REGULATION OF PITUITARY THYROTROPIN PRODUCTION IN RED DRUM

A Senior Scholars Thesis

by

ELIZABETH DRONE

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

UNDERGRADUATE RESEARCH SCHOLAR

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Approved by:

Research Advisor: Duncan MacKenzie
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Regulation of Pituitary Thyrotropin Production in Red Drum. (April 2009)

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Department of Biology

Thyroid stimulating hormone (TSH), also known as thyrotropin, is a pituitary hormone which stimulates the thyroid gland to synthesize and secrete the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). In tetrapod vertebrates, the regulation of pituitary hormone production and secretion is accomplished by a portal system which delivers thyrotropin-releasing hormone to positively stimulate the pituitary to release more TSH. However, in teleost fish such as the red drum (*Sciaenops ocellatus*), TSH production appears to be negatively controlled through direct neurosecretory innervations of the pituitary by the hypothalamus. Further research has yet to establish the importance of precise regulation of TSH expression in the red drum pituitary gland via hypothalamic TSH inhibitory factors (TIFs). Examining pituitary glands in *in vitro* incubations should provide a direct method for testing possible TIFs while controlling for other factors. Unfortunately, the pituitary gland of the red drum provides a very small amount of tissue, and it can be difficult to extract enough RNA from single pituitary

glands to use in common mRNA expression analysis techniques such as Northern blotting. My objective is to determine if more sensitive techniques, dot blot and real-time PCR, are suitable for analyzing mRNA expression of red drum TSH. Dot blot was found to be successful in determining relative quantification of TSH mRNA in samples of at least two pooled pituitary glands following *in vitro* incubation. Real-time PCR with TaqMan probes was also successful at amplifying a TSH mRNA signal from 50ng of red drum pituitary mRNA. Thus, real-time PCR should provide a sensitive technique to measure mRNA expression of TSH in single pituitary glands and allow further investigation of the existence of hypothalamic TSH inhibitory control factors.

ACKNOWLEDGMENTS

Thank you to my advisor Dr. Duncan MacKenzie for all his direction and support. Thank you also to Richard Jones, Thomas Miller and Dr. Bill Cohn for their patience and guidance in helping me learn the techniques necessary to complete my project.

NOMENCLATURE

TSH	Thyroid Stimulating Hormone
TIF	Thyrotropin Inhibiting Factor
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
T_3	Triiodothyronine
T_4	Thyroxine
TH	Thyroid Hormone
MMI	Methimazole
^{32}P	Radioactive Phosphorus Isotope
cDNA	Reverse Transcribed Deoxyribonucleic Acid

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Thyroid stimulating hormone (TSH), also known as thyrotropin, is one of several hormones produced by the pituitary gland. TSH is a 30 kilodalton glycosylated dimer which is manufactured by thyrotrophs in the pars distalis of the pituitary gland. It comprises two subunits, an α subunit which is common to other glycoprotein hormones and aids in receptor affinity and binding stabilization, and a β subunit which is unique to TSH and specifies TSH binding to thyroid receptors. TSH acts via membrane receptors to stimulate the thyroid gland to synthesize and secrete the thyroid hormones (THs), thyroxine (T_4) and triiodothyronine (T_3). TSH also activates other vital aspects of thyroid function including the transportation and concentration of iodide, manufacture and processing of the TH precursor thyroglobulin, and the growth and differentiation of the thyroid gland. Thyroid hormones in turn are involved in controlling a host of genes which regulate metabolic rate throughout the body and maintain essential metabolic tone in cells. Thyroid hormones are also critical factors in the correct growth and development of many organs and tissues, especially of the central nervous system. Thus, the precise regulation of these hormones via TSH is vitally important in all vertebrate animals.

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

The distribution of pituitary hormones in the vertebrate lineage indicates that the shared α subunit is conserved all the way back to Cephalochrodata (e.g. *Amphioxus*) whereas the TSH β gene is present in Chondrichthyes (elasmobranch species such as sharks and rays) onward. Although TSH genes are highly conserved, the control of TSH production varies among vertebrates. In tetrapod vertebrates, the regulation of pituitary hormone production and secretion is accomplished by a portal system, called the hypothalamo-hypophysial portal system, which delivers hormones directly from the hypothalamus to stimulate the pituitary's pars distalis. By releasing thyrotropin-releasing hormone (TRH) into the portal circulation, the hypothalamus positively stimulates the pituitary to release more TSH. However, in teleost fish, such as the goldfish *Carassius auratus*, TSH production appears to be negatively controlled through direct neurosecretory innervations of the pars distalis by nerves of the hypothalamus. This inhibitory control was illustrated by *in vivo* studies of goldfish in which lesions were created in the hypothalamus to disrupt its control over the pituitary and consequently thyroid function was activated (Peter, 1970). Increases in TSH levels in isolated pituitaries *in vivo* or with lesion studies have also been seen in other teleost species (Ball *et al*., 1963; Olivereau and Ball, 1966). Further experiments by Dr. Peter demonstrated that autotransplanted pituitaries, those moved away from the brain and out of proximity to the hypothalamus, also increased production of TSH in goldfish (Peter, 1972). It can thus be inferred that the hypothalamus had been exerting some inhibitory effect on the pituitary to down-regulate the expression of TSH. Peter proposed that the hypothalamus produced a novel regulatory factor responsible for inhibition of TSH release from the pituitary gland:

thyrotropin inhibitory factor (TIF). However, a TIF molecule has never been identified. In a similar case, another important pituitary hormone, growth hormone (GH), has been experimentally proven to be inhibited by the hypothalamus in many species, particularly goldfish, by dopamine and somatostatin (Kwong and Chang, 1997).

GH inhibition is common throughout vertebrates, but TSH inhibition in teleost fish is unique. This unique inhibition may be due to the unusual system of direct innervation of the teleost fish pituitary instead of a portal system. However, little is known about the physiological mechanism through which this inhibition is achieved. The objective of this project is to determine whether techniques developed for the study of pituitary hormone production in other fish species are suitable for the identification of inhibitory factors controlling TSH release in a common agricultural fish species, the red drum. The red drum provides an excellent species to study because extensive research on its thyroid system by the MacKenzie lab has shown dynamic daily changes of T_3 and T_4 (Leiner and MacKenzie, 2003). The studies on goldfish TSH inhibition can be used as background for examining the same effects in the red drum. The decreased presence of TSH and the feedback mechanisms of thyroid hormones led Eales and Brown to propose that in teleost fish peripheral control of thyroid hormone levels via deiodoniases are more crucial to thyroid system regulation than central control through the hypothalamo-pituitary axis (Eales and Brown, 1993). In contrast, the discovery of hypothalamic TSH inhibitory factors would provide evidence that precisely-regulated TSH expression is

important in controlling a dynamically regulated thyroid system. If inhibition of TSH expression can be established for this teleost species, then the red drum can be used to conduct further research to discover novel factors important in TSH regulation and to characterize possible hypothalamic inhibitory control factors of TSH, particularly a candidate for thyrotropin inhibitory factor.

When working with such a small amount of tissue as the pituitary gland of the red drum, extremely sensitive techniques must be developed for measuring hormones. Various methods have been used to successfully measure TSH mRNA expression in fish pituitaries, including dot blots, northern blots, radioimmunoassay, and most recently quantitative polymerase chain reaction (PCR). In the Coho salmon, TSH radioimmunoassay and RNAse protection assay were used to observe T_3 inhibition of TSH *in vivo* and to evaluate stimulatory effects of molecules such as corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH) on TSH expression *in vitro* (Moriyama *et al*., 1997, Larsen *et al*., 1998, Larsen *et al*., 1997). Additionally, northern blots have been used successfully in the past to examine T_3 inhibition of TSHβ expression *in vitro,* for example in the European eel and goldfish (Schmitz *et al*., 1998, Sohn *et al*., 1999a). Because of its high sensitivity, real-time PCR has become the most commonly used method to measure TSHβ expression both *in vivo* and *in vitro* in many fish species, including the Japanese eel, turbot, Senegalese sole and bighead carp (Han *et al*., 2004,

Manchado *et al*., 2008, Chatterjee *et al*., 2001, Chowdhury *et al*., 2004). The MacKenzie lab has developed cDNA probes for red drum TSH α and β subunits by cloning and sequencing their genes. Using northern and dot blot techniques, the amount of RNA in pituitary samples can be detected with these probes labeled with radioactive ^{32}P. However, the amount of RNA that can be obtained from a single pituitary can be highly variable depending on the size of the fish. Occasionally, the RNA obtained from one pituitary is not enough to use in northern or dot blot techniques. Thus, to maximize the number of data points that can be obtained from a limited number of fish specimens by ensuring that a single pituitary can be used, this project will incorporate the design and standardization of a quantitative PCR, or real-time PCR, technique for measuring red drum TSH α and β using TaqMan probes.

In vitro cultures of pituitary cells provide a more controlled environment for examining very precise TSH mRNA level changes which may be of very small magnitude. This study therefore will utilize an *in vitro* pituitary incubation system to evaluate regulation of TSH production. The *in vitro* incubation protocol for isolated red drum pituitaries was developed based on the experiences of members of the MacKenzie lab and on previous culture techniques published in several laboratories (Li *et al.,* 2002, Larsen et. al, 1998, Schmitz *et al*., 1998, Han *et al*., 2004, Sohn *et al*., 1999b, Chatterjee *et al*., 2001, Chowdhury *et al*., 2004). Additionally, preliminary work on the time course of TSH β mRNA expression has allowed me to establish basal TSH levels in single red drum

pituitaries as related to the size of the fish. We thus have preliminary evidence that TSH is expressed and detectable in *in vitro* red drum pituitaries. These *in vitro* experiments support the hypothesis that TSH expression will increase when pituitaries are removed from inhibitory hypothalamic control.

Further *in vitro* experiments will include hormone-response tests in an effort to identify potential regulators of TSH production and possibly TIF. Both *in vitro* and *in vivo* studies of TSH production in teleost pituitaries have shown that thyroid hormones inhibit TSH mRNA expression (Schmitz *et al*., 1998; Pradet-Balade *et al*., 1997). Furthermore, studies in the bighead carp identified various neuropeptides, such as TRH and leptin, as potential regulators of TSH expression in cultured carp pituitary cells (Chowdhury *et al.,* 2004). To examine possible methods of increasing TSH expression in red drum, isolated pituitaries will be treated with thyroid hormones and hypothalamic hormones that are known to influence TSH expression and may exert stimulatory control on TSH production in vertebrates (Denver and Licht, 1989; Canosa *et al.*, 2007). Pituitaries will also treated with agonists of dopamine, a known inhibitor of the pituitary hormones prolactin, growth hormone (GH) and gonadotropin; somatostatin, another GH inhibitor; and T_3, which is a feedback inhibitor of TSH (Peter, 1971). These experiments will simulate physiological doses of these hormones ($10^{-6} – 10^{-10}$ M). Overall, this project will strive to establish an inhibitory effect of the hypothalamus on TSH production in the red drum by demonstrating that TSH expression increases when pituitaries are removed

and examined *in vitro.* Once baseline TSH mRNA levels are established, it will be possible to identify specific neuropeptides that inhibit TSH production. These hormone-response tests will validate the *in vitro* system and possibly identify the most potent neuropeptide for TSH regulation.

CHAPTER II

METHODS

Animals and animal procedures

Red drum (*Sciaenops ocellatus*) were obtained from the Aquacultural Research and Teaching Facility at Texas A&M University. The red drum were maintained in a 4000 liter recirculating artificial seawater system at a salinity of 5-6 parts per thousand, a temperature of 25^{o}C, and fed a commercial diet. Fish used in these experiments had a body weight ranging from 200-700g. Before pituitary glands were extracted using forceps, the fish were anesthetized using tricaine methanesulfonate (MS-222) and then sacrificed by severing the spinal cord at the base of the skull.

In vitro incubation of pituitary glands to test for RNA integrity

Preparation of reagents

Fresh incubation medium was prepared the day of each experiment according to the following recipe: 0.4755g Minimum Essential Medium (MEM), 0.11g Sodium bicarbonate, 0.238g HEPES, 0.5ml Pen-Strep antibiotic, and buffered to a pH of 7.2-7.4 using 1N Sodium hydroxide. Finally, the medium was QS to 50ml total volume.

Pituitary gland extraction and incubation

For measurement of mRNA expression, pituitary glands were removed from three red drum and placed in 1ml TRIzol each. All three samples were homogenized individually

by gentle aspiration through a 20g needle attached to a 3cc syringe. Homogenates were snap frozen on dry ice and stored at -80^{o}C until further analysis. For *in vitro* incubation studies, pituitary glands were removed from three fish and were cut with a razor blade into at least four fragments. While being sliced, pituitary glands were placed on a flat glass plate and bathed in 100µl of medium. After this medium was pipetted into one well on a 24-well culture plate, another 100µl of medium was used to wash the surface of the glass dish and was also pipetted into that well to catch any remaining fragments. Each fragmented pituitary gland was thus placed in one well of a standard 24-well plate with 1 ml of medium. The plate was then placed in an incubator at 28^{o}C under 4% CO_2 and incubated for 20 hours. Samples were then transferred to 1.5ml conical tubes and centrifuged at 12,000 x g and 4^{o}C for 5 minutes. The supernatant was discarded and the pellet was homogenized in 1ml TRIzol, snap frozen on dry ice, and stored in -80^{o}C. RNA samples were later extracted using the TRIzol Reagent procedure and then analyzed by RNA Gel Electrophoresis according to the protocols below.

In vitro incubation of pituitary glands with T_3 and T_4 treatments

Preparing medium containing T_3 and T_4 treatments

Medium was prepared using the same recipe above. 1µl of a 1mM thyroxine (T_4) stock solution, containing T_4 free acid in saline (1.8g NaCl, 0.2g Bovine Serum Albumin, QS to 200ml) was added to 999µl of media. This preparation provided medium with a 1µM concentration of T_4. The medium containing triiodothyronine (T_3) was prepared in the same way, except using a 1mM T_3 stock solution, containing T_3 free acid and saline.

Pituitary gland extraction and incubation

Pituitary glands were removed from 18 red drum and placed into a small beaker of medium on ice. The medium had been placed on ice before beginning the experiment so that the pH could be adjusted to physiological levels of red drum (7.2-7.4) at the colder temperature. The pituitary glands underwent a one hour pre-incubation period while waiting on ice as they were being collected. Two pituitary glands were selected at random from the beaker and sliced into at least two pieces using the method described under "*In vitro* Incubation of Pituitary Glands to test for RNA integrity." These two pituitary glands were then placed in the same well of a standard 24-well plate which contained 1ml of media and no hormone. This same procedure was used for all of the remaining pituitary glands. This gave a total of nine different samples which were separated into three groups of three. The first three samples had no hormone added and acted as a control group. The next three samples each had 1µl of a T_4 stock solution added (as described in the media preparation section above). The final group had 1µl of a T_3 stock solution added (also described above). All samples were incubated for 20 hours at 28^{o}C under 4% CO_2. At the end of the incubation period, the samples with their medium were placed into conical tubes and centrifuged in a cold room for 5 minutes. 1ml of TRIzol Reagent was then added and the samples were gently homogenized using 20 g needles attached to 3cc syringes. Finally, the samples were snap frozen in liquid nitrogen and stored at -80^{o}C. Later, RNA samples were extracted using the TRIzol Reagent procedure and analyzed by dot blot according to the protocols below.

RNA extraction using TRIzol reagent

Phase separation

Samples frozen in TRIzol were quickly thawed in a hot water bath for 10 seconds and then incubated at room temperature (15-30^{o}C) for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Then, 0.2ml of chloroform was added to each TRIzol sample. Tubes were shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2-3 minutes. Samples were next centrifuged at top speed for 15 minutes which allows the sample to separate into a lower phase containing phenol-chloroform and DNA or protein contaminants, an organic interphase, and a colorless upper aqueous phase containing RNA.

RNA precipitation

The aqueous phase was then transferred to a fresh 1.5ml tube and 0.67µl of glycol-blue was added so that the RNA would be visible when precipitated. 0.5ml of isopropanol was then added to each sample in aqueous phase and incubated at room temperature for 10 minutes. The samples were then centrifuged at top speed for 10 minutes and the supernatant was discarded. The RNA pellet was then washed once by adding 0.5ml of 100% ethanol and centrifuging at top speed for 5 minutes. The ethanol was aspirated and decanted by inversion onto paper towels. The pellet was allowed to air dry for 5-10 minutes.

Re-dissolving RNA and measuring concentration

7µl of DEPC water was used to re-suspend the pellet. The pellet was pipetted repeatedly and occasionally was briefly heated in the 65^{o}C water bath to assure that it was dissolved. If necessary, an additional 5µl of DEPC water was used if the pellet still had not dissolved. The concentrations of the samples were then measured using a Nanodrop ND-100 spectrophotometer. The concentration in nanograms per milliliter, the absorbance value at a wavelength of 260nm, and the ratio of the absorbance at 260nm to the absorbance at 280nm were recorded for each sample.

RNA gel electrophoresis

Preparing an RNA gel

A 1.0-1.2% SDS gel was prepared by dissolving 0.5-0.6g of agarose in 45ml of double deionized water (ddH_2O) and heating it in a flask for 10-20 seconds in a microwave. The gel was then cooled to approximately 60^{o}C by submerging the base of the flask in running tap water. Once the agarose was cooled, 5ml of 10X formaldehyde gel buffer was added and mixed by swirling the flask. The gel was then immediately poured into a gel box with the teeth of the comb positioned 1-2mm from the bottom of the gel tray. After the gel had solidified it was placed in the gel box and the box was filled with 10X MOPS buffer (21g MOPS, 3.4g NaOAC, 1 ml 0.5M EDTA, 400ml ddH_20). The gel was allowed to soak for 15 minutes.

Preparing the samples

Using no more than 6µl of each sample, the amount of that corresponded to 5 µg of RNA was transferred to a new tube. RNA loading buffer (from Ambion) was then added in a 3:1 ratio to the volume of each sample. The samples were then vortexed, pulsed, and heated at 60-65^{o}C for 10-15 minutes. Immediately after heating, the samples were pulse spun in a microcentrifuge and quenched on ice.

Running the gel

The samples were loaded into the wells of the gel. The gel was run at 80-100 volts until the first dye front was two-thirds of the way down to the end of the gel. After it was finished running, the gel was removed from the running buffer and placed into a staining solution (25µl of concentrated ethidium bromide in 500ml of ddH_2O). The gel was gently rocked in the covered staining solution tray for 30 minutes and then moved to a destaining solution of 500ml of H_2O and gently rocked overnight. A picture of the gel was then taken using a UV transilluminator.

Dot blot analysis of RNA content

A volume of each sample that corresponded to 1.1µg was obtained and brought up to 80µl with DEPC ddH_2O. 240µl of master mix for each sample was then prepared with 500 parts formamide, 162 parts formaldehyde, and 100 parts 10X MOPS. The 240µl of master mix was added to each sample, heated at 65^{o}C for 15 minutes, and then chilled on ice. Finally, 80µl of 20X SSC was added to each sample and they were vortexed, pulse

spun, and placed on ice. The dot blotter was then prepared by cutting a piece of Whatman filter paper and a blotting membrane to the correct size to cover all of the dots that would be used for the samples. The two pieces were briefly rinsed in ddH2O and then 20X SSC. The Whatman paper was placed on the blotter first and then the membrane on top. Any unused dots were covered with Parafilm and the blot was clamped down. A vacuum was applied and the samples were carefully loaded to their corresponding dots. After the entire sample had passed through, each dot was washed with 500µl of 20X SSC. The RNA was fixed to the membrane by placing it in a UV crosslinker for 12 seconds and then by soaking the membrane in 2X SSC. The blot can then be analyzed.

Validation of real-time PCR technique

Sample preparation

In order to validate the first use of the real-time PCR technique, samples were used from a previous *in vivo* experiment conducted by Richard Jones which had been previously analyzed by Northern blot and shown to have a large difference in TSHβ expression. Jones used injected treatments of methimazole (MMI), T_3, and saline as a control treatment. MMI is a known thyroid hormone synthesis inhibitor which increases TSH expression by decreasing negative feedback of thyroid hormones at the level of the pituitary. T_3 decreases TSH expression by increasing negative feedback to the pituitary. Red drum were injected with these three treatments and then pituitary glands were pooled into a single sample for each treatment group.

DNAse treatment using DNA-free kit

To remove any DNA contamination, the RNA samples were treated with DNAse enzyme. 10µg of RNA from each sample was added to 5µL of buffer, 1µL rDNAse I, and brought up to a volume of 50 µL using DEPC ddH_2O. These samples were then incubated at 37^oC for 20-30 minutes. Then 5µL of DNAse Inactivation Reagent is added to each sample and mixed well by vortexing. The samples are incubated at room temperature for another 2 minutes while being occasionally mixed as the inactivation reagent stops the reaction. Centrifuging at 10,000g for 1.5 minutes separates the inactivation reagent from the sample; the aqueous RNA phase can then be removed to a new tube. After DNAse treatment, RNA samples were at a concentration of 0.2µg/µL.

High-capacity cDNA reverse transcription kit

Using the Applied Biosystem High-Capacity Reverse Transcription (RT) Kit specifically designed for use preparing real-time PCR samples. 400ng of RNA from each treatment group was used in separate reverse transcription experiments to prepare enough DNA for 8 samples of 50ng each for use in real-time PCR. Thus, 2µL of each sample was added to 10µL of master mix. The master mix was made using 2µL 10X RT Buffer, 0.8µL 25X dNTP Mix, 2µL 10X TR Random Primers, 1.0µL Reverse Transcriptase, and 4.2µL DEPC ddH_2O for each reaction. Once combined with master mix, the samples were mixed by pipetting up and down and then loaded into the thermocycler and run at the following conditions: Step 1: 25^oC for 10 minutes, Step 2: 37^oC for 120 minutes, Step 3:

85°C for 5 minutes, Step 4: Hold at 4°C. After the RT reaction is complete, the RNA samples have now been converted to cDNA and are ready for real-time PCR.

Real-time PCR run using TaqMan reagents

Real-time PCR was run using TaqMan reagents because the combination of primer and probe that it utilizes ensures higher specificity for the target sequence than other real-time methods. As polymerization begins, the probe with a fluorescent reporter dye (R) and quencher dye (Q) attached as shown in Figure 1 anneals to the target gene sequence. As the Taq Polymerase synthesizes a new strand of target DNA from the template, it reaches the probe and displaces it from the original DNA strand. The reporter dye is then cleaved from the probe and begins to fluoresce once it is out of proximity to the quencher. Thus, fluorescence increases as amplification increases.

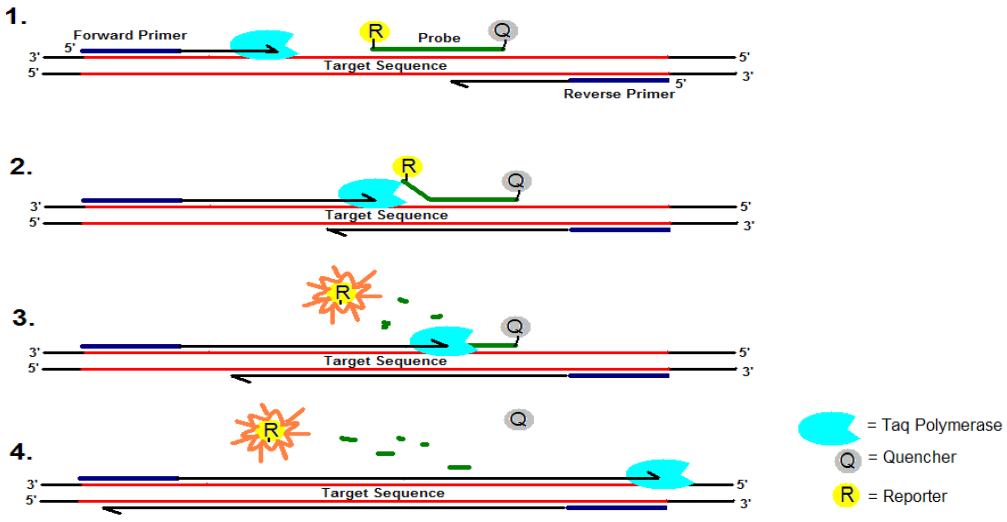

Figure 1. Process of real-time PCR amplification using TaqMan probes. Step 1: Polymerization begins. Step 2: Taq Polymerase displaces probe. Step 3: Reporter dye is cleaved from the probe and begins to fluoresce. Step 4: Polymerization of the target sequence is complete.

To set up the real-time PCR reaction, each sample was prepared according to the following formula: 12.5µL TaqMan Universal PCR Master Mix (2X), 2µL of 12X concentrated TaqMan Primer/Probe Mix, 2µL cDNA sample at a concentration of 0.025µg/µL which gives 50ng cDNA, and 10.5µL nuclease-free ddH_2O. Four replications of each sample type were run and each replication had its own endogenous control. The replications were created to test the precision of the real-time PCR technique. Thus, for the control saline injected sample, eight wells of a standard optical 96-well plate were prepared containing reverse transcribed cDNA from the control sample and primer/probe mix for TSHβ was added to four of the wells and primer/probe mix for 18S ribosomal RNA was added to the other four wells to measure a housekeeping gene and serve as endogenous controls. The expression of 18S is measured in order to standardize for the amount of RNA in each sample. Eight more wells were prepared in the same manner for each of the remaining two samples, MMI treated and T_3 treated. The plate was then loaded into an Applied Biosystems 7500 Real-Time PCR thermocycler and run at the following conditions: Step 1: 50^oC for 2 minutes, Step 2: 95^oC for 10 minutes, Step 3: 40 cycles of 95^oC for 15 seconds and then 60^oC for 1 minute. Data was collected and analyzed using Applied Biosystems SDS Software 1.3.1 which uses the comparative Ct method of relative quantification of real-time PCR data.

CHAPTER III

RESULTS

In vitro incubation of pituitary glands to test for RNA integrity

Incubation and RNA extraction protocols were successful in quantifying the RNA content of individual pituitary glands (Table 1). For pituitary glands incubated for 0 hours, the TRIzol Reagent RNA extraction technique extracted an average of 3.533µg of RNA from each gland. The average amount of RNA extracted from single pituitary glands incubated for 20 hours was 3.4502µg. The large variation in RNA content of individual glands incubated for the same amount of time may be attributed to a large variation in mass of the individual fish between approximately 200g and 800g. Additionally, variation in the efficiency of the RNA extraction technique for each sample may have introduced variability in RNA content of various single pituitary glands.

Table 1. Amount of RNA obtained from individual pituitary samples. T0 represents the Time 0 samples which were not incubated. T20 represents the Time 20 samples which were incubated in medium for 20 hours. A-260 indicates the absorbance of the sample at 260nm wavelength and is an indicator of sample quality. The 260/280 value is a ratio of absorbance at 260nm to the absorbance at 280nm and is an indicator of contaminants in the sample when the ratio is above ~2.30.

Sample	Total Amount of RNA (µg)	A-260	260/280
T0-1	1.6212	6.754	2.00
T0-2	1.9092	7.821	1.96
T0-3	7.0686	29.452	1.94
T20-1	1.887	7.861	2.07
T20-2	3.1212	13.004	2.01
T20-3	5.3424	22.259	1.75

Samples were successfully visualized using gel electrophoresis of the entire samples (Figure 2). The differing RNA content of the samples, which can be observed by brighter bands containing more RNA, may be due to variability in the TRIzol RNA extraction protocol but also reflects that the amount of RNA loaded per well was not the same for each sample. Although the same volume of every sample was used, the RNA concentration of each sample varied. The amount of RNA was unimportant and the gel results still verify that RNA molecules in each pituitary gland remained intact during the incubation process.

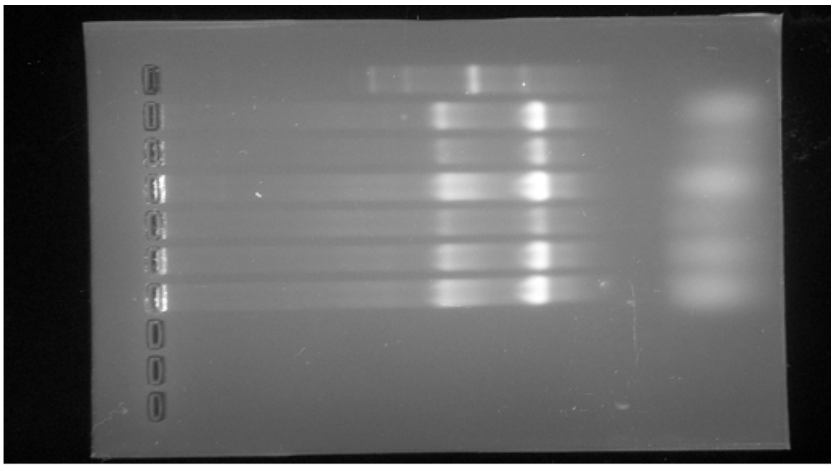

Figure 2. UV exposed image of the RNA gel electrophoresis of incubated samples. From top to bottom, the sample in each well is as follows: 100 bp ladder, T0-1, T0-2, T0-3, T20-1, T20-2, and T20-3.

In vitro incubation of pituitary glands with T_3 and T_4 treatments

Dot blot analysis of the extracted from the samples (Table 2) was successful at visualizing the content of TSHβ mRNA. 1µg of RNA from each sample was blotted to the membrane. Two pituitary glands incubated in the same well yielded an average of 2.23µg of RNA using the TRIzol RNA extraction technique.

Table 2. Amount of RNA obtained from each sample of two pooled pituitary glands. Control samples without hormone treatment in incubation medium are indicated by the letter "C." T_3 and T_4 treated samples are indicated as such. A-260 indicates the absorbance of the sample at 260nm wavelength and is an indicator of sample quality. The 260/280 value is a ratio of absorbance at 260nm to the absorbance at 280 nm and is an indicator of contaminants in the sample when the ratio is above ~2.30

Sample	**RNA Concentration (µg/µL)**	**A-260**	**260/280**	**µL RNA sample used for dot blot**
C1	0.6808	17.020	1.86	1.6157
C2	2.8029	70.072	1.90	0.3924
C3	0.9964	24.910	1.86	1.10324
T_3-1	2.9982	74.954	1.77	0.3668
T_3-2	2.1635	54.087	1.91	0.5084
T_3-3	2.6184	65.460	1.92	0.4201
T_4-1	1.1115	27.788	1.79	0.9865
T_4-2	2.78365	72.043	1.92	0.3951
T_4-3	1.9232	37.992	1.27	0.5719

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Dot blot analysis results show a statistically significant decrease in TSHβ mRNA expression in samples treated with T_4 as compared with the control samples (Figure 3). The data also show some decrease in TSHβ mRNA expression in T_3 treated samples which might have been statistically significant had this experiment used a larger number of samples.

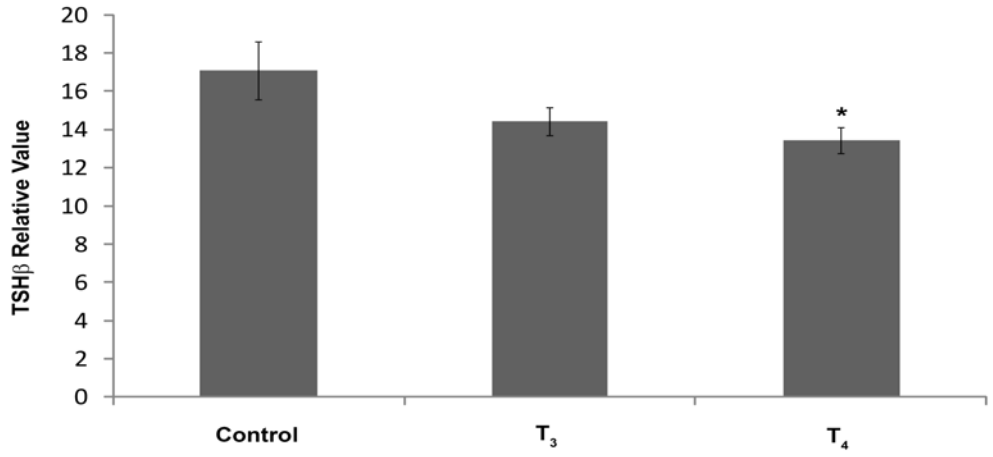

Figure 3. TSHβ expression based on strength of radioactivity on dot blot. Radioactivity was measured in pixels by a phorsphoimager. The Control bar represents the average value of the three samples which were incubated in media without hormone treatment. The T_3 bar represents the average value of the three samples incubated in media containing T_3. The T_4 bar represents the average value of the three samples incubated in media containing T_4. * denotes statistical relevance at $P \leq 0.05$.

Validation of real-time PCR technique

Real-time PCR was successful in amplifying TSHβ expression in each sample well (Figure 4). Three distinct clusters of curves corresponding to the three sample groups can be observed in the amplification plot. The first group to cross the threshold value of magnitude of fluorescence corresponds to the MMI samples and indicates that they had the highest TSHβ expression and thus the highest amount of fluorescence. The middle group corresponds to the control samples and the last group to cross the fluorescence threshold is the T_3 treated samples.

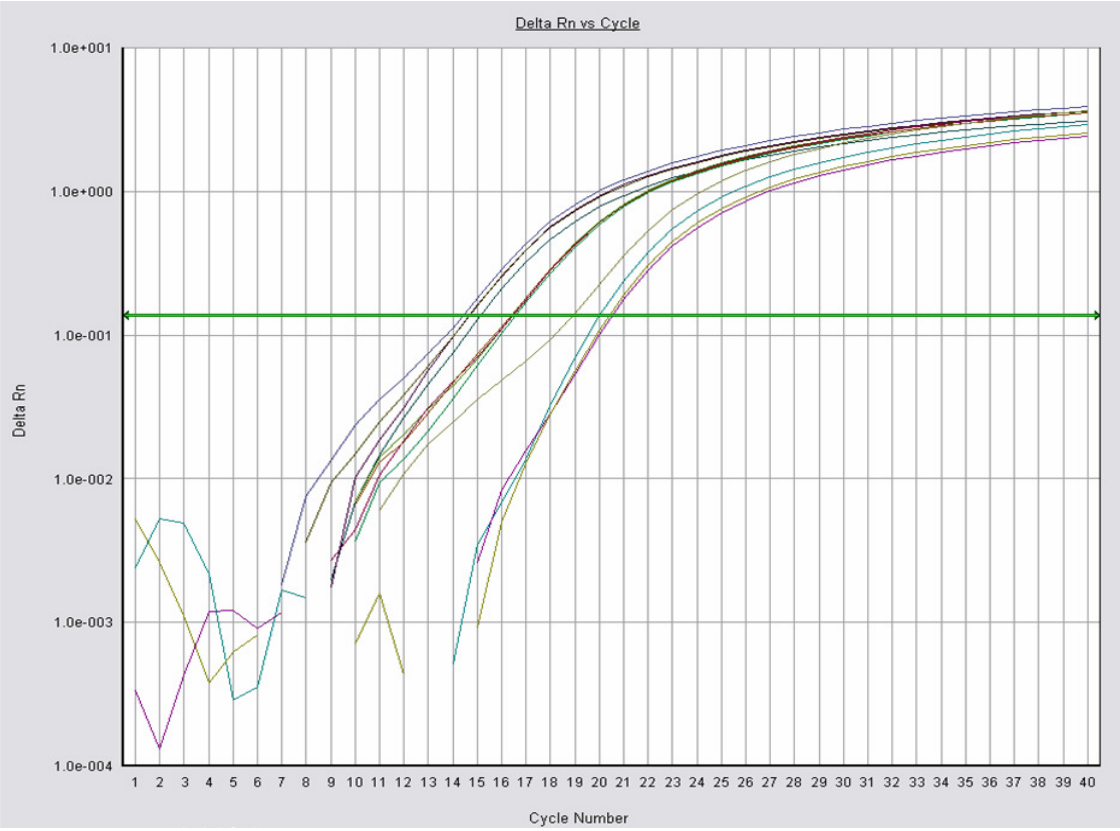

Figure 4. Amplification plot of RNA samples. Graph shows change in fluorescence of each sample over time. Each line represents an individual sample. Green line represents threshold values above which fluorescence levels begin to be measured. Threshold value is set at the standard 0.200000 ΔRn.

In accordance with previous analysis of these samples, data show an increase in TSHβ mRNA expression of MMI treated samples compared to the saline treated control (Figure 5). The data also show a decrease in TSHβ mRNA expression of T_3 treated controls. Additionally, precision of sample replicates seems intact as seen by the small deviation in expression value among replicates of the same sample.

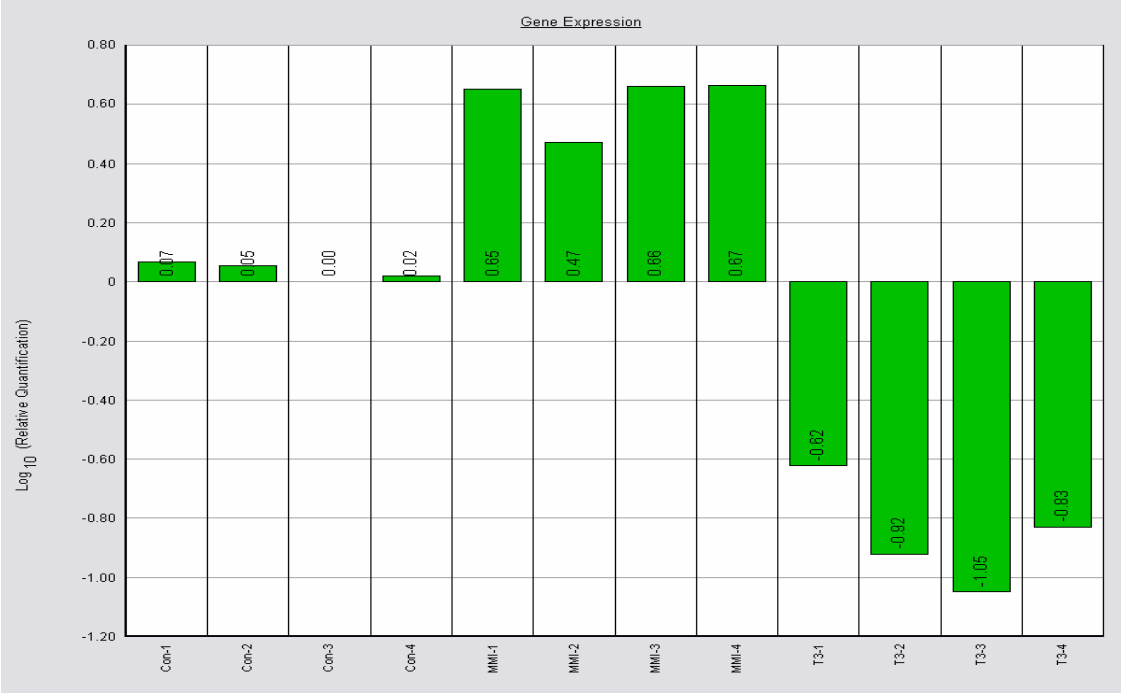

Figure 5. Relative TSHβ mRNA expression per sample. Expression values were standardized against 18S expression levels. Values shown are relative to lowest control group sample expression, sample Con-3. Labels are as follows: Con: control group treated with saline and no hormone. MMI: treated with MMI. T3: treated with T_3.

CHAPTER IV

CONCLUSIONS

In order to successfully test for TIFs *in vitro,* red drum pituitary gland incubations and RNA extraction techniques must be optimized to achieve the most sensitive results. The high yield of RNA and the preserved integrity of extracted RNA samples from an overnight *in vitro* incubation shows that our extraction and incubation methods will be effective methods to obtain RNA from further *in vitro* experiments. Although the average RNA obtained was 3.4502µg, the RNA content ranged from 1.6212µg to 7.0686µg depending on fish size which suggests that very sensitive analysis techniques may be necessary to measure TSH expression in single pituitaries especially when using small fish.

The dot blot technique was successful in quantifying TSHβ mRNA extracted from two pooled pituitary glands incubated *in vitro*. Two pituitary glands incubated in the same well yielded an average of 2.23µg of RNA, but only 1µg of that RNA was needed to analyze each sample. The data shows a statistically significant decrease in the amount of TSH in the T_4 treated group reflecting the negative feedback of T_4 on TSH expression. The T_3 treated group also showed some decrease in TSH expression although it was not statistically significant. The demonstration of negative feedback on TSH expression via thyroid hormones illustrates that *in vitro* incubations can reproduce the physiological conditions present in the fish. Although the dot blot adequately analyzed TSH levels,

more sensitive techniques are necessary if it is desirable to measure the TSH content of a single pituitary gland.

Real-time PCR should provide a sensitive measure of mRNA expression of TSHβ in single pituitary glands. Running a single well used only 50ng of RNA for TSHβ measurement and another 50ng for 18S standardization for a total of 100ng of RNA needed per sample, which was 1.5µg less than the smallest amount obtained in any extraction of a single gland from an *in vitro* incubation. Real-time PCR was also shown to replicate the physiological data we expected from samples which had been previously analyzed by another technique; MMI treated samples had high TSHβ expression and T_3 treated samples had lower TSHβ expression compared to control saline-treated samples. Therefore, real-time PCR should allow further *in vitro* investigation of the existence of hypothalamic TSH inhibitory control factors.

In conclusion, we have successfully tested an *in vitro* incubation system for red drum pituitary glands which can be sensitively analyzed individually by real-time PCR. This system should allow us to observe the effects of various compounds on TSHβ expression which will hopefully lead to the discovery of TIF and further the understanding of the teleost fish thyroid axis.

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