted with cyclohexane for GC-MSanalysis, as described in the previous paragraph. Iodate concentration was then calculated by difference using the iodide concentrations before and after $Na_2S_2O_5$ treatment (Liu, et al. 2013).

qPCR

qPCR was used to measure mRNA expression of the red drum NIS based on methods previously developed in our lab (Jones 2012). qPCR step 1 RT reactions and step 2 PCR reactions were performed using the high-capacity cDNA Reverse Transcription Kit and TaqMan Universal PCR Master Mix (Invitrogen) with red drum specific qPCR primers/probes (Integrated DNA Technologies) following the manufacturer's protocol. qPCR primers/probes were designed using Primer Quest software from Integrated DNA Technologies and designed to cross a predicted intron-exon boundary (Bustin 2000). Step 2 of the qPCR was performed and analyzed using the QuantStudio Fast Real-Time PCR machine following the manufacturer's protocol for

relative quantification. Expression of the 18S ribosomal subunit was amplified as a reference control gene for qPCR (Bustin 2000).

The ΔΔCt method was used to calculate qPCR results. Relative values correspond to the mRNA expression of the gene-of-interest/18S Ribosomal subunit and the average relative value corresponds to the mean of all relative values within the same experiment independently determined for each gene-of-interest. PCR efficiency was above 90% for all assays. Reference gene (18S) mRNA levels were not detectably different among treatments. To validate the qPCR assay for NIS, we created a standard curve using known concentrations of red drum RNA product including concentrations within the range of expected red drum RNA concentrations and a range of values exceeding the expected range. qPCR for NIS and 18S was performed on all concentrations and graphed. The standard curve was linear in the range of expected values.

Statistics

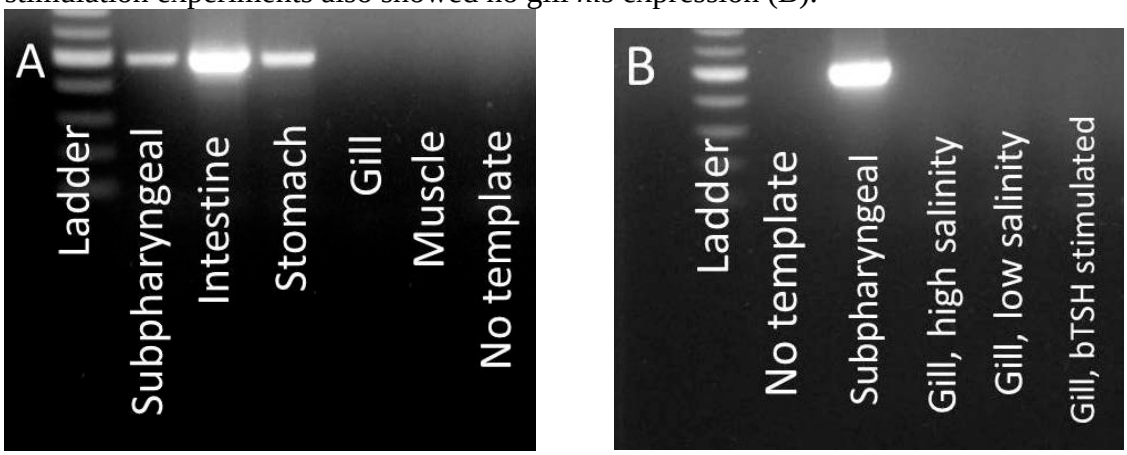
Data were checked for homogeneity of variance and non-parametric data were transformed using log or square root transformations. Post-hoc Tukey's tests were performed to determine differences within groups with statistical significant differences.

Results

nis identification

PCR products were sequenced and confirmed by BLAST analysis to be NIS. Gel electrophoresis showed a 500 bp PCR product, the size expected based on sequencing results (Figure 8). These 500bp NIS bands were present following RT-PCR with RNA from the subpharyngeal region, intestine, and stomach of red drum (Figure 8). Gel bands were brightest for the intestine and subpharyngeal tissues. No expression was found in red drum muscle negative control or the no template control (Figure 8A). No evidence of *nis* expression was seen initially in gill tissue. In an attempt to determine if gill *nis* expression would appear under different physiological conditions we performed RT/PCR and gel electrophoresis on RNA from gill filaments of fish in high salinity, low salinity and bTSH stimulated experiments but still did not see any gill bands (Figure 8B). A partial sequence for red drum NIS was submitted to Genbank (accession number MF802867).

Figure 8. Gel electrophoresis of PCR products using sodium iodide symporter (*nis*) primers in red drum tissues. *nis* expression is seen in the subpharyngeal, intestine and stomach tissues but not in the gills. No *nis* expression was seen in the muscle or the no template negative control (A). Fish from high and low salinity and fish from bTSH stimulation experiments also showed no gill *nis* expression (B).



qPCR validation

We created a standard curve using serial dilutions of known concentrations of RNA obtained from RNA isolation of subpharyngeal, intestine, stomach, gill and muscle tissues. Dilutions included the maximum and minimum expected concentrations of RNA we previously obtained when performing RNA isolation from these tissues. Standard curves were linear within expected concentrations for all tissues (Figure 9). Due to consistent high yields of RNA and a sensitive range of linear dilutions, intestine was determined to be most appropriate for an internal positive control tissue for relative comparison across all qPCR experiments. Consequently, 200 ng of intestine was included in all subsequent qPCR determinations to confirm stability of the assay.

Figure 9. Standard curves of red drum RNA isolated from muscle, stomach, gill, intestine and subpharyngeal tissues diluted before qPCR.

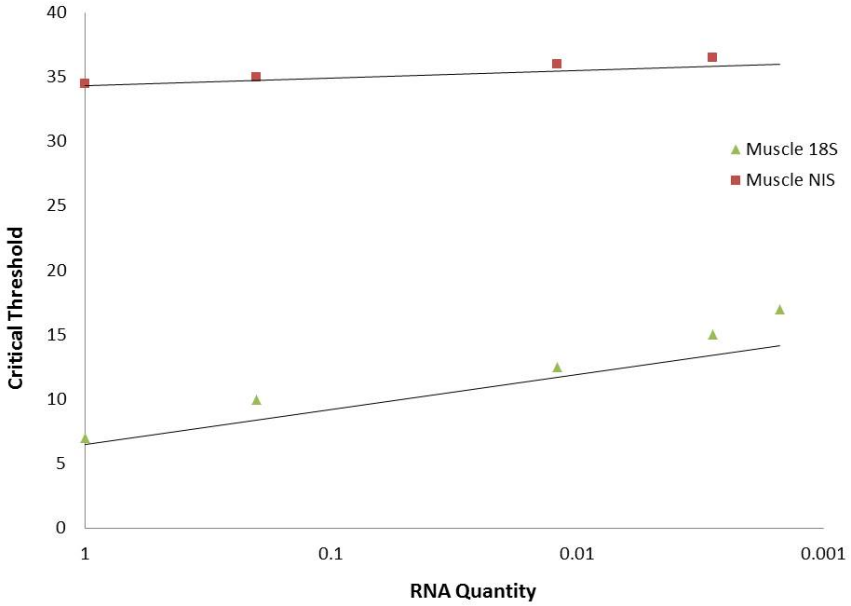
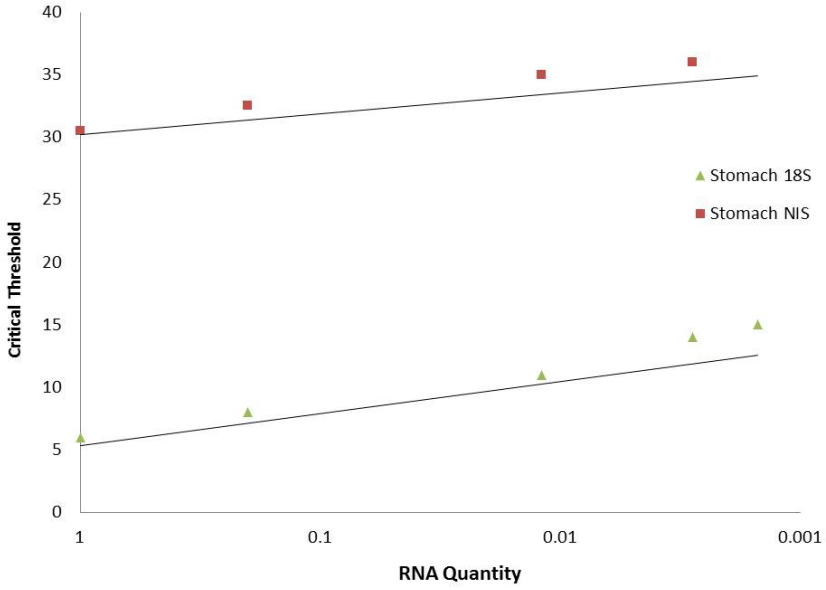


Figure 9 continued



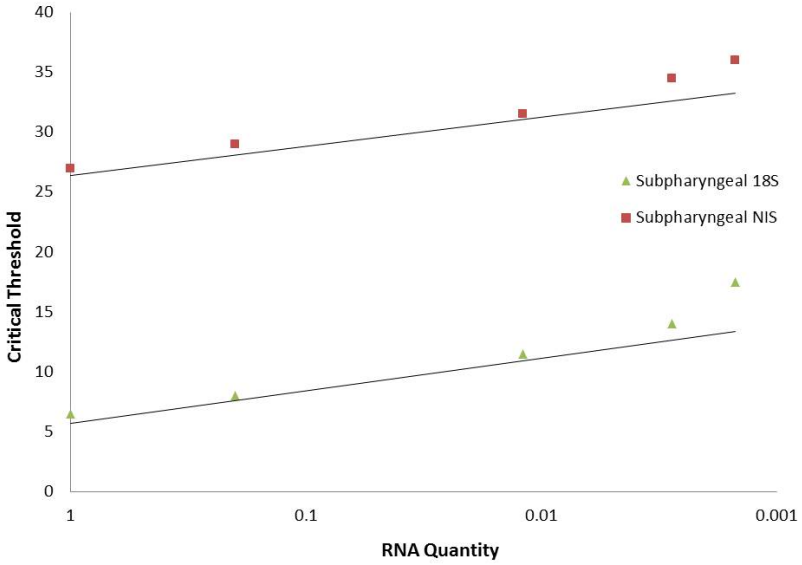
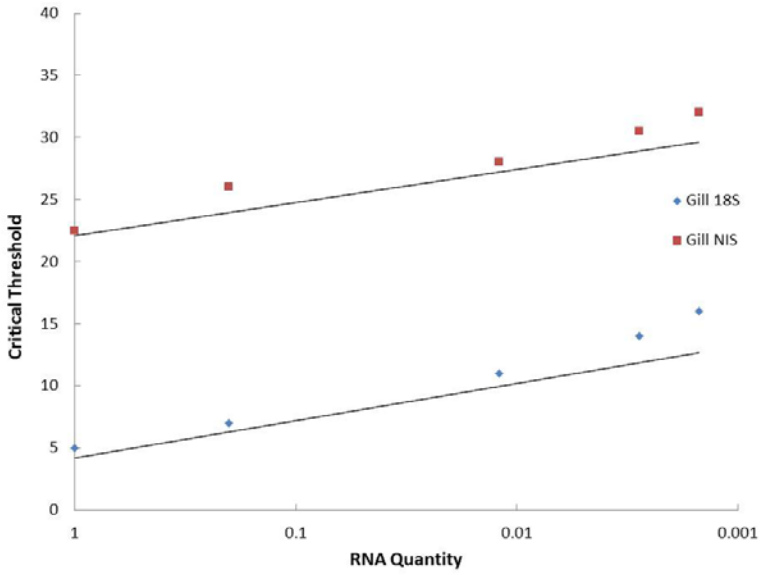
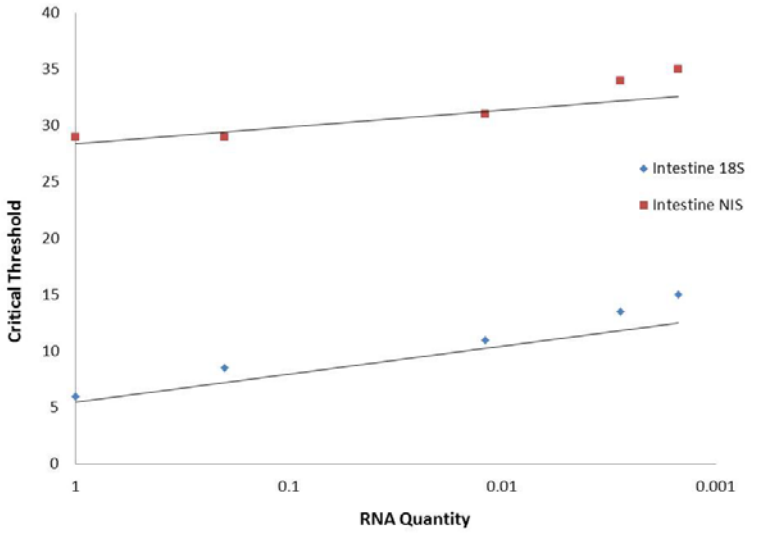


Figure 9 continued





bTSH injection studies

bTSH injections increased circulating T_4 above levels seen in fish injected with control BSA saline. There was no significant difference between circulating thyroxine levels of fish injected with 0.157, 0.625, or 2.5 mU/g body weight bTSH (P = 0.2). Red

drum injected with bTSH had a significant increase in circulating plasma T_4 levels (P = 0.003) compared to red drum injected with control BSA solution (Figure 10). The highest dose (2.5 mU/g body weight) of bTSH also caused a significant increase in subpharyngeal *nis* expression (p = 0.04) over saline-injected fish. There was no change in *nis* expression in any other tissue at any TSH dose (Figure 11).

Figure 10. Circulating T_4 levels in red drum injected with 0.157, 0.625, or 2.5 mU/g body weight of bovine bTSH or control BSA saline. bTSH injection cause an increase in circulating T4. Different letters represent significant difference between values.

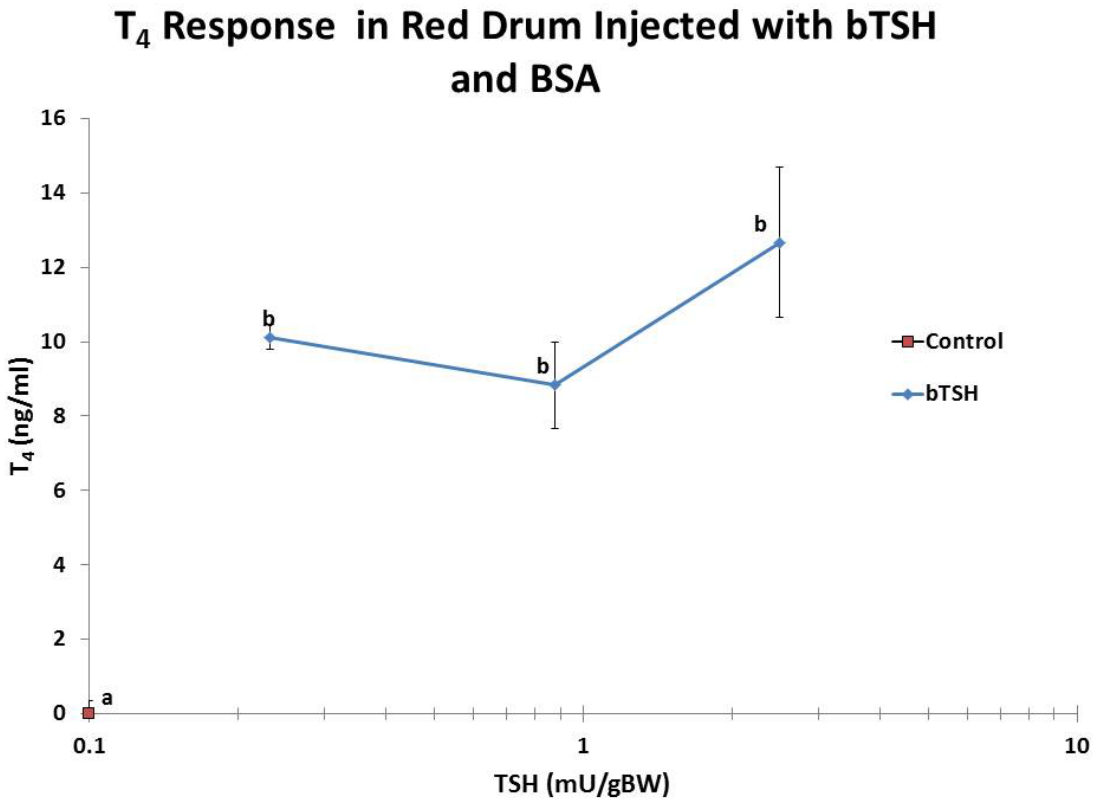
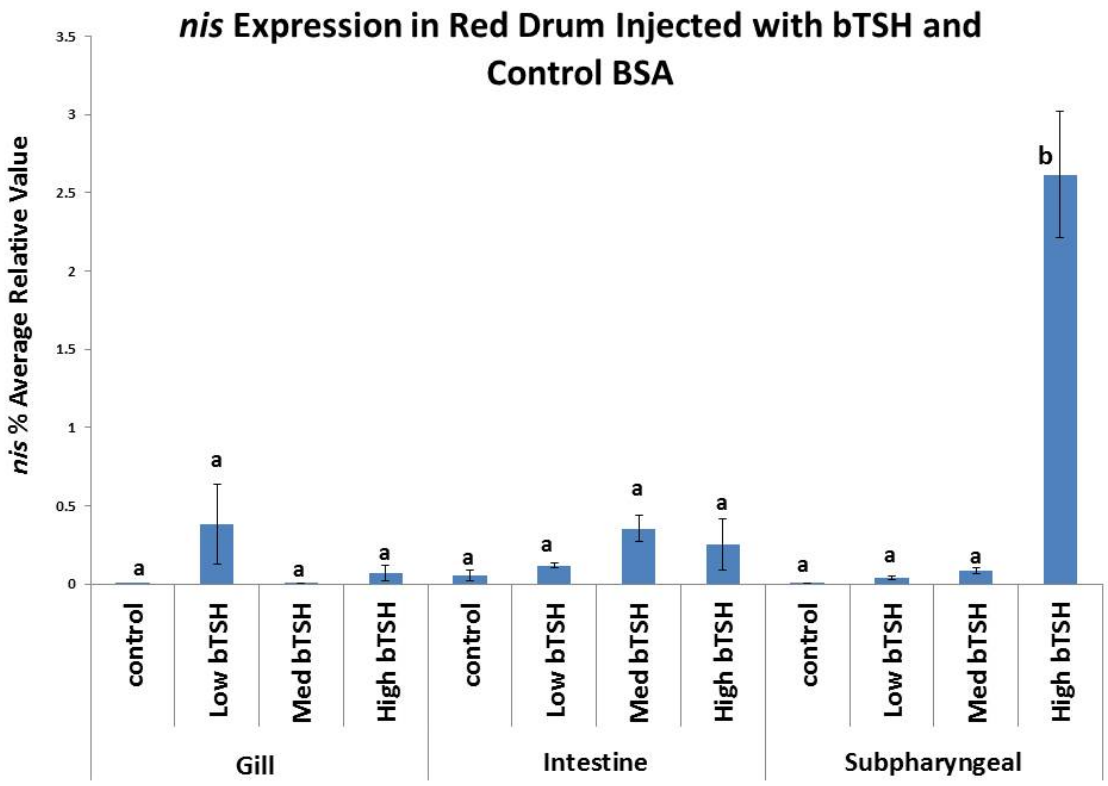


Figure 11. *nis* expression in red drum injected with 0.157, 0.625, or 2.5 mU/g body weight of bovine TSH (bTSH) or a control BSA solution. Different letters indicate significant difference in *nis* expression.

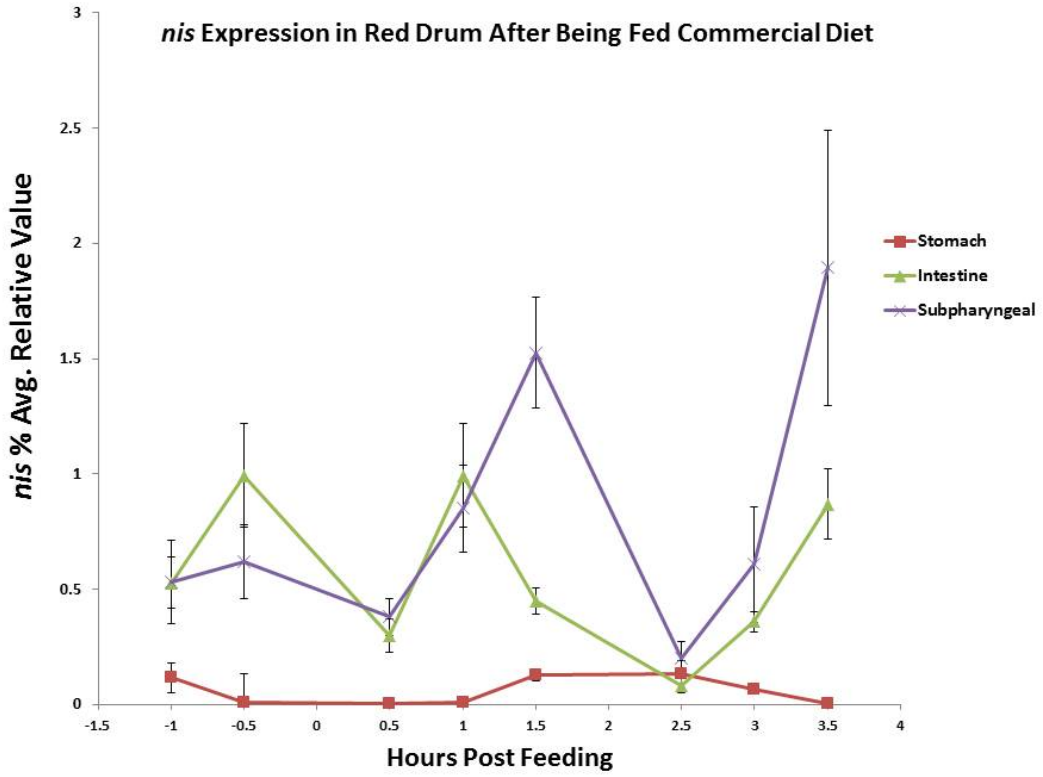


Postprandial nis expression

No clear trends in *nis* expression were found in any tissue following a single meal. Within each tissue, although expression levels were variable there was no significant change in expression over time (Figure 12). Values for each tissue over the total 4.5 hour experiment were then combined for analysis. Average subpharyngeal values over the entire experiment were significantly different ($p = 0.01$) from stomach.

Similarly, average intestinal expression over the entire experiment was significantly different (p = 0.002) from stomach. Stomach *nis* expression remained low and unchanged across all time points (p = 0.1).

Figure 12. *nis* expression in stomach, intestine and subpharyngeal tissues of red drum following a single meal. There was no significant different within any of the three tissues at any time point.



Ambient iodide measurements

Because fish in these studies were held at a variety of salinities, we measured iodide and iodate levels at different salinities of Fritz Super Salt Concentrate© mixed with Morton's Salt© diluted with RO water and confirmed that a decrease in salinity

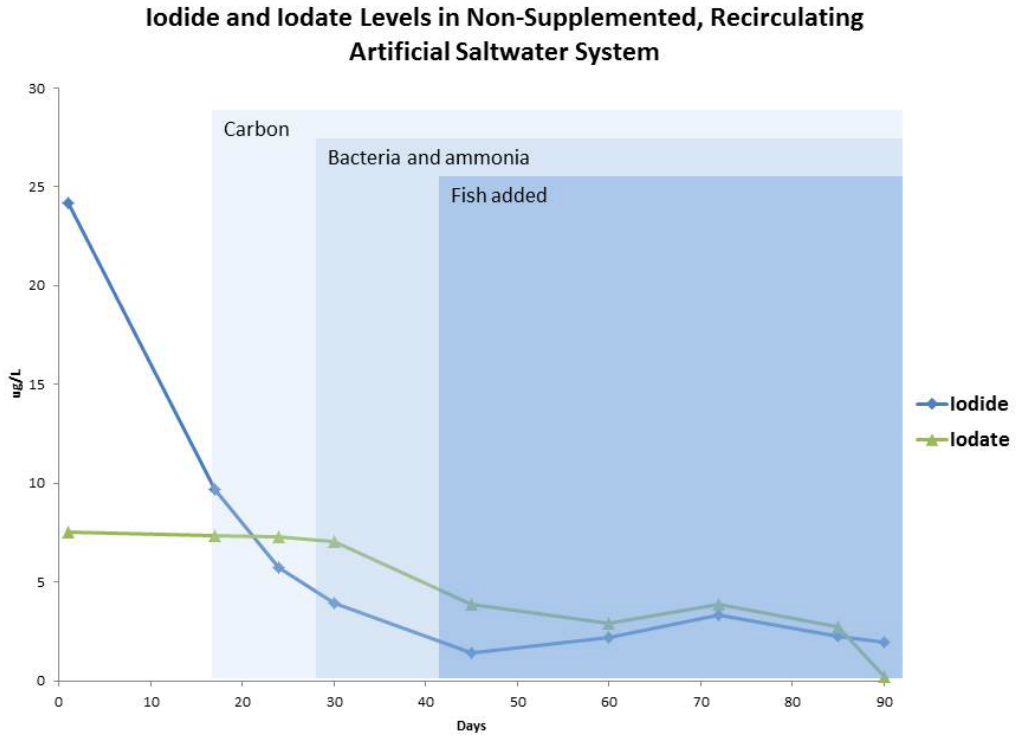
caused a decrease in iodide and iodate (Table 2). Similarly, diluted artificial saltwater made from the same Fritz Concentrate© mixed with Morton's Salt© from the Texas A&M Aquaculture and Teaching Facility also had low iodide and iodate levels at low salinity (Table 2). Artificial saltwater for the TAMU Biology reef tank created by dissolving Instant Ocean Salt Concentrate© into RO water to 31ppt had higher levels of iodide and iodate than Fritz Super Salt Concentrate© mixed with Morton's Salt©. However, once the Instant Ocean saltwater was added to a biologically filtered recirculating system the salinity remained at full strength seawater but iodide decreased (Table 2). Similarly, we found that 24 hours after adding activated carbon to the recirculating red drum system iodide decreased from 9.68 to 5.73 ug/L and iodate from 7.33 to 4.12 ug/L. Since all of these systems used artificial saltwater, we also measured iodine in a natural sea water system. TAMUG Sea Life Center natural seawater was 29ppt with 1.3 ug/L iodide and 11.9 ug/L iodate.

Table 2. Iodide and iodate levels measured in TAMU recirculating systems. Different commercial salts at similar salinities have varying iodine levels. Decreases in salinity corresponded with decreases in iodide and iodate levels.

System	Salinity (ppt)	Iodide (ug/L)	Iodate (ug/L)
Redfish Salinity Experiments	35	72.6	24.1
	20	15.5	11.9
	5	6.8	1.8
Redfish Saltwater Reservoir	35	88.4	<0.14
Aquaculture Research and Teaching Facility	7	2.68	1.55
Biology Department Reef Tank Reservoir	31	134.2	5.1
Biology Department Reef Tank	31	8.9	11.8
TAMUG Sea Life Center	29	1.3	11.9

Because iodine content varied across different systems, we evaluated temporal changes in iodide and iodate levels in our biologically filtered, recirculating artificial saltwater red drum systems by tracking iodine levels in one system from initial set up through addition and acclimation of fish (Figure 13). Freshly-made full strength seawater in the holding reservoir had high levels of iodide and low levels of iodate. Twenty four hours after reservoir water was added to the recirculating system iodide levels decreased to 24.2 ug/L iodide while iodate levels increased to 7.5 ug/L. Iodate levels continued to decrease until day 40 when they stabilized through the rest of the experiment. Iodate levels remained stable from day 0 to day 30 and then until day decreased for the rest of the experiment. Iodide and iodate levels in our recirculating system thus continually decrease as the water remains in the system.

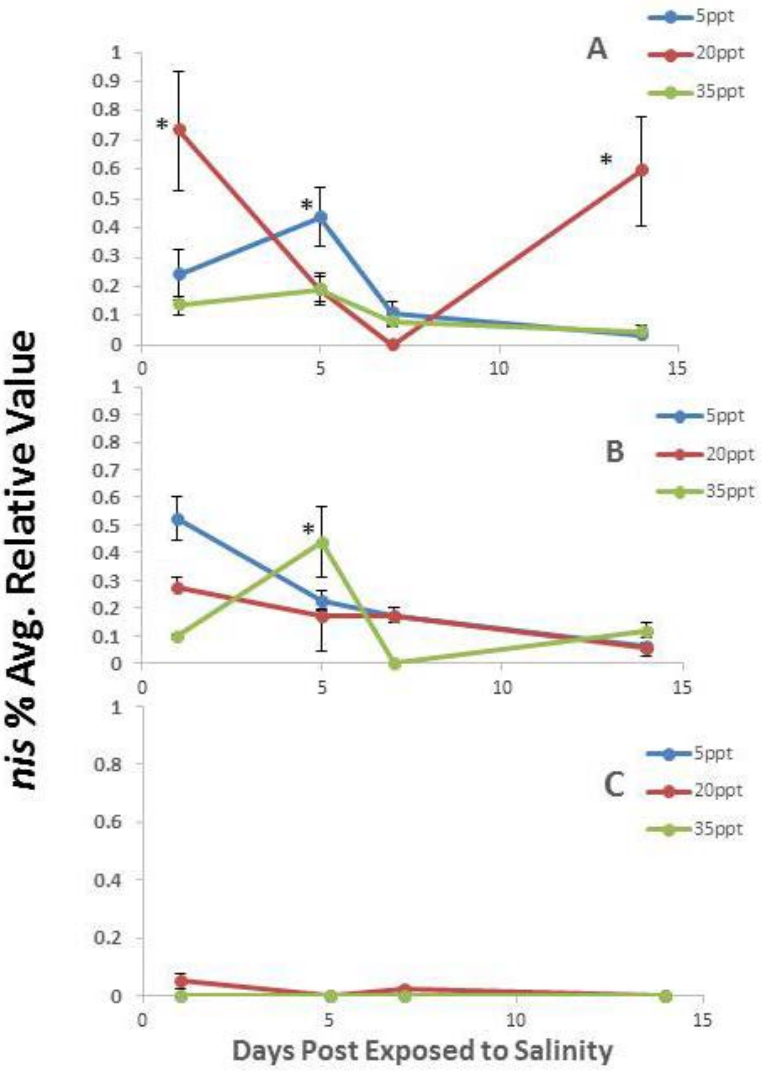
Figure 13. Iodide and iodate decreases over time in a recirculating aquatic system with biological and carbon filtration.



salinity mediated nis expression

Subpharyngeal and intestine were the only tissues that showed a significant *nis* response to salinity change (Figure 14). Subpharyngeal, intestine and gill *nis* expression remained unchanged in the control 35ppt transfer group across all time points. However, subpharyngeal *nis* expression at 20 ppt was higher than 35ppt one day and 14 days after exposure to new salinity ($p < 0.0001$). Similarly, subpharyngeal *nis* expression at 5ppt was higher than 35ppt 5 days after exposure to new salinity ($p < 0.0001$). Salinity had no effects on intestine or gill *nis* expression.

Figure 14. *nis* expression in subpharyngeal, intestine and gill tissue of red drum moved to a treatment salinity and held for 14 days. Subpharyngeal and intestine were the only tissues that showed changes in *nis* expression in response to exposure to a new salinity. The asterisks denote significant difference between tissues within a time point.



Iodine supplementation

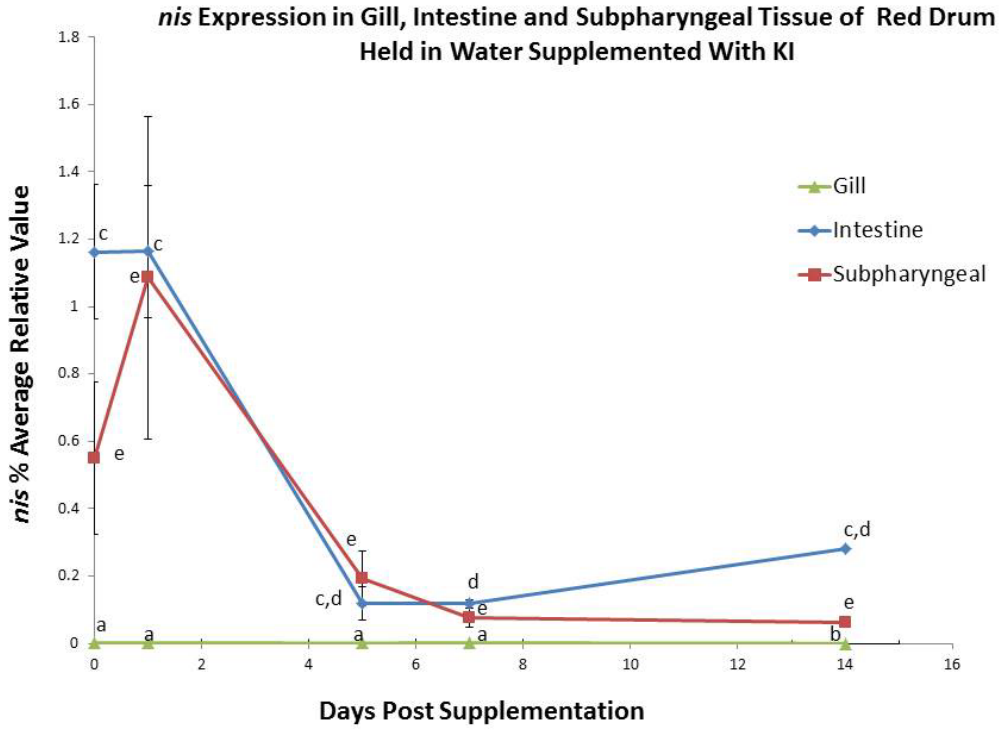
To examine how ambient iodide levels influenced *nis* expression, we supplemented 5ppt artificial seawater with KI (Table 3) to restore iodide and iodate to full strength seawater levels (cf. Tables 2 and 3). Although iodide and iodate decreased

30% and 60% from day 1 to day 5, levels remained stable until the end of the experiment at day 14. Subpharyngeal *nis* expression was variable at the initiation of the experiment and did not change significantly ($p = 0.054$) over 14 days (Figure 15). Intestine *nis* expression at 5 days was significantly lower than before supplementation ($p < 0.0001$). However, all other time points were not significantly different. Iodide supplementation had no effect on gill *nis* expression until a significant decrease of 80% 14 days post supplementation ($p = 0.0001$). Subpharyngeal *nis* expression across all time points was not significantly different than intestine ($p = 0.39$). However, gill *nis* expression at each time point was significantly lower than intestine or subpharyngeal *nis* expression ($p = 0.005$).

Table 3. Iodide and iodate levels in an artificial saltwater recirculating system supplemented with 0.3g KI. Supplementation caused an increase in both iodide and iodate that then decreased after 5 days and remained stable during the rest of the experiment.

Days Post Supplementation	Iodide (ug/L)	Iodate (ug/L)
0	5.3	7.2
1	36.2	14.1
5	25.2	5.7
7	26.6	17.9
14	26.8	2.5

Figure 15. *nis* expression in subpharyngeal, intestine and gill tissue of red drum in 5ppt water supplemented to with addition of 0.3g KI. Supplementation had no effect on subpharyngeal *nis* expression ($p = 0.054$). Intestine *nis* expression decreased 5 days post supplementation ($p < 0.0001$). Gill *nis* expression remained unchanged until it increased at day 14 ($p < 0.0001$).



Discussion

It is believed that both mammalian and non-mammalian vertebrates have similar mechanisms for accumulating environmental iodide and concentrating it in the thyroid. Whereas NIS presence and regulation are well characterized in mammals, the presence of NIS in teleost fish has only been examined in one species, the zebrafish (Porazzi et al. 2009). We can now confirm that NIS is present in lower jaw thyroid follicles of an

additional species, the red drum. BLAST results confirmed that our amplified sequence belongs to NIS. Additionally, the increase of subpharyngeal *nis* expression in response to bTSH stimulation provides further support that the gene we sequenced codes for NIS. We have shown that red drum NIS is expressed and hormonally regulated by TSH in thyroid tissue similar to mammals (Dohan et al. 2003; Nicola et al. 2009; Portulano et al. 2014) suggesting its fundamental function of iodine uptake into thyrocytes is conserved between mammals and fish.

We are the first to show *in vivo* bTSH stimulation causes an increase in red drum subpharyngeal *nis* expression. Since TSH rapidly activates mammalian thyroid *nis* expression (Alotaibi, et al. 2017; Kogai et al. 1997), our research supports findings that this TSH function is conserved across vertebrates (Sherwood et al. 2012) including fish (MacKenzie et al. 2009). Although we did see an increase in circulating T_4 in response to all three doses of bTSH, *nis* expression only increased in response to the highest dose of bTSH. TSH doses were based on previous research that also indicated red drum held for greater than six weeks in our recirculating system develop insensitivity to bTSH stimulation (Miller 2011). The lack of T_4 dose response combined with low *nis* expression in the low and medium doses of bTSH may suggest that our bTSH concentrations were too low. Repeating this experiment with a wider range of bTSH concentrations may provide better insight on how red drum upregulate *nis* expression in response to TSH stimulation.

Our fish did not show any significant changes in stomach or intestine *nis* expression before or after a scheduled meal. If *nis* expression is activated by feeding we

might expect to see an increase in intestine *nis* expression in fed fish compared to fasted fish. To maximize nutrient uptake, mammals entrained to specific feeding schedules optimize digestive processes by increasing cell proliferation, gene expression and enzymatic activity in anticipation of food (Scheving, et al. 1983; Strubbe and van Dijk 2002). For example, feeding regulates the expression of intestinal cotransporters in rats (Saito, et al. 2008) and goldfish increase gastric enzymes in anticipation of entrained feeding (Vera, et al. 2007). This suggests that red drum might upregulate *nis* expression in response to or in anticipation of regularly scheduled feeding, yet we did not observe this. This lack of change suggests red drum may not regulate NIS based on anticipated meals but may instead maintain constitutive intestinal *nis* expression to facilitate iodide uptake once iodide is ingested. Because our experiment only included fish fed a single meal without any control for possible circadian changes in expression further studies including unfed controls are needed to help determine if *nis* expression displays a circadian component. Additionally, variability in our data suggests we may have lost statistical power due to low sample size. An increase in the number of fish sampled at each time point should allow for more robust statistics in future studies. In contrast to the intestine, stomach *nis* expression was low and unchanged during the feeding experiment. It is possible that stomach NIS does not play a role in dietary iodide absorption and instead functions as a recycling pathway whereby the stomach transports iodide from the blood into the gastric lumen for reabsorption by intestinal NIS (Portulano et al. 2014). This has been observed in both mammals and amphibians (Carr, et al. 2015).

While feeding might be expected to activate an increase in mammalian intestinal symtransporter expression, *nis* expression has been shown to decrease in response to high dietary iodine (Nicola et al. 2009). Rangen© commercial red drum diet iodide levels fall within the standard range of 1-5mg/kg (Lewbart 1998; Lovell 1979; Watanabe, et al. 1997) which may not have been high enough to cause any postprandial decrease in *nis* expression. Iodine is supplemented in fish food and yet there is little uniformity on the defined minimum iodine levels for fish diet (Lewbart 1998; Lovell 1979; Watanabe et al. 1997). While it is assumed that the iodide levels in commercially manufactured fish food are sufficient, there is not enough information about fish intestine and stomach NIS to determine if fish are regulating *nis* expression in response to low or high dietary iodide. Future studies of gastrointestinal iodine transport would help to better identify *nis* response to dietary iodine levels and thus and help establish optimal dietary iodine content of aquaculture feeds.

Intestine and subpharyngeal *nis* expression decreased in response to increased ambient iodide suggesting that red drum NIS is downregulated when ambient iodide levels increase. If subpharyngeal and intestine *nis* expression is being downregulated by increased ambient iodide levels this further suggests that the regulation of NIS may be conserved between mammals and red drum. Mammalian *nis* expression is inhibited in response to high iodine, a process known as the Wolff-Chaikoff effect (Eng, et al. 1999). Even though we only increased ambient iodide to levels of full strength seawater, the decrease observed in intestinal *nis* expression suggests NIS is regulated within the normal range of iodide levels encountered in red drum natural habitats. This is the first

time euryhaline fish *nis* expression has been measured in response to naturally encountered iodine levels. It suggests that additional research on how iodide uptake mechanisms respond to natural iodine levels will help clarify how euryhaline fish regulate iodide uptake when moving between salinities.

Mammalian studies suggest the gut is the primary location for environmental iodide uptake, however there is considerable evidence for a unique uptake location in fish gills. Early studies in which brook trout goiter was treated by supplementing pond water with iodide suggested that fish can obtain iodide via direct absorption from the environment (Marine and Lenhart 1910). Immersing fish in radioiodide showed iodide enters through the gills and is transported via circulation to iodide-concentrating organs (Baker 1958; Geven et al. 2007; Gregory and Eales 1975; Hunn and Reineke 1964; Hunt and Eales 1979; Tarrant Jr 1971). Reduced iodide uptake following exposure to perchlorate, a known NIS inhibitor, has suggested that NIS is responsible for gill iodide uptake (Hunn and Fromm 1964) and iodide excretion via gills has been observed in rainbow trout (Fromm 1965; Hunn and Fromm 1964) but cellular mechanisms of gill iodide transport have not been investigated. Whereas it has been suggested that NIS is the transport protein responsible for iodide uptake in fish gills (Geven et al. 2007), we were unable to detect *nis* expression using RT/PCR and found very low levels of *nis* expression using qPCR suggesting that the red drum gill has very low *nis* expression. However, this does not preclude an iodide transport mechanism in fish gills. Pendrin and the apical iodide transporter (AIT) are additional iodide transporters found in the apical membrane of thyrocytes (Lacroix, et al. 2004; Rodriguez, et al. 2002). With a 70%

similarity in sequence identity to NIS, AIT is also sensitive to perchlorate (Wright and Turk 2004). Therefore it is possible that red drum gills are utilizing an alternative iodide uptake mechanism besides NIS. To understand how iodide uptake through the gills is achieved we will need to determine if other iodide transport mechanisms are expressed in red drum gills.

It is possible that the low iodine levels observed in our recirculating systems may contribute to low or absent gill *nis* expression. Fish have gill stem cells that can differentiate into freshwater absorptive ionocytes or saltwater excretory ionocytes (Dymowska, et al. 2012; Hwang, et al. 2011; Takei and McCormick 2013) in order to regulate chloride ion transfer. If fish gills use this mechanism for osmoregulatory ions then it may be possible that a similar mechanism of cell differentiation exists for iodide uptake and excretion. Red drum have been reported to physiologically adapt to ion deficient water by gill remodeling and decreasing gill absorptive ion transport mechanisms (Watson et al. 2014). We supplemented iodine deficient water and tested for changes in *nis* expression in order to test if low *nis* expression is a result of low ambient iodine, but gill *nis* expression remained low and stable and only increased very slightly 14 days after supplementation. Unlike the inhibitory effects we saw in subpharyngeal and intestine tissues, we did not see a significant decrease in gill *nis* expression in red drum exposed to iodide supplemented water further suggesting that red drum are not regulating gill *nis* expression in response to changes in ambient iodide. The lack of changes in gill *nis* expression in low ambient iodide or after supplementation suggests it is unlikely gill NIS is regulated by ambient iodide levels and further supports

the contention that an *nis*-mediated transport system in gills is not an important source of ambient iodide for red drum.

Further complicating the ability to understand red drum gill *nis* expression, over the course of two weeks our recirculating system had a 90% decrease in iodide, the biologically important form of iodine, and a 99% decrease in iodate. It has been suggested that in recirculating aquatic systems iodide organified by bacteria could be removed by activated carbon in filters (Dr. Peter Santschi, Texas A&M University at Galveston, personal communication). This may explain why our recirculating systems had an iodide range of <.14-88.4 ug/L compared to published ranges of 45-60 ug/L (Moreda-Piñeiro, et al. 2011). Low ambient iodide levels may force freshwater fish to be more dependent on a dietary source of iodine (Watanabe et al. 1997). Low ambient iodide, lack of gill *nis* response, and robust *nis* expression in the intestine all suggest that red drum may also be more reliant on dietary iodide and intestinal iodide uptake. Since euryhaline fish ingest saltwater to maintain ion balance (Barton 2007), red drum are likely drinking to maintain osmoregulatory balances and therefore intestine may be the primary location of iodide uptake. If red drum are more dependent on dietary iodide instead of ambient iodide then maintaining ambient iodide may not be as important as dietary iodide content. Further understanding how fish *nis* expression responds to changes in ambient and dietary iodide should help to define minimum iodine levels in food required for cultured fish and may help prevent hypothyroidism. Hypothyroidism, as evidenced by simple goiters, has been noted in a number of captive teleost fish species and attributed to either low ambient iodide or environmental goitrogens

(Leatherland 2005). Preventing hypothyroidism is vital for the commercial production of flatfishes (Ribeiro et al. 2011). For example, Atlantic halibut fed cultured *Artemia* are hypothyroid and have incomplete or problematic metamorphoses (Solbakken, et al. 2002). Therefore, understanding how NIS functions in dietary and ambient iodide uptake will not only help us to understand conserved mechanisms of iodide transport in vertebrates, but may also help refine husbandry procedures serving to maintain thyroid function in aquacultured fish.

Aside from understanding the environmental regulation of iodine uptake in fish, NIS has been studied as a mechanism for thyroid health and cancer research. For several decades NIS and radioiodine have been used in the diagnosis and treatment of thyroid cancer (Dohan et al. 2003; Mandell, et al. 1999). Mammalian NIS cloning and characterization has allowed for the development of *in vitro* NIS gene therapy experiments revolved around targeted NIS gene expression in order to create radioiodine uptake within cancer cells (Shimura, et al. 1997). Radioiodide therapy is one of the most promising treatments of cancer in that not only is it effective but it is free of serious adverse effects that are usually associated with cancer treatments (Dohan et al. 2003). Because zebrafish embryos are transparent with rapid, external development, zebrafish have become popular *in vitro* systems for genetics (Dahm and Geisler 2006), toxicology (Hill, et al. 2005) and cancer research (Amatruda, et al. 2002). These embryos have already been used to study the effects of chemicals on thyroid and thyroid cancer development (Bourque and Houvras 2011). Application of powerful molecular genetic techniques to the examination of thyroid function in zebrafish should provide novel

information on the effects of iodine, drugs and other cancer treatments on *nis* expression. A better understanding of the expression and regulation of fish NIS may allow medical researchers to create and implement these *in vitro* methodologies for targeted radioiodide uptake in cancer cells without destroying non-target, healthy tissue.

CHAPTER IV

CONCLUSIONS

Thyroid epithelial cells utilize iodide to synthesize the hormone thyroxine (T_4) which is secreted into circulation, transported to target tissues, translocated to the nucleus and bound to thyroid hormone receptors that regulate fundamental physiological processes including metabolism, endocrine function, reproduction and development (Moreno, et al. 2008; Yen 2001). Sufficient iodine supply is critical for normal endocrine function and iodide deficiency results in an impaired ability to synthesize T_4 (Goldsmith 1949; Leblond 1951; Zoeller, et al. 2007) leading to hypothyroidism and other serious developmental, metabolic, and reproductive disorders (Pearce, et al. 2013). In mammals, iodine uptake from the environment is achieved by the sodium iodide symporter (NIS), synthesized in enterocytes of the mammalian small intestine for dietary iodide absorption and in the stomach for iodide recycling (Carrasco 1993; Kotani, et al. 1998; Nicola, et al. 2009; Portulano, et al. 2014). Because the basic biochemical pathways for thyroid hormone synthesis and receptor activation appear conserved throughout vertebrates (Eales 1979), it has been assumed that fish have mechanisms of iodide uptake similar to humans and yet few studies have identified the NIS gene and characterized the regulation of its activity in fish. Evidence from a single teleost species, zebrafish, confirmed that the NIS gene is present in the thyroid follicles in the lower jaw (Alt, et al. 2006; Porazzi, et al. 2009). However, these studies did not examine NIS expression at extra-thyroidal locations, nor did they investigate how NIS expression might be regulated. I sought to determine if NIS is present and regulated in a manner

similar to mammals in red drum, *Sciaenops ocellatus*, a euryhaline perciform fish easily obtained from hatcheries and used for nutritional and thyroid hormone research (MacKenzie, et al. 2009; Pohlenz and Gatlin III 2014).

To successfully determine if NIS exists in red drum thyroid tissue, I first needed to create a reliable way to locate active thyroid follicles in fish. Fish thyroid glands are typically found in subpharyngeal areas along the ventral aorta and lower branchial arteries, and non-encapsulated thyroid follicles can be found in multiple locations adjacent to the heart or within the head kidney, and kidney itself. I developed a fast, non-lethal methodology that used PET/CT imaging of 124I injected fish to produce three dimensional images showing areas of active radioiodide uptake in the subpharyngeal region of red drum (*Sciaenops ocellatus*) and tilapia (*Tilapia mossambicus*). Not only did this technique produce high resolution three-dimensional images but the imaging software allowed me to histologically verify the location of tissues with active iodide uptake. I was able to use this information to create a methodology to consistently remove active subpharyngeal thyroid tissue for red drum NIS research.

Additionally, this is the first study to use PET/CT to find areas of iodide uptake in fish thus confirming that red drum and tilapia have concentrated regions of active thyroid follicles in the subpharyngeal region, between the second and fourth gill arches. This confirms previous research identifying iodide concentrating thyroid tissue in tilapia subpharyngeal region (Geven, et al. 2007) and refining previous histological research that red drum have discrete clusters of thyroid follicles distributed along the ventral aorta between the third and fifth gill arches (MacKenzie 1988). Furthermore, comparing SUVs

of heterotopic and renal thyroid tissue provides quantifiable data on thyroid tissue activity beyond indirect histological measures of cell height. PET/CT provides a new approach to quantify how thyroid status in fish responds to ecological, chemical and developmental stimuli without laborious and lethal histological methods.

The development of a reliable thyroid tissue collection procedure for red drum allowed me to isolate RNA from red drum subpharyngeal thyroid follicles in order to confirm the presence of *nis* expression in red drum. My work is the first to confirm that NIS exists in red drum thyroid, stomach and intestine and that *nis* expression in red drum subpharyngeal thyroid tissue is regulated by thyroid stimulating hormone (TSH) similar to mammalian thyroid NIS. Combined with previous work on vertebrate thyroid development, confirmation of NIS in red drum tissues reinforces that the basic pathways for thyroid hormone synthesis and receptor activation may be conserved in vertebrates (Eales 1979).

nis expression in red drum subpharyngeal, stomach and intestine tissues suggests that in these fish iodine uptake, thyroid hormone manufacture and iodide recycling are similar to mammals. My results support the hypothesis that red drum utilize NIS to uptake dietary iodide from their diet. Iodide then enters into circulation where it travels to the subpharyngeal thyroid follicles. Here I found that the same NIS concentrates iodide in the basolateral membrane of thyroid epithelial cells for the manufacture of thyroid hormone. In contrast, stomach NIS in red drum is less actively expressed, and if it resembles other vertebrates may more likely play a role in dietary iodide recycling by pumping excess iodide from circulation into the gastric lumen to be reabsorbed by

intestinal NIS. Utilization of intestinal NIS for dietary iodide absorption and stomach NIS for iodide recycling in red drum further would further support conserved iodide uptake mechanisms throughout vertebrates.

nis expression did not change in anticipation of or in response to regularly scheduled feeding suggesting red drum may not regulate NIS based on anticipated meals but may instead maintain constitutive intestinal *nis* expression to facilitate iodide uptake once iodide is ingested. Iodine is supplemented in fish food and yet there is little uniformity on the defined minimum iodine levels for fish diet (Lewbart 1998; Lovell 1979; Watanabe, et al. 1997). While Rangen© commercial red drum diet iodide levels fall within the standard range of 1-5mg/kg (Lewbart 1998; Lovell 1979; Watanabe et al. 1997) iodide levels may not have been high enough to cause any postprandial decrease in *nis* expression. It is assumed that the iodide levels in commercially manufactured fish food are sufficient; however, there is not enough information about fish intestine and stomach NIS to determine if fish are regulating *nis* expression in response to low or high dietary iodide. Future studies of gastrointestinal iodine transport would help to better identify *nis* response to dietary iodine levels and thus and help establish optimal dietary iodine content of aquaculture feeds.

Intestine and subpharyngeal *nis* expression decreased in response to increased ambient iodide suggesting that red drum NIS is downregulated when ambient iodide levels increase. Even though I only increased ambient iodide to levels in full strength seawater, the decrease in *nis* expression suggests NIS is being regulated even within the normal range of iodide levels encountered in red drum natural habitats. Understanding

how NIS functions in dietary and ambient iodide uptake will not only help us to understand conserved mechanisms of iodide transport in vertebrates, but may also help refine husbandry procedures serving to maintain thyroid function in aquacultured fish while also explaining how euryhaline fish regulate iodide uptake when moving from one environment to another.

While there is evidence for a unique uptake location in fish gills, I did not find robust *nis* expression or regulation in red drum gills. The lack of changes in gill *nis* expression in low ambient iodide or after supplementation suggests it is unlikely gill NIS is regulated by ambient iodide levels and calls into question whether gills are an important source of ambient iodide for red drum. Their euryhaline abilities combined with seasonal movements across variable salinities ensure red drum encounter different habitats with variable iodine levels. Low ambient iodide levels force freshwater fish to be more dependent on a dietary source of iodine (Watanabe et al. 1997). Since our red drum are maintained in low iodide and low salinity, my red drum may not utilize gill NIS and instead be dependent on dietary iodide. Branchial iodide transport in eels was not blocked after application of perchlorate, a known NIS blocker (Leloup and Delignieres 1972) suggesting there may be other gill iodide transport mechanisms. Pendrin and the apical iodide transporter (AIT) are iodide transporters in the apical membrane of thyrocytes (Lacroix, et al. 2004; Rodriguez, et al. 2002). Red drum gills might utilize one of these alternative iodide transport mechanisms. Fish gill stem cells differentiate into freshwater absorptive ionocytes or saltwater excretory ionocytes (Dymowska, et al. 2012; Hwang, et al. 2011; Takei and McCormick 2013) in order to

regulate chloride ion transfer. This plasticity allows for changes in salt uptake in response to variable salinity. Therefore it might be possible that similar non-NIS mechanisms exist in red drum. To understand how branchial iodide uptake is achieved we will need to determine if other iodide transport mechanisms are expressed in red drum gills or if iodide uptake mechanism are capable of differentiation in order to maintain circulating iodide levels in response to changes in ambient iodide.

Mammalian NIS and radioiodine have been used in the diagnosis, treatment, and study of thyroid cancer (Dohan, et al. 2003; Mandell, et al. 1999). NIS cloning and characterization has allowed for the development of *in vitro* NIS gene therapy experiments to enhance NIS expression to endow cancer cells with a radioiodine uptake pathway (Shimura, et al. 1997). Zebrafish embryos are transparent with rapid, external development and have become popular *in vitro* systems for genetics (Dahm and Geisler 2006), toxicology (Hill, et al. 2005) and cancer research (Amatruda, et al. 2002). These embryos are already used to study specific cell lineages and the effects of chemicals on thyroid and thyroid cancer development (Bourque and Houvras 2011). Applying *in vitro* NIS gene therapy techniques developed in mice to zebrafish embryos may make it possible to assess the regulatory effects of radioiodine, drugs and other cancer treatments on *nis* expression. Better control over *nis* presence and regulation in zebrafish may allow for more targeted radioiodide treatment of cancer cells without destroying non-target, healthy tissue.

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