DIFFERENTIAL EXPRESSION OF THE SODIUM-IODIDE SYMPORTER IN TWO SPECIES OF TELEOST FISH, RED DRUM (*SCIAENOPS OCELLATUS*) AND ZEBRAFISH (*DANIO RERIO*)

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ABSTRACT

Differential expression of the sodium-iodide symporter in two species of teleost fish, red drum (*Sciaenops ocellatus*) and zebrafish (*Danio rerio*)

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In all vertebrates thyroid hormones are essential for normal growth, metabolism, and development. Thyroid hormones are unusual in that they require iodine to function. While the pathway of iodide uptake in terrestrial vertebrates has been established to be strictly dietary, and is mediated by the same sodium-iodide symporter (NIS) protein that concentrates iodine in the thyroid, the method of iodine uptake in fish remains controversial. In this study, I attempted to contrast the expression of a potential orthologue of NIS in two species of teleost fish, the red drum (*Sciaenops ocellatus*), a marine species living in an iodide rich environment, and the zebrafish (*Danio rerio*), a freshwater species living in an iodide poor environment. I applied RT-PCR to the digestive tract, the subpharyngeal region, and the gill, to determine whether fish in low iodine environments may possess a more active branchial iodide uptake pathway. I found that red drum held at higher salinities lack significant expression of *nis* in the gill but did not successfully identify the expression pattern of *nis* in zebrafish. Further research is needed to accomplish this goal.

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CHAPTER I

INTRODUCTION

Iodine is essential for the synthesis of thyroid hormones. Iodine is transported into thyroid follicle cells by the sodium-iodide symporter (NIS), a protein which transports one iodide anion and two sodium cations down a sodium concentration gradient. The transported iodide is then used in the synthesis of the primary circulating thyroid hormone thyroxine (T_4) which is released into the blood, where it is transported throughout the body. T_4 diffuses across target cell membranes where it is monodeiodinated by deiodinases. This product, 3-5-3'-triiodothyronine (T_3), binds to receptors inside the nucleus, where it regulates numerous physiological processes, including growth, metabolism, and development (Hadley and Levine, 2007). T_3 is further metabolized by progressively removing the remaining iodines from the hormone. The removed iodine quickly returns to its ionic state, and most of it is taken back up by the thyroid (Dohán et al., 2003; Hadley and Levine, 2007). The iodide that is not recycled is excreted and in terrestrial vertebrates must be replaced via dietary uptake. The same *nis* expressed in mammalian thyroid has been reported in the gut and other secretory tissues, indicating that this protein is used for both thyroidal and extrathyroidal iodine transport. (Carr et al., 2015; Nicola et al., 2009; Perron et al., 2001; Spitzweg et al., 2001; Spitzweg et al., 1998). Therefore, as the protein both enabling iodine uptake from the environment and iodine concentration in the thyroid, NIS is essential for normal thyroid function.

Like terrestrial vertebrates, fish are dependent on external sources of iodine for thyroid hormone synthesis (Eales, 1979). Unlike terrestrial vertebrates, however, the mechanism of extrathyroidal iodine transport in fish is not well established. While diet is undoubtedly a source of iodine for fish (Davis and Gatlin, 1996), a study nearly fifty years ago (Hunn and Fromm, 1966) suggested that rainbow trout (*Oncorhynchus mykiss*) and possibly other teleost fish take up iodine from their environment through their gills or opercular membrane, and therefore are not dependent on dietary iodine like terrestrial vertebrates. However, this study was never replicated nor the hypothesis the authors formed rigorously tested. Despite this it has become accepted scientific dogma that fish take up iodine through a branchial pathway (Eales, 1979; Norris and Carr, 2013). Recent studies in our lab (Butler, 2013) suggest there may be low *nis* expression in the gills of a marine teleost fish, the red drum (*Sciaenops ocellatus*), but none at all in another buccal transport tissue, the opercular membrane, whereas there is significant expression in the thyroid and throughout the entire intestine. Subsequent experiments have failed to fully support branchial *nis* expression (Allison Wilkes, Biology TAMU, unpublished results). As a marine species, however, the red drum is adapted to an environment high in iodine (Moreda-Piñeiro et al., 2011), and may obtain sufficient iodine through their digestive tract, particularly since they constantly drink seawater to counter water loss to their environment (Evans et al., 2005). As the gill is both a respiratory and osmoregulatory organ, branchial chloride cells in saltwater fish pump ions out of the organism. By contrast, freshwater fish inhabit an environment low in iodine and other salts, so their chloride cells pump ions into the organism (Dymowska et al., 2012; Hadley and Levine, 2007; Randall et al., 2002; Zadunaisky, 1996). Previous studies on gill iodine transport were conducted in freshwater fish, where branchial iodine transport may be more prominent if

nis resembles other ion transporters. Therefore, I hypothesized that if *nis* is present in the gill, it is more likely to be evident in freshwater fish.

Today, we have techniques available that the authors of the 1966 study did not. Existence of a protein in a tissue can be inferred, though not conclusively proven, by measuring the expression of its corresponding mRNA in the tissue. We have identified a potential ortholog for *nis* in two species of teleost fish, the red drum (*Sciaenops ocellatus*), a marine fish (Butler and Wilkes, unpublished results), and zebrafish (*Danio rerio*), a freshwater species (NCBI accession number: NM_001089391.1). The red drum has been used in our lab for over twenty years for detailed studies of thyroid function. As such, we have developed a variety of molecular tools to use to study thyroid-related gene expression in this species. In addition to being easy to obtain, they are an important game fish along the Texas coast, and their ability to survive in various salinities makes them useful for iodine research. The zebrafish has become a common model organism for developmental work in recent years. As a freshwater fish, it cannot survive large variations in salinity, but its genome has been sequenced and a region of active thyroid follicles has been described (Alt et al., 2006; Patiño et al., 2003). The overall goal of my project is to contrast the locations of iodine uptake between these organisms to determine how low-iodine environments may impact the extrathyroidal expression of *nis*. In addition to answering the fundamental fish physiology question of how iodine accumulation is achieved, my project will help aquaculturists to better understand how environmental iodine may serve to maintain normal thyroid function in their animals. Therefore, I used *S. ocellatus* as a representative saltwater fish and *D. rerio* as a representative freshwater fish. I applied molecular techniques to these organisms to examine how expression of *nis* varies between aquatic environments of different salinities.

In order to accomplish this, I first developed techniques to qualify the expression of *nis* in both zebrafish and red drum. The MacKenzie lab already has developed techniques for amplification of *nis* from red drum (Butler, 2013), but not for zebrafish. PCR primers for zebrafish *nis* were taken from the literature (Alt et al., 2006). Once I determine the efficacy of these primers, I will be able to compare the expression of *nis* in the gill, lower jaw, gut, and muscle of these organisms, by qualitative PCR.

CHAPTER II

METHODS

Experimental animals

Red drum

Red drum were obtained from the Texas Parks and Wildlife Department Sea Center hatchery at Lake Jackson, Texas. They were kept in a recirculating system of artificial seawater with salinity between 12 and 30 ppt, with a temperature of 25°C, and a 12L:12D photoperiod. Reverse osmosis water was supplemented with Morton Salt (Morton Salt, Chicago, IL) and Fritz Super Salt Concentrate (Fritz Aquatics, Mesquite, TX). They were fed commercial diet (Rangen, Angleton, TX) once daily shortly after lights on.

Zebrafish

Zebrafish were obtained from Texas A&M University BSBE surplus breeding stock. They were kept in recirculating tanks of reverse osmosis water supplemented with 0.5% Instant Ocean (Instant Ocean, Blacksburg, VA), at a temperature of 27°C, on a 14L:10D photoperiod. They were fed commercial diet (Wardley, Secaucus, NJ) twice daily, supplemented with brine shrimp once daily. These fish were provided under a tissue-sharing program after euthanasia following previous use in genetic studies.

Tissue collection

Red drum

Previous work has shown that active thyroid follicles are located in the vascularized tissue in the subpharyngeal region between the 2nd and 4th gill arches in red drum (Wilkes and Browning, unpublished results). The entire lower jaw, including the gill arches, was removed in order to access this area. Gill arches and surrounding musculature were removed, leaving only thyroid follicle-enriched tissue. Gill tissue was collected by removing the thin gill filaments from their arches. The stomach, pyloric caeca, and intestine were collected. A small amount of muscle was collected as a negative control. All samples were placed in 1 mL RNALater (Ambion) and stored at -80°C until processing.

Zebrafish

Similar to red drum, active thyroid follicles in zebrafish are concentrated in the subpharyngeal region, between the 1st and 3rd gill arches (Alt et al., 2006). Due to the small size of the animal, the cranium was first removed from the head before the gill arches were removed. The head was then removed just posterior to the main body cavity, as certain teleost fish have active follicles anterior to the head kidney (Eales, 1979). The entire digestive tract was collected from zebrafish, as they lack pyloric caeca and the stomach and intestine are not clearly delineated as they are in red drum. Muscle was additionally collected as a negative control. Due to initial low yields of RNA and low A260/A280 ratios, four fish were pooled into each sample, with a total of three separate pooled samples per tissue type. All samples were placed in 1 mL RNALater (Ambion) and stored at -80°C until processing.

RNA isolation and quantification

Tissues were thawed until the RNA Later was completely liquid so samples could be gently shaken to remove excess RNA Later. They were then placed in a pre-cooled mortar and pestle and homogenized under liquid nitrogen. This homogenate was then treated with 15 µL of Proteinase K in a 600 µL solution containing 300 µL of DNA/RNA Shield (Zymo Research) and 300 µL of 2x Digestion Buffer (Zymo Research) to denature as many of the proteins in the sample as possible. Half (300 µL) of this treated homogenate was then added to 300 µL of RNA Lysis Buffer (Zymo Research). RNA extraction was completed via the ZR RNA MiniPrep Kit (Zymo Research) based on the manufacturer's protocols. Of the homogenate in buffer, 400 µL was first centrifuged in a ZymoSpin IIIC column at 8,000*g* for 30 seconds to extract any polypeptides, using a Microfuge 18 Centrifuge (Beckman Coulter). 320 µL of ethanol was added to the flow-through, and this solution was centrifuged at maximum speed (18,000 rcf) in a ZymoSpin IIC column. Flow-through was discarded and the column was washed by adding 300 µL of RNA wash buffer and centrifuging at max speed for 30 seconds. In-column DNAse digestion was accomplished using a cocktail of 5 µL 2U/µL DNAse I, 80 µL RNA Wash Buffer, and 5 µL Reaction Buffer, which was added to the column and incubated at 37°C for 15 minutes. Flow-through was discarded and 400 µL RNA Prep Buffer was spun through the column, and two additional washes of 700 µL and 300 µL RNA Wash Buffer were performed. The column was then spun for an additional 2 minutes to remove any excess buffer. Finally, 20 µL of DEPC-treated autoclaved water were added to the column to extract the RNA. RNA was stored at -80°C until quantification, which was achieved using a NanoDrop (Thermo Scientific, Asheville, NC). For RNA to be considered pure, a sample needed an A260/A280 ratio between 1.8 and 2.2. Autoclaved DEPC-treated water was used as a blank.

Reverse transcription

The extracted RNA was used as a template to make complementary DNA (cDNA). The High-Capacity cDNA Reverse Transcription Kit (Invitrogen, NY, USA) was used according to the manufacturer's protocol, with the following modifications: 5 µL 10x RT buffer, 2 µL dNTP mix, 8 µL 10x random primers, 2.5 µL oligo dT, a volume equivalent to 2 µg of RNA, and was brought up to a total of 47.5 µL with autoclaved double-distilled water (ddH2O) before 2.5 µL of reverse transcriptase was added for a total of 50 µL. The reaction was incubated in a thermocycler at 25°C for 10 minutes, 37°C for 2 hours, and finally the enzyme was denatured by incubating at 95°C for 5 minutes. The samples were cooled to 4°C and held for up to one hour before being placed in a -20°C freezer.

PCR

Red drum

5 µLs of cDNA from the RT reaction was added to 10 µLs of GoTaq Green (Promega, WI, USA), 2 µL of primer and 3 µLs of autoclaved double-distilled water. No template controls used 5 µLs of ddH2O instead of cDNA. The forward *nis* primer was 5'-GCT GTG ATC TGG ACT GAT GTG TTC CA-3' and the reverse *nis* primer was 5'-TCC CGC TGT ATG CAC AGG CAA GAA-3' (Butler, 2013). Thermocycler conditions were as follows: a hot start of 95°C for 2 minutes, 32 cycles with 30 seconds at 95°C, 30 seconds at 58°C, and one minute at 72°C, and finally 5 minutes at 72°C before being cooled to 4°C and held for up to one hour or until transferred to a -20°C freezer.

Zebrafish

5 µLs of cDNA from the RT reaction was added to 10 µLs of GoTaq Green (Promega, WI, USA), 2 µL of primer and 3 µLs of autoclaved double-distilled water. No template controls used 5 µLs of ddH2O instead of cDNA. The forward *nis* primer was designed as 5'-TGA CAG ACC ACC GGG CTT CTG-3' and the reverse primer was designed as 5'-TTA CAG CCT TCA TGC CAC CCA-3'. Thermocycler conditions were as follows: a hot start of 95°C for 2 minutes, 32 cycles with 30 seconds at 95°C, 30 seconds at 55°C, and one minute at 72°C, and finally 5 minutes at 72°C before being cooled to 4°C and held for up to one hour or until transferred to a -20°C freezer.

DNA gel electrophoresis

The 1% agarose gel was made by melting .5g of agarose in 49 mL of ddH2O. Once the solution was cooled to about 60°C, 1 mL of 50x TAE and 2 µL of ethidium bromide were added and swirled in under a fume hood. This solution was poured into a taped 8cm x 10cm gel tray containing a 10-well comb and left at room temperature until fully solidified, about 30 minutes. While the gel solidified, 10 mL of 50x TAE was added to 490 mL ddH2O and mixed by inversion to make 500 mL of running buffer. Once the gel solidified, the tape and comb were removed and the gel tray was placed in the gel box. The running buffer was poured over the gel. 10 µL of PCR product was added directly to the wells, as the GoTaq Green contains a thermally stable loading buffer and dye. A DNA ladder was made by mixing 1 µL of 100bp ladder (New England BioLabs, MA, USA) with 2 µL of 6x DNA loading buffer (New England BioLabs, MA, USA) and 9 µL of autoclaved ddH2O. The gels were run at 103 V for up to 90 minutes and were visualized under UV light.

CHAPTER III

RESULTS

RNA yield

Red drum

RNA yield from red drum tissue samples is shown in Table 1. A 260nm/280nm ratio of 2.0 was chosen as ideal for RNA samples, but any ratio between 1.8 and 2.2 was considered acceptable. The highest yields were obtained from the intestine and pyloric caecum, with the muscle and stomach having the lowest yields.

Table 1. RNA Extraction Results for Red Drum

Sample	RNA (ng/µL)	A260/A280
Muscle 1	705.4	2.08
Muscle 2	333.3	2.00
Muscle 3	383.3	2.03
Muscle 4	621.9	2.11
Gill 1	1113.1	2.08
Gill 2	903.1	2.09
Gill 3	786.9	2.09
Gill 4	782.7	2.10
Subpharyngeal Region 1	789.0	2.10
Subpharyngeal Region 2	766.4	2.09
Subpharyngeal Region 3	833.7	2.09
Subpharyngeal Region 4	869.1	2.10
Stomach 1	637.2	2.08
Stomach 2	767.5	2.06
Stomach 3	182.6	2.02
Stomach 4	850.6	2.00
Intestine 1	1325.1	2.10
Intestine 2	729.9	2.12
Intestine 3	604.6	2.08
Intestine 4	1901.0	2.06

Table 1. RNA Extraction Results for Red Drum (continued)

Sample	RNA (ng/µL)	A260/A280
Pyloric Caecum 1	1924.8	2.06
Pyloric Caecum 2	1447.9	2.07
Pyloric Caecum 3	916.8	2.09
Pyloric Caecum 4	1210.9	2.10

Zebrafish

RNA yield from zebrafish tissue samples is shown in Figure 2. A 260nm/280nm ratio of 2.0 was chosen as ideal for RNA samples, but any ratio between 1.8 and 2.2 was accepted. The samples outside of this range were considered contaminated. The yields of these samples were significantly lower than those from red drum, likely due to the significantly smaller amounts of tissue from zebrafish. The ratios were much lower too.

Table 2. RNA Extraction Results for Zebrafish

Sample	RNA (ng/µL)	A260/A280
Muscle 1	53.1	1.59
Muscle 2	47.1	1.72
Muscle 3	132.6	1.51
Digestive Tract 1	211.6	1.71
Digestive Tract 2	131	1.67
Digestive Tract 3	229.2	1.69
Gill 1	79.7	1.57
Gill 2	194.9	1.62
Gill 3	209.2	1.47
Subpharyngeal Region 1	265.8	1.6
Subpharyngeal Region 2	197.8	1.73
Subpharyngeal Region 3	199.7	1.62

DNA Gels

Red drum

The DNA gels are presented below (Figure 1). Expression of putative *nis* was not found in the muscle or the no-template control. *Nis* was strongly expressed in the subpharyngeal region in all animals. It was also weakly expressed in the stomach of all animals. Strong *nis* expression was found in the intestine of two fish, with weak expression in the pyloric caecum of three animals. *Nis* was not expressed in the gill. The reference gene actin was expressed in all of these samples except for one muscle, one thyroid, and one stomach sample. (Figure 2). *Nis* was previously sequenced by Butler (2013).

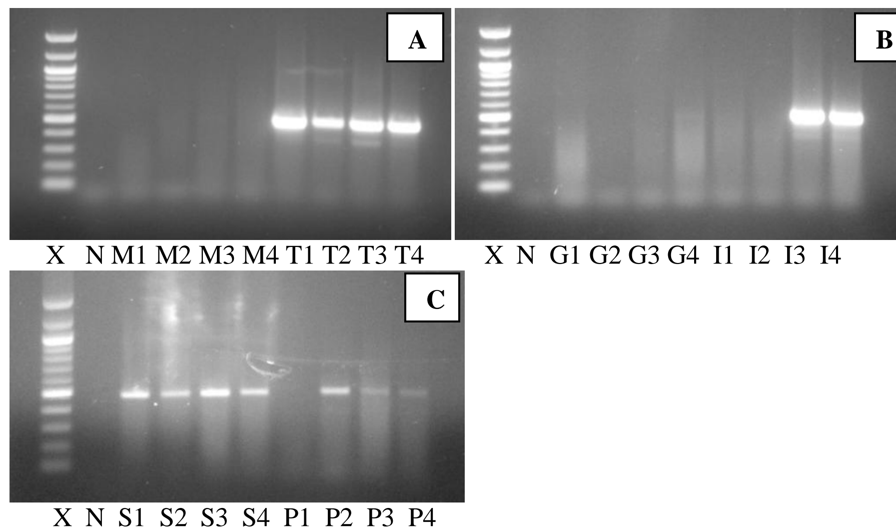

Figure 1. Red Drum *nis* DNA Gels Panel A: Muscle and subpharyngeal region. Panel B: gill and intestine. Panel C: stomach and pyloric caecum. Legend: X, ladder; N, no template; M, muscle; T, subpharyngeal region; G, gill; I, intestine; S, stomach; P, pyloric caecum. Numbers 1-4 denote which organism the tissue sample came from.

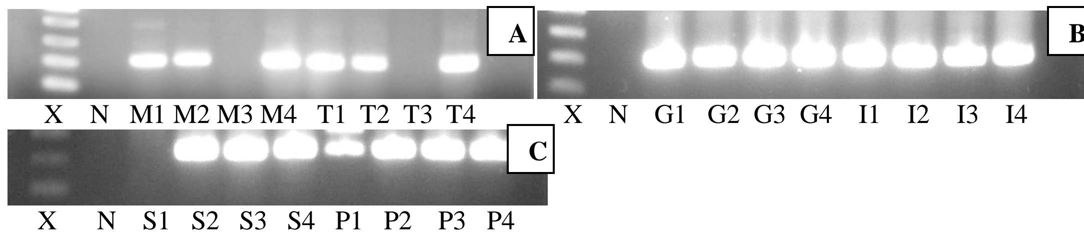

Figure 2. Red Drum actin DNA Gels Panel A: Muscle and subpharyngeal region. Panel B: gill and intestine. Panel C: stomach and pyloric caecum. Legend: X, ladder; N, no template; M, muscle; T, subpharyngeal region; G, gill; I, intestine; S, stomach; P, pyloric caecum. Numbers 1-4 denote which organism the tissue sample came from.

Zebrafish

The DNA gels are presented below (Figure 3). Between 5 and 7 bands were observed in the subpharyngeal region and intestine, with no bands in the gill or muscle. Actin controls were positive for all samples (Figure 4).

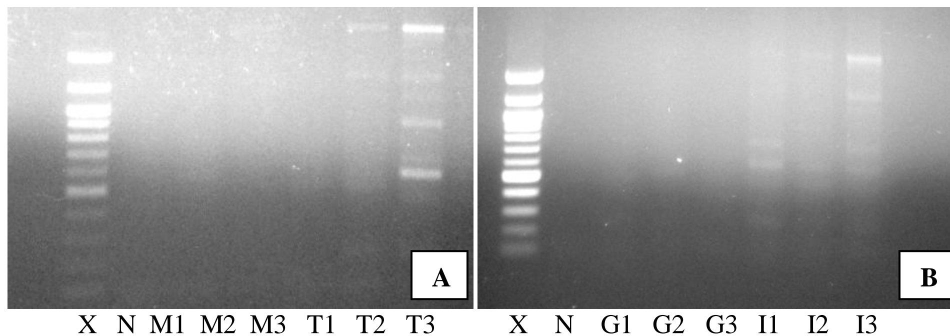

Figure 3. Zebrafish *nis* DNA Gels Panel A: Muscle and subpharyngeal region. Panel B: gill and digestive tract. Legend: X, ladder; N, no template; M, muscle; T, subpharyngeal region; G, gill; I, digestive tract. Numbers 1-3 denote which pool the tissue sample came from.

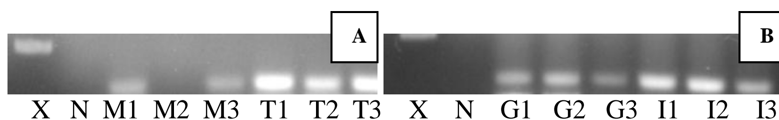

Figure 4. Zebrafish actin DNA Gels Panel A: Muscle and subpharyngeal region. Panel B: gill and digestive tract. Legend: X, ladder; N, no template; M, muscle; T, subpharyngeal region; G, gill; I, digestive tract. Numbers 1-3 denote which pool the tissue sample came from.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

My objective for red drum was to characterize the expression of *nis* at a higher, more natural salinity than used by Butler (2013) to determine if *nis* is normally expressed branchially in red drum. Overall, my results were similar to those obtained by Butler (2013), with the important exception of the gill. I confirmed that *nis* is present in the digestive tract and subpharyngeal region under different conditions. I found some variation between animals in the expression of *nis,* most clearly in the intestine. All of these samples showed expression of actin. Therefore, it is possible that some as yet undetermined physiological mechanism, such as nutritional alteration in *nis* expression (Eng et al., 1999; Eng et al., 2001) may be acting to cause variability in intestinal *nis*, but further experiments are needed before a clear hypothesis can be formed. Like the intestine, all of my gill samples showed expression of actin. However, unlike the intestine, there was no variability in the expression of *nis* in the gill: *nis* expression was uniformly absent across all gill samples.

There are several possible physiological explanations for this lack of gill nis expression. The most obvious is that iodide uptake through the digestive tract is sufficient at the salinity at which these animals were kept. Marine teleost fish, such as the red drum, osmoregulate by drinking saltwater, which contains iodide (Evans et al., 2005; Moreda-Piñeiro et al., 2011). It is therefore possible that the branchial pathway, contrary to established scientific dogma (Eales, 1979; Hunn and Fromm, 1966) is only active when iodide is deficient from the diet, as may occur in a low salinity environment such as that used by Butler (2013). It is also possible that *nis* is not the

primary protein responsible for branchial iodide transport, as is the case in several non-chordates that concentrate iodine for thyroid function (Heyland et al., 2006; Miller and Heyland, 2013). If this is the case, such a protein would represent a novel form of iodide transport in a vertebrate and would disprove the hypothesis that NIS is solely responsible for iodide transport in all vertebrates (Carr et al., 2015; Norris and Carr, 2013).

As in the red drum, my objective was to describe the expression of *nis* in zebrafish. I hoped to compare the expression of *nis* in the digestive tract and gill in zebrafish to that of red drum to determine if there is a difference in the expression of *nis*, and therefore potentially different iodide uptake, between freshwater and marine teleost fishes. However, I was not successful at this because my PCR reactions failed to produce a single band for zebrafish, meaning that no information could be determined from these reactions other than to say that PCR amplification is possible in these samples and needs to be optimized to produce useful data.

There are several possible reasons my reactions did not work. The most likely cause is that the primers were not run at the appropriate annealing temperature, leading to non-specific binding. Low A260/A280 ratios were a persistent issue with the zebrafish samples, and I believe that this is primarily an artifact of the method of extraction failing to remove contaminants more problematic in zebrafish such as pigments or residual RNALater, which was more difficult to remove from the zebrafish samples than the red drum samples because the zebrafish samples were smaller and in multiple parts. Also, it is possible that the proteinase digestion step is leading to amino acid contamination, lowering my ratios and adversely affecting my reactions, again due to the smaller zebrafish tissue samples.

In the future, I would like to optimize my primers to resolve the issue with multiple bands. Finding the appropriate annealing temperature should result in a single band, allowing me to sequence the band and determine if it is *nis*. If the band is not *nis*, then I will design new primers and optimize them. I would also like to resume using Trizol extractions for zebrafish, as Trizol is an older and more tested method of extracting RNA from a wide variety of tissue types.

Despite my difficulties with the zebrafish samples, I believe the comparison between red drum and zebrafish is worth pursuing further. Previous work in iodide transport was done in a different freshwater fish, the rainbow trout (Hunn and Fromm, 1966), but this work has been extended to apply to all marine and freshwater fish (Eales, 1979; Norris and Carr, 2013). There are numerous genetic techniques available in zebrafish, such as labeling NIS with a fluorescent protein, which would provide a clear visual of where *nis* is expressed, that cannot be as easily applied to the rainbow trout. While these were outside the scope of my study, they could be applied to iodide transport in zebrafish at a later date. Although like the rainbow trout, many of the genetic techniques available in zebrafish are difficult to apply to red drum, it remains one of the few salt-tolerant fish in which iodide transport has been characterized (Butler, 2013, Wilkes, unpublished results) and as such is a good organism for variable salinity iodide transport studies. We can learn about how salinity affects the locations of iodide transport by comparing data from these two species, and should be able to determine if branchial expression of the sodium iodide symporter is up-regulated in low salinities like other branchial ion transporters (Dymowska et al., 2012; Hadley and Levine, 2007; Randall et al., 2002; Zadunaisky, 1996).

Ultimately, I can only draw conclusions about the red drum, but not about the zebrafish or differences between the two species. My results suggest that in red drum, branchial expression of *nis* is only present under low salinities, while *nis* is expressed in the subpharyngeal region and throughout the digestive tract but not in the muscle. My first goal moving forward is to successfully produce a single band through PCR in zebrafish that can then be sequenced and identified as *nis* so the comparison I have proposed in this paper can come to fruition.

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