THYROID HORMONE REGULATION OF DEIODINASE IN RED DRUM (*SCIAENOPS OCELLATUS*)

A Senior Scholars Thesis

by

LAURA ANN RON

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2011

Major: Biology

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Approved by:

Research Advisor:	Duncan MacKenzie
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ABSTRACT

Thyroid Hormone Regulation of Deiodinase in Red Drum (*Sciaenops ocellatus*).
(April 2011)

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The thyroid gland is known to be important in the regulation of metabolism, growth, and reproduction in both mammalian and non-mammalian species. However, little information is available how thyroid hormones act on target tissues in non-mammalian species such as fish. Fish are difficult to study because we do not currently have an effective, non-invasive method for administering thyroid hormone to them or for studying the effects of altered thyroid hormone levels on their tissues. As a result, research done on fish often utilizes pharmacological dosages of stressfully-administered thyroid hormone, causing experimental artifacts. In my study, I developed a thyroid hormone immersion system to generate physiological increases of the thyroid hormone thyroxine (T_4) in the red drum (*Sciaenops ocellatus*), a commercially important fish species. Immersion experiments were performed, where red drum were kept in 20-gallon glass tanks – with either T_4-treated or control solution-treated tank water, and the system recirculating water flow turned off – for the course of the set immersion period.

Immersion in T_4-treated tank water for 40 hours induced an increase in circulating T_4 from 8.3 ng/mL (in control fish) to 28.7 ng/mL (in T_4-treated fish), within the physiological range. This physiological elevation of T_4 did not induce significant changes in liver IRD gene expression, considered to be a marker of pharmacological elevation of thyroid hormones. These results showed that thyroid hormone immersion should be an effective, non-invasive method for administration of physiological levels of thyroid hormone to red drum.

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CHAPTER 1

INTRODUCTION

In both mammalian and non-mammalian vertebrates, the thyroid gland is an endocrine gland that synthesizes and secretes the hormone thyroxine (T_4) into the animal's bloodstream (Norris *et al.* 2007). Thyroxine is carried by the blood to all tissues of the body. When T_4, the "pro-hormone" form of thyroid hormone, reaches a target cell, it is transported inside the cell and converted to triiodothyronine (T_3), the active form of thyroid hormone, by an enzyme called outer-ring deiodinase (ORD). T_3 can remain in the target cell where it binds to a receptor and stimulates the expression of a variety of genes, promoting growth, metabolism and reproduction (Lema *et al.* 2009). T_3 also can return to the circulation, where it travels through to other body tissues.

If, however, there is an excess of T_4 in the circulation, T_4 can be converted to reverse-T_3, the inactive form of thyroid hormone, by an enzyme called inner-ring deiodinase (IRD). Reverse-T_3 is incapable of binding to the thyroid hormone receptor, and therefore, cannot simulate expression or effects in the target tissues.

This thesis follows the format of *General and Comparative Endocrinology*.

This alternative pathway for T_4 allows target cells to protect themselves from overstimulation by excessive, pharmacological levels of T_4 (Eales & Brown, 1993).

Effects of thyroid hormone on growth, metabolism, and reproduction have been found in both mammalian and non-mammalian vertebrates, but our understanding of the mechanism of thyroid hormone action comes primarily from mammals. Although modern bony fish (belonging to the order Teleosteii) are commonly used for thyroid research because of their importance in commercial aquaculture and their significance as the most abundant of all aquatic vertebrates, little information exists on the actions of thyroid hormone in fish target cells. This is because several unique characteristics of the fish thyroid have made it difficult to study. For example, surgical removal of the thyroid (thyroidectomy) was traditionally used to identify thyroid hormone actions. Teleost fish cannot be successfully thyroidectomized because their thyroid is not a single, encapsulated organ, but instead is broadly distributed throughout their lower jaw (Norris *et al.* 2007). It is also challenging in fish to administer thyroid hormone, the most common method of triggering thyroid responses in a laboratory setting. Thyroid hormone is typically administered to laboratory animals via injection, but this is invasive, possibly stressing the fish and altering hormone results. Although incorporation of thyroid hormones in feed has been used successfully in the past to activate thyroid hormone responses in fish, this technique is applicable only to T_3, not the circulating hormone T_4 (Moon *et al.* 1994). Furthermore, because of variable food

consumption by fish, adding hormone to food cannot ensure consistent administration of hormone among fish in a tank, or even to one fish over time. If we are to better understand the mechanism of thyroid hormone action in commercially-important fish species, we need a more effective, non-invasive method of administering controlled thyroid hormone doses, yielding predictable circulating hormone levels that remain within physiological ranges. In my study, I have developed a technique which allows administration of physiological levels of thyroid hormone to fish accurately and non-invasively, in a form appropriate for studies of thyroid hormone action. This system is suitable for evaluating the relationship between increased T_4 blood levels and thyroid hormone-induced gene expression in target tissues. The immersion system of thyroid hormone administration is useful because it allows hormone to be distributed in the fish's environment, causing minimal disruption to the fish themselves. A study using this technique, performed by a previous graduate student in our laboratory, confirmed that T_3 immersion can alter circulatory levels physiologically, but that study did not characterize any target tissue responses to thyroid hormone manipulation to demonstrate that hormone administered in this manner is truly physiologically active (Leiner & MacKenzie, 2003).

In my experiment, I attempted to prove that T_4 added to fish tank water will be able to raise circulating hormone levels within physiological ranges. To quantify this increase in hormone levels, I measured the expression of a known thyroid hormone

target gene. The target gene I evaluated codes for the IRD enzyme, previously established as a sensitive target for thyroid hormone regulation (Gereben *et al*. 2008). If the thyroid hormone immersion technique results in physiological increases of biologically active thyroid hormone in the circulation, it should be capable of increasing levels of circulating T_4 without increasing the expression of the IRD enzyme gene, since it would only be necessary for the target cell to increase expression of this enzyme if circulating T_4 was present in extreme, pharmacological levels. Therefore, success in this experiment would lead to the conclusion that thyroid hormone immersion is an effective method for producing physiological increases of blood T_4 in a laboratory setting and that blood T_4 levels within physiological ranges do not stimulate thyroid hormone inactivation in fish target tissues.

The model species I used for this experiment is the red drum (*Sciaenops ocellatus*), a common aquaculture and research fish species. Red drum have been used in our laboratory for studies of thyroid function for over 20 years, providing extensive reference data on physiological thyroid hormone levels.

CHAPTER II

MATERIALS AND METHODS

T_4 time-course immersion experiment, water only

Prior to obtaining fish, an experiment was performed by adding T_4 solution to our aquaria to determine the stability of T_4 in tank water over time. The aquaria, water pipe system, and hormone solutions used for this experiment were the same as those used for the actual T_4 immersion experiments; the only difference was the absence of red drum in the tanks. The experimental aquaria consisted of 20-gallon glass tanks connected to a common recirculating water system. Thirty minutes before hormone was added to the water system, 1.0mL water samples were taken in labeled 1.5mL microcentrifuge tubes from all aquaria and from two other locations in the recirculating system (the raceway and biofilter). At the start of the experiment, the circulating water flow was turned off and hormone solutions were added to each of the tanks: 3mL of 0.1N NaOH to each of four control tanks and 3mL of T_4 solution (30mg T_4 free acid in 15mL of 0.1 NaOH) to each of four T_4 tanks. The circulating water flow remained off in these static tanks for four hours. Water samples were taken from all eight tanks, the raceway and the biofilter at 30 minutes, 2 hours and 4 hours after addition of the hormone solutions. After the 4-hour water samples were taken, the circulating water flow was turned back on, and water samples (from all eight tanks, as well as the raceway and biofilter) were taken 10 minutes, 2 hours and 4 hours after the flow was restored. All water

samples were analyzed with a T_4-specific radioimmunoassay (Coat-a-Count, Siemens, Inc.) at the Endocrine Diagnostic Laboratory of the Texas Veterinary Medical Diagnostics Laboratory.

Experimental animals

We obtained juvenile red drum (*Sciaenops ocellatus*) from the Texas Parks and Wildlife Stock Enhancement Program at Sea Center Texas, in Freeport, TX. The red drum were housed in a 1600 L system of recirculating 25°C artificial sea water (4.0 ppm salinity), with a 12 hour light/12 hour dark photoperiod. They were fed a commercial diet until they grew to an average of 20 grams (range of 15-56 grams).

T_4 time-course immersion experiments on red drum

Two thyroid hormone immersion experiments were performed, on two different dates, to obtain all of the desired experimental time points. The first experiment involved a 4-hour immersion period (zero-hour to 4-hour) and yielded 4-hour (immediately after conclusion of immersion) and 18-hour (14 hours after conclusion of immersion) time points. The second experiment involved a 40-hour immersion (zero-hour to 40-hour) and yielded 22-hour (during immersion) and 40-hour (immediately after conclusion of immersion) time points. Although the experiments were performed on different dates, the same procedures and equipment were used for each. Red drum were randomly assigned to 20-gallon glass tanks on a common circulating water system. (The number of fish per tank ranged from 7-

13.) Each tank had the outside faces of the glass painted black to minimize visual environmental stress on the fish and was supplied with an air stone. At the start the experiment, the circulating water flow in the glass tanks was turned off and hormone solutions were added to the tank water: 3mL of vehicle solution (0.1N NaOH) in each control tank and 3mL T_4 solution (2mg T_4 per 1mL 0.1N NaOH) in each T_4 tank. The circulating water flow remained off for the duration of the experiment. Hormone concentrations were based on preliminary studies in our laboratory (Richard Jones, personal communication) which established that 100ng T_4 per 1.0mL of tank water was effective at producing an increase in red drum blood T_4 levels within a physiological range, and my earlier experiment with T_4 in water alone which illustrated that approximately 50% of added T_4 was lost from static tank water over a 4-hour period. Therefore, the target concentration for diluted T_4 in tank water was 50ng/mL at the end of immersion. At specific time points (4 hours, 18 hours, 22 hours, 40 hours), 5 fish from one control tank and 5 fish from one T_4 tank were collected for liver sampling. The fish were anesthetized in tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, diluted in tank water to 200mg/L) before having their spinal cords severed near the base of the skull. From each fish, a 0.5-1.0cm tissue sample from the right lobe of the liver was taken and placed in a 1.5mL microcentrifuge tube containing 1.0mL of the RNA extraction reagent Triazol (Invitrogen). The samples were stored at -80°C from time of sampling until immediately before RNA extraction.

RNA extraction

To extract RNA, liver samples were first quickly defrosted by placing the sample tubes (straight from the -80°C freezer) into a 50°C water bath, just until the Triazol melted to liquid (approximately 1-2 minutes). The liver tissue in each tube was homogenized by repeatedly drawing up into a 3cc syringe attached to a 18Gx1-1/2'' needle and then ejecting back into the original tube. Once homogenized, the sample tubes incubated at room temperature (15-30°C) for 5 minutes, allowing the nucleoprotein complexes to dissociate. After 5 minutes, 0.2mL of chloroform was added to each tube (under a fume hood) and each tube was shaken vigorously by hand for 15 seconds. The tubes then incubated at room temperature again for 2-3 minutes before being centrifuged (14000rpm) for 15 minutes. This separated the contents of the sample tube into an upper aqueous phase (containing RNA) and a lower phenol-chloroform phase (containing DNA and proteins). A pipettor (P1000 with RNase-free tip) was used to remove the upper aqueous phase and transfer it to a sterile, labeled 1.5-mL microcentrifuge tube. Isopropanol (0.5mL) was added to each tube before incubation at room temperature for 10 minutes, then centrifugation (14000rpm) for 10 minutes. This step caused the RNA to precipitate into a compact pellet at the base of the sample tube, which was visible upon removal of the sample tubes from the centrifuge. Taking care not to dislodge this pellet, the supernatant was decanted from each tube into a glass waste container (under a fume hood). However, because the RNA pellet was derived from liver tissue, it must be precipitated again to remove glycogen contamination. To do this, the pellet was

resuspended in 0.5mL DEPC-treated, double-distilled water. (Often, the pellet did not readily go into solution with simple vortexing alone. When this occurred, the sample tube in question was briefly heated at 45°C for 30 seconds to 1 minute, then vortexed again. If the pellet still had not gone into solution after heating, it was not heated again, to minimize the risk of degrading the RNA. Instead, the pellet was carefully pulverized against the walls of the microcentrifuge tube with the side of a pipette tip, taking care not to trap pellet fragments within the pipette tip, and then vortexed – for the samples used in this experiment, the maximum number of times the pulverization/vortex step had to be repeated was three times.) Once the pellet was in solution, 0.5mL of 10M LiCl was added to each tube before centrifuging (14000rpm) for 20 minutes. Upon removal from the centrifuge, another pellet was visible at the base of the sample tube. The supernatant was again decanted into a glass waste container (under a fume hood). The pellet was then washed by adding 1.0mL of 75% RNA-grade ethanol to each tube and centrifuging (14000rpm) for 10 minutes. Then, the ethanol was decanted into a glass waste container and the tubes were allowed to dry inverted on paper towels until as much ethanol was removed as possible. (If small droplets remain after 15-20 minutes of drying time, they were carefully removed with a pipette, without dislodging the pellet.) The pellet was then re-dissolved by adding 40uL of DEPC-treated, double-distilled water to each tube and vortexing (or pulsing with a centrifuge) to mix. The extracted RNA solution was kept at -80°C until immediately before its conversion to cDNA.

RNA conversion to cDNA

The extracted RNA was converted to complementary DNA (cDNA) using the Omniscript RT Kit (Qiagen), following the manufacturer's protocol.

DNA gel

The complementary DNA products obtained were subjected to electrophoresis on 4% agarose gels. To make the gel, 2.0g of agarose was combined with 49mL of double-distilled water in a 125mL Erlenmeyer flask, using a 100mL glass beaker as a lid. The mixture was swirled by hand, then heated in a laboratory microwave until clear and just beginning to bubble/boil (approximately 1 minute, stopping to swirl the flask after 20 seconds, 32 seconds, 40 seconds, and 5-second intervals after 40 seconds). The flask was then cooled in room temperature-water for approximately 30 seconds to 1 minute, until the base of the flask was warm to the touch. Once cooled, 1.0mL of 50xTAE (per liter of 50xTAE: 242g Tris, 57.1mL of 17.4N HoAc glacial acetic acid, 18.6g EDTA, and approximately 900mL double-distilled water) and 2uL of ethidium bromide was added to the agarose mixture. The mixture was swirled until red coloration (from the ethidium bromide) was no longer visible. Then, the agarose mixture was quickly poured into a gel box that had been washed, dried, taped at the sides, and had a comb inserted in the proper location. The gel was allowed to set for approximately 30 minutes before the comb and tape are removed. 600mL of 50xTAE running buffer (containing 145g of Tris, 34mL of 17.4N glacial acetic acid, and 11g of EDTA in approximately 540mL of

double-distilled water) was poured into the gel box over the gel. For each red drum liver sample, 20uL of its cDNA solution (with 2uL of GoTaq Green Mastermix DNA loading dye adding added per 20uL of sample) was injected into a well of the gel using a pipette. Each gel contained ten lanes, with multiple gels run to accommodate all samples. The gels were run (at 80-100 volts for 1.5-2 hours) until the first dye front reached approximately two-thirds of the length the gel. Afterwards, each gel was destained by submerging in double-distilled water in a rocking water bath overnight. They were each visualized with a UV transilluminator to identify gel band location and intensity.

Gel band sequencing & IRD confirmation

From each lane on the gel, I extracted the single band believed to contain the IRD gene (based on DNA fragment size) using the Qiaquick Gel Extraction kit (Qiagen), following the manufacturer's protocol. The extracted bands were sent to the Texas A&M University Gene Technologies Laboratory for DNA sequencing.

IRD quantification

When the DNA sequence found in the bands matched the DNA sequence of IRD, the identity of our isolated product was confirmed to be IRD. The amount of IRD gene product generated over time was quantified with two-step qPCR, using Applied Biosystems High Capacity cDNA Reverse Transcription Kit and TaqMin Universal PCR Mastermix according to the manufacturer's protocol. This

specifically measured IRD gene mRNA content in the liver samples from each of the experimental groups: T4-treated and control-treated. Once we quantified the IRD gene product content in all samples from both of the experimental groups, a Mann-Whitney U-test (non-parametric statistical test) was used to test for significant differences between the groups.

CHAPTER III

RESULTS

T_4 time-course immersion experiment, water only

Treatment of tank water with 3.0mL of100ng/mL T_4 solution did cause increased water T_4 levels, which differed significantly from water T_4 levels in the untreated tanks and remained relatively stable throughout the 4 hour static immersion period (Figure 1). At most, 50% of the administered dosage of T_4 was detectable in the tank water (around 50 ng/mL when 100 ng/mL was added), which could be due to hormone absorption to tank walls or degradation from temperature or light. After the circulating water flow was turned back on, T_4 levels in the T_4-treated tanks rapidly declined (50.3 ng/mL to 16.6 ng/mL after 30 minutes of circulating flow, 5.3 ng/mL after 1 hour of circulating flow). At the same time, T_4 levels in the untreated tanks increased slightly (0.13 ng/mL to 0.45 ng/mL after 30 minutes of circulating flow, 2.15 ng/mL after 1 hour of circulating flow), indicating that traces of the administered T_4 can be transported through the actively circulating water system.

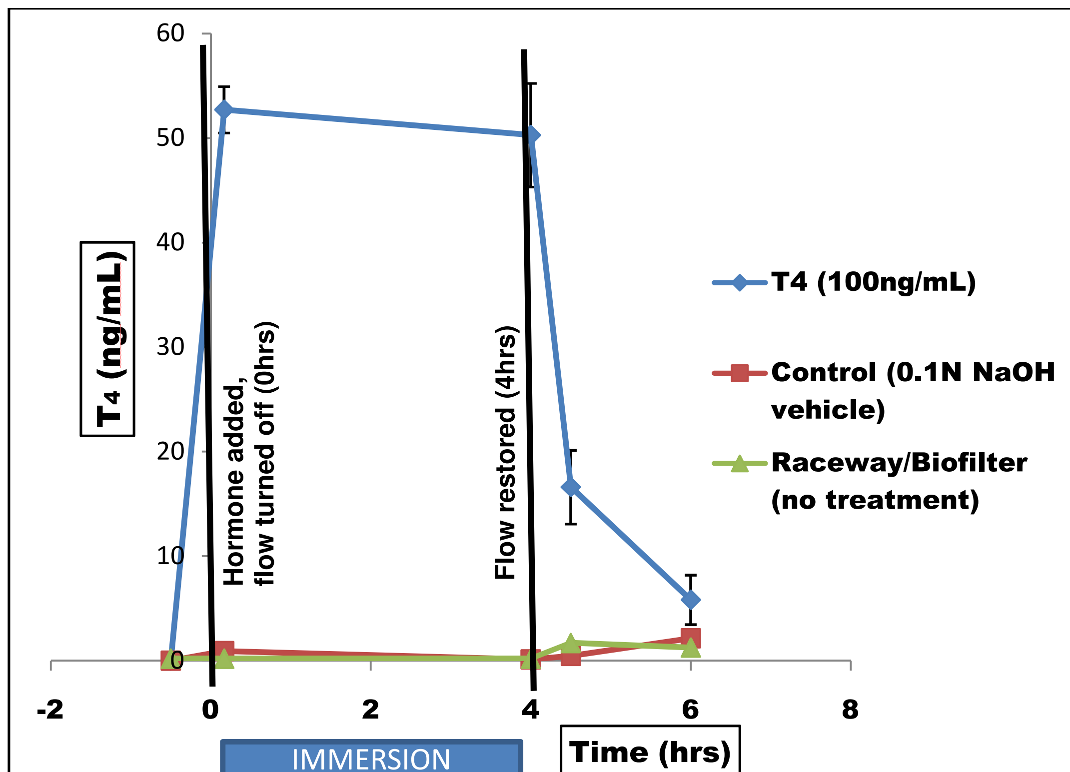

Figure 1 Results from T_4-specific radioimmunoassay of tank water samples. Data for each time point represent the average of 2 samples.

T_4 time-course immersion experiments on red drum

Treatment of tank water with 3.0mL of100ng/mL T_4 solution did cause increased blood T_4 levels in red drum, which differed significantly from blood T_4 levels in red drum from untreated tanks (Figure 2, Figure 3). Two different lengths of T_4 immersion were also studied: 4 hour immersion (with samples taken at 4-hour and 18-hour time points) and 40-hour immersion (with samples taken at 22-hour and 40-hour time points). A longer immersion time increased the total concentration of T_4 in the blood at the end of immersion (18.5ng/mL blood T_4 at end of 4-hour

immersion, 28.7ng/mL blood T_4 at end of 40-hour immersion). Blood T_4 levels decreased steadily after the end of immersion (when circulating water flow is restored); for the 4-hour immersion, differences between the T_4-treated and untreated groups were statistically insignificant 14 hours after the end of immersion.

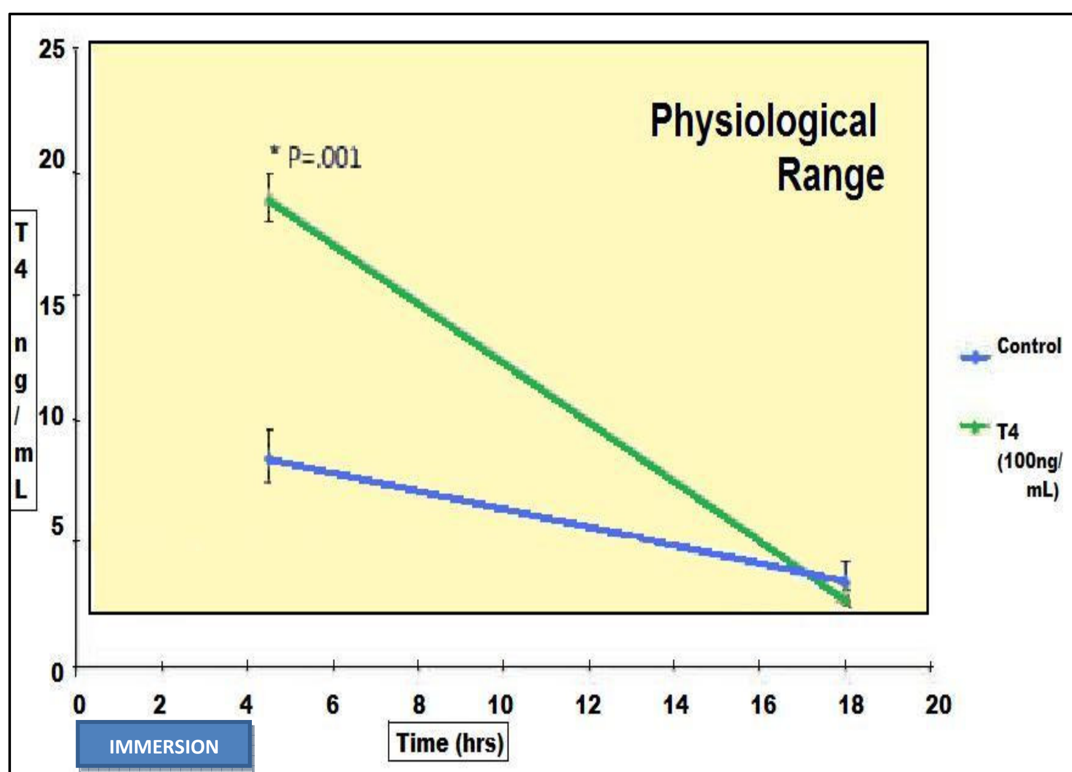

Figure 2 Results from T_4-specific radioimmunoassay of blood samples from control and T_4-treated red drum, 4.5-hour and 18-hour time points, with T_4 immersion from 0-4 hrs. Data values for each time point represent the average of 4-5 samples. The yellow box represents the normal physiological range of T_4 in red drum blood.

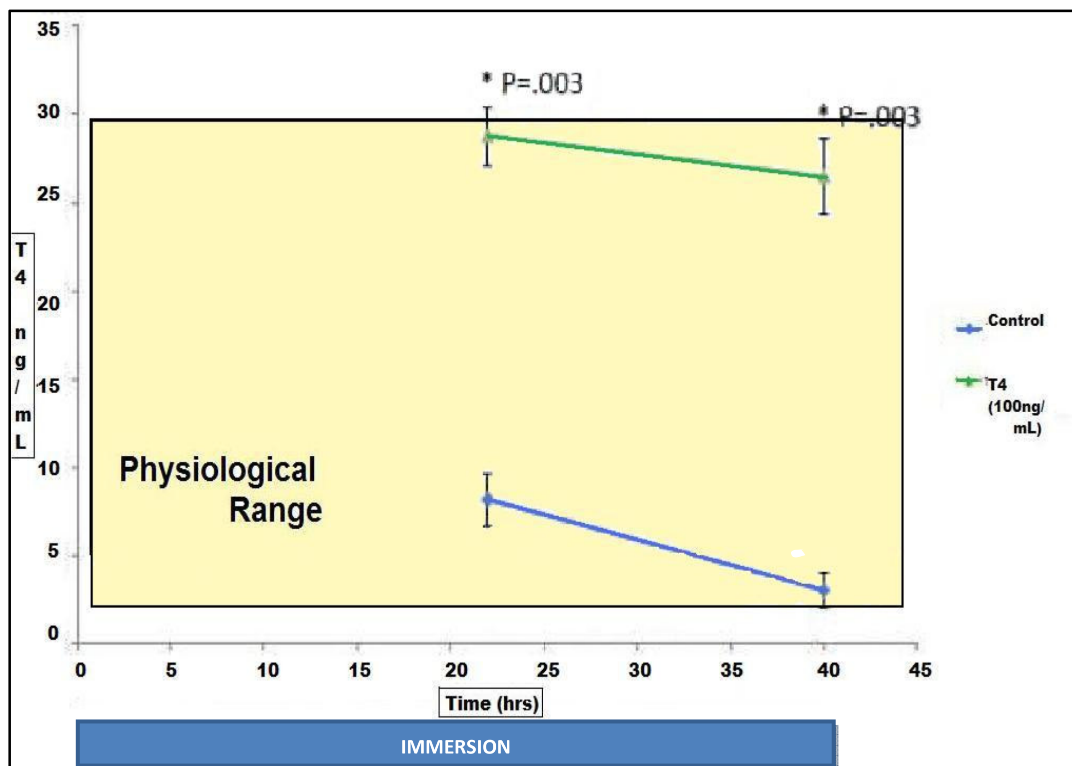

Figure 3 Results from T_4-specific radioimmunoassay of blood samples from control and T_4-treated red drum, 22-hour and 40-hour time points, with T_4 immersion from 0-40hrs. Data values for each time point represent the average of 4-5 samples. The yellow box represents the normal physiological range of T_4 in red drum blood.

IRD quantification with real-time qPCR

Treatment of tank water with 3.0mL of100ng/mL T_4 solution – for both 4-hour immersion and 40-hour immersion – did not cause a statistically significant difference in red drum liver IRD gene expression, when compared to liver IRD gene expression in red drum from untreated tanks (Figure 4). Levels of liver IRD gene expression were statistically indistinguishable for all sampling time points in

both experiments (4-hour and 18-hour sampling times for 4-hour immersion experiment, 22-hour and 40-hour sampling times for 40-hour immersion experiment).

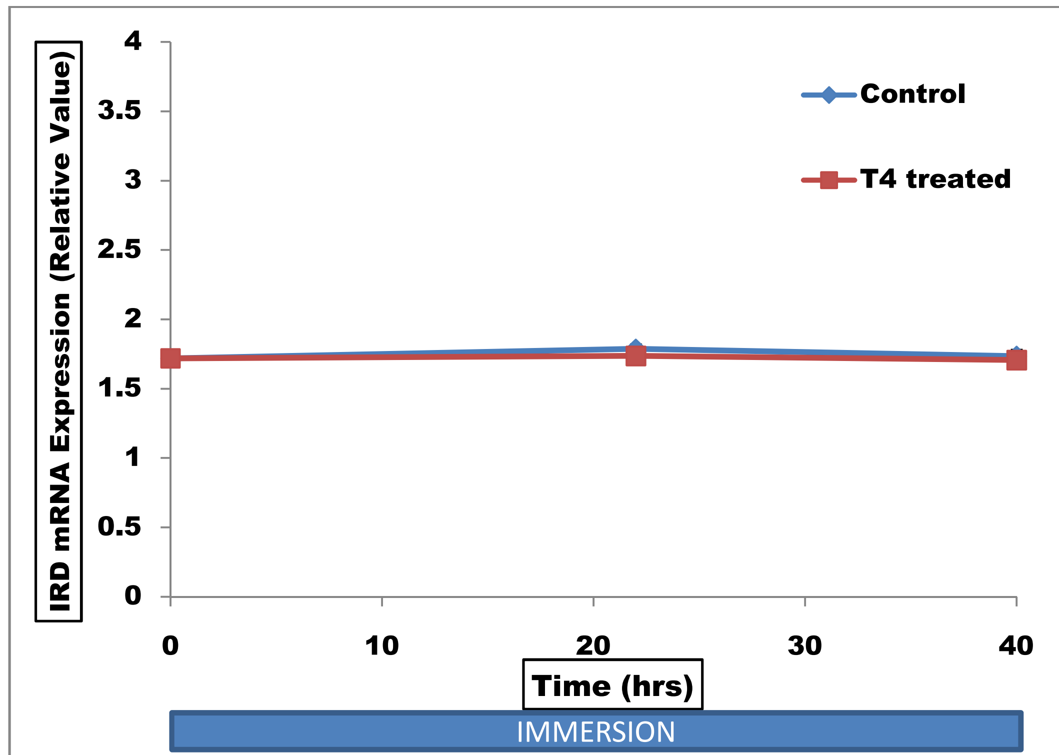

Figure 4 Results from IRD-specific real-time qPCR of liver samples from control and T_4-treated red drum, 22-hour and 40-hour time points, with T_4 immersion from 0-40hrs. Data values for each time point represent the average of 4-5 samples. Errors bars are shown, but too small to be visible.

CHAPTER IV

CONCLUSIONS

Results from the T_4 immersion experiments performed on water alone confirmed that T_4 administered to tank water remained stable over the duration of immersion, and could be removed from the water (decreased to levels with no significant difference from the control) within 2 hours of the system recirculating flow being restored.

Since the tank water concentration of T_4 was about 50% of the added concentration of T_4, we can estimate that, for these immersion experiments, the concentration of T_4 in the tank water during immersion was 50ng/mL. Results from the T_4 immersion experiments performed on red drum demonstrated that environmental levels of 50ng/mL T_4 over as little as 4 hours could yield elevations of T_4 blood levels within the established physiological range for red drum. We can further conclude that this increase in T_4 blood levels was most likely within the physiological range for red drum because there was no elevation of liver IRD gene expression. This could be confirmed by obtaining a positive control – administration of pharmacological levels of T_4 through immersion, with the goal of elevating liver IRD gene expression – through further experimentation with the T_4 immersion method.

In conclusion, we propose that T_4 immersion is a useful method for examining the physiological function of T_4 in red drum. This method could be utilized in the future both by commercial aquaculture, to stimulate beneficial physiological effects in red drum through thyroid hormone administration, and by laboratory researchers, to manipulate thyroid hormone levels in red drum as part of the ongoing study of thyroid function evolution.

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