BIOLOGICAL ACTIVITY OF THYROTROPIN IN TWO TELEOST FISH, RED DRUM (*Sciaenops ocellatus*) AND GOLDFISH (*Carassius auratus*)

A Dissertation

by

THOMAS CHARLES MILLER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Major Subject: Zoology

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Approved by:

Chair of Committee,	Duncan MacKenzie
Committee Members,	Scott Jaques
	Delbert Gatlin
	Gil Rosenthal
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ABSTRACT

Biological Activity of Thyrotropin in Two Teleost Fish, Red Drum (*Sciaenops ocellatus*) and Goldfish (*Carassius auratus*). (May 2011)

Thomas Charles Miller, B.S. North Carolina State University

Chair of Advisory Committee: Dr. Duncan S. MacKenzie

Thyrotropin (TSH) is a glycoprotein hormone released from the pituitary gland to promote the synthesis and secretion of thyroid hormone. The existence of well-established peripheral mechanisms for regulation of thyroid hormone delivery to targets has called into question the significance of TSH as a primary regulator of circulating thyroid hormone concentrations in fish. However, relatively little is known about the regulation or action of endogenously secreted teleost TSH, largely due to lack of purified TSH suitable for biological testing and immunoassay development. I developed a red drum *in vivo* bioassay to aid in the production and purification of recombinant TSH from the red drum, a perciform fish demonstrating dynamic daily thyroxine (T_4) cycles hypothesized to be driven by TSH. Exogenous bovine TSH injection resulted in a time and dose-dependent increase in circulating TSH and T_4 in red drum. However, the sensitivity of the red drum thyroid gland to stimulation by bovine TSH was lost during growth under controlled laboratory conditions, even when circulating levels of exogenously-administered mammalian TSH remained elevated. The insensitivity of the thyroid was not due to prior TSH injection or feed source. Because insensitivity of the

red drum thyroid precluded their use as a bioassay species, the plasma TSH and T_4 response to exogenous TSH was next characterized in goldfish. The T_4 response in goldfish was stable and repeatable, with T_4 levels peaking at 5 hours and remaining elevated for more than 11 hours after bovine TSH injection. Plasma TSH peaked from 2-5 hours following TSH injection with more than 90% cleared by 11 hours. The goldfish bioassay was further utilized to evaluate the effects of structural modifications on TSH biological activity. Substitution of four positively charged amino acids at the n-terminal of the glycoprotein hormone subunit α (GSUα), which increase the potency of recombinant human TSH, had the same effect in goldfish. The heterothyrotropic potency of mammalian follicle stimulating hormone in goldfish was also enhanced by the same amino acid substitutions. Finally, the importance of oligosaccharides to TSH bioactivity was also examined in goldfish. Deglycosylation abolished TSH bioactivity, even when immunoreactivity persisted in circulation. Furthermore, recombinant canine TSH was less potent when produced in cell lines generating insect-type glycosylation than when produced in a cell line capable of mammalian-type glycosylation. These studies utilizing recombinant mammalian demonstrated conservation of mammalian TSH hormone-receptor interactions in goldfish, suggesting TSH function might likewise be conserved. Thus, I have established goldfish as a sensitive and stable bioassay which can now be utilized to monitor the biological activity of teleost TSH expressed *in vitro* as well as to evaluate how structural modifications of the TSH molecule influence its vivo biological activity.

DEDICATION

I dedicate this dissertation to my wife, Jocelyn Miller, for the loving support that made this possible.

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I would like to thank my committee chair, Dr. Duncan MacKenzie, and my committee members, Dr. Scott Jaques, Dr. Delbert Gatlin, and Dr. Gil Rosenthal, for their guidance and support throughout the course of this research.

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CHAPTER I

INTRODUCTION

Thyroid hormones regulate many important physiological processes including development, growth, metabolism, and reproduction (Norris, 2007). The hormone predominantly released from the thyroid is thyroxine (tetraiodothyronine, T_4). T_4 is a prohormone converted to 3,5,3'-triodothyronine (T_3) within peripheral tissues through the cleavage of iodine from its outer phenolic ring by outer-ring deiodinase (ORD). Intracellular T_3 can bind to nuclear receptors to regulate transcription of genes or travel to other tissues by reentering the circulation. T_4 and T_3 not bound by blood transport proteins (free T_4 and free T_3) are available for uptake by cells and increase proportionally with the amount of thyroid hormone released by the thyroid (Eales and Brown, 1993). Thus, T_4 secretion drives thyroid hormone stimulation of target tissues by increasing the intracellular substrate for T_3 synthesis.

In mammals, thyrotropin (thyroid stimulating hormone, TSH) stimulates the biosynthesis and release of thyroid hormone from the thyroid gland (Szkudlinski et al., 2002). Thyrotropin is a glycoprotein comprising a hormone-specific β subunit coupled to an α subunit (glycoprotein subunit α, GSUα) which is shared with the two other

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This dissertation follows the style of Integrative and Comparative Biology.

pituitary glycoprotein hormones, the gonadotropins (GTHs). Thyrotropin release in mammals is controlled by the hypothalamus, predominantly through stimulatory actions of thyrotropin releasing hormone (TRH), and through T_4 and T_3 negative feedback at the hypothalamus and pituitary. These central regulatory mechanisms of TSH release ensure appropriate amounts of thyroid hormone are maintained within the circulation. Plasma TSH concentrations reflect circulating thyroid hormone levels to the degree that measurement of TSH is often used to diagnose clinical hypothyroidism and hyperthyroidism and can be used to determine appropriate thyroid hormone doses in clinical hypothyroidism (DeGroot and Jameson, 2001). Moreover, it has been demonstrated that injections of recombinant human TSH elevate T_4, T_3, free T_4, and free T_3 (Nielsen et al., 2004b), and that plasma TSH shows a circadian rhythm which corresponds with an increase of free T_3 in humans (Russell et al., 2008). These findings suggest that in mammals TSH centrally promotes thyroid hormone action by providing prohormone for intracellular T_3 production.

Although it is known that TSH can activate thyroid hormone biosynthesis and secretion in non-mammalian vertebrate species, Eales and Brown (1993) hypothesized that TSH may not be as important in promoting peripheral T_3 production in teleost fish as it is in mammals. Control of thyroid hormone secretion by the pituitary gland appears to have evolved after agnathans, ancient vertebrates which have functional thyroids but lack TSH (Sower et al., 2006; Uchida et al., 2010). This led Eales and Brown (1993) to suggest that thyroid function arose independent of hypothalamic-pituitary control, and may still be regulated independently of central control in more ancient vertebrate groups,

including actinopterygian fish. They suggested that hypothalamic inhibition of the thyroid axis in teleost fish (Ball et al., 1963; MacKenzie et al., 1987; Olivereau and Ball, 1966; Peter, 1970) results in low pituitary TSH secretion, with TSH consequently playing a minor role in maintaining thyroid function of teleosts. Additionally, Eales and Brown (1993) argued that if TSH drives T_3 formation in teleost fish through the provision of T_4 substrate, then it would be expected that an increase in plasma T_4 would cause an increase of plasma T_3. The resultant increase in T_3 should then negatively feedback on TSH secretion to limit further formation of T_3. However, large exogenous doses of T_4 or TSH fail to increase plasma T_3 in teleosts, and there is evidence that exogenous T_3 does not reduce TSH secretion (Eales and Brown, 1993). Therefore, Eales and Brown (1993) proposed that teleost fish utilize peripheral mechanisms such as deiodination, blood binding proteins, and plasma clearance to regulate thyroid hormone provision to target cells.

Since Eales and Brown (1993) proposed that central mechanisms may play a secondary role in regulating plasma thyroid hormones, evidence has accumulated which demonstrates that TSH in teleosts is robustly produced under dynamic control of the hypothalamus. It was first demonstrated nearly 50 years ago that homologous TSH purified from pituitaries increases T_4 production in eels (Fontaine, 1964). This has since been verified in other teleost species (Bandyopadhyay and Bhattacharya, 1993; Byamungu et al., 1991; Kuhn et al., 1986; Marchelidon et al., 1991; Ng et al., 1982; Roy et al., 2000a). More recent studies have found easily detectable TSH mRNA expression in a number of teleost species (Chatterjee et al., 2001; Cohn et al., 2010; Ito et al., 1993;

Larsen et al., 1997; Schmitz et al., 1998; Yoshiura et al., 1999). It has also been demonstrated in teleost fish that T_3 as well as T_4 reduces TSH mRNA expression *in vivo* and *in vitro* (Chatterjee et al., 2001; Chowdhury et al., 2004; Cohn et al., 2010; Larsen et al., 1997; Manchado et al., 2008a; Pradet-Balade et al., 1999; Pradet-Balade et al., 1997; Schmitz et al., 1998; Sohn et al., 1999b; Yoshiura et al., 1999). TRH has been shown to increase TSHβ mRNA expression *in vitro* in the Japanese eel (Han et al., 2004) while corticotropin releasing hormone (CRH) stimulates TSH secretion *in vitro* in the coho salmon (Larsen et al., 1998). In the bighead carp leptin and β-endorphin as well as TRH have been shown to increase while neuropeptide Y and galinin decrease TSHβ mRNA expression *in vitro* (Chowdhury et al., 2004). These results suggest stimulatory and inhibitory hypothalamic factors interact with T_4 and T_3 negative feedback to precisely regulate teleost TSH production and secretion. Additionally T_3 negative feedback on TSH further suggests central TSH secretion is important in driving thyroid hormone delivery to target cells in teleost fish (MacKenzie et al., 2009).

Other studies of TSHβ mRNA expression in teleost fish further suggest that TSH secretion may play an important role in promoting cellular actions of thyroid hormones. Increases in TSHβ mRNA expression correlate with increases of circulating thyroid hormone known to stimulate developmental changes (Han et al., 2004; Manchado et al., 2008b; Martin et al., 1999). In contrast, there is an inverse relationship between TSHβ mRNA steady state levels and thyroid hormones during seasonal cycles in goldfish, suggesting that TSHβ mRNA depletion reflects an increase in translation and secretion (Sohn et al., 1999a). Although these studies suggest TSH can regulate thyroid hormone

delivery to targets in teleosts, it will not be possible to conclusively establish the role of central mechanisms in driving thyroid hormone supply to targets in teleost fish without knowledge of TSH secretion rates and circulating levels.

Only one immunoassay, a coho salmon (*Oncorhynchus kisutch*) TSH αβ - α subtractive radioimmunoassay (RIA), has been developed to measure piscine TSH (Moriyama et al., 1997). This RIA was used to demonstrate that negative feedback of thyroid hormone on TSH is functional in the coho salmon, as T_3 decreased plasma TSH while methimazole, a thyroid hormone synthesis inhibitor, increased plasma TSH. The coho salmon TSH RIA was also used to demonstrate that CRH increased TSH secretion *in vitro* (Larsen et al., 1998). These studies successfully demonstrated that a piscine TSH immunoassay can be used effectively to study regulation of TSH secretion and aid in determining the relationship between plasma TSH release and thyroid hormone levels. However, the salmon RIA was not used to examine the role of TSH in driving dynamic changes in thyroid hormone, such as during smoltification. Low basal TSH and thyroid hormone levels and a relatively low magnitude T_4 response to exogenous TSH (Moriyama et al., 1997) suggest that coho salmon may have limited utility as a model species for understanding central regulation of teleost fish thyroid function. Furthermore, the assay was created as a subtractive RIA due to the difficulty encountered in obtaining large enough quantities of purified TSH to generate a TSH antibody. The lack of purified TSH suitable for biological testing and immunoassay development has been a major obstacle to improving our understanding of teleost fish TSH physiology. The objective of this study was therefore to develop techniques to

enable the production, purification, and characterization of biologically active teleost TSH produced using an *in vitro*, recombinant system.

Glycosylation of TSH in expression systems

Although TSH has been sequenced in many teleost species, expression systems have yet to be utilized for developing recombinant teleost TSH. Recently, piscine gonadotropins from a number of fish species were produced using *in vitro* expression systems (Levavi-Sivan et al., 2009). These hormones have successfully been used in the creation of piscine gonadotropin immunoassays (Aizen et al., 2007b) and were biologically active *in vitro*. However, the same hormones had diminished potencies *in vivo* (Levavi-Sivan et al., 2009). It was hypothesized that the reduced *in vivo* activity of recombinant piscine gonadotropins could have been due to the limited glycosylation capabilities of the non-mammalian expression systems that were used to produce them (Kazeto et al., 2008).

In mammals, glycosylation of TSH is important for its production, decreases its clearance rate, and increases receptor binding and activation (Szkudlinski et al., 2002). Two asparagine-linked, biantennary oligosaccharide structures are attached to GSUα and one to TSHβ by an N-acetylglucosamine. The terminal sequences of endogenously secreted TSH glycans are most often sulfonated N-acetylgalactosamine ($S0_4$-GalNAc) or galactose and sialic acid (Green and Baenziger, 1988). Thyrotropins with glycans that terminate in sialic acid, such as recombinant human TSH produced in mammalian cell lines, escape removal by the liver and instead are cleared more slowly by the kidneys, thereby increasing the duration of *in vivo* biological activity (Szkudlinski et al., 1993;

Szkudlinski et al., 1995a). In contrast, human TSH produced in insect cells contains oligosaccharides which terminate in highly branched mannose residues and have increased *in vitro* activity but reduced *in vivo* activity (Grossmann et al., 1997).

Compared to mammals, relatively little is known about the regulation or utilization of glycans on endogenously secreted teleost glycoprotein hormones. Molecular cloning of TSH from a number of teleost species demonstrated conservation of N-glycosylation sites throughout vertebrate evolution (MacKenzie et al., 2009). Thyrotrophic fractions from the pituitaries of several fish species bind to lectin columns and elute with buffers containing 0.15M α-methylglucopyranoside (α-MG), suggesting that, like mammals, teleost TSH glycans have α-D-glucopyranosyl end groups or internal 2-o-linked-D-mannopyranosyl residues (Ng et al., 1982). Enzymes which transfer $S0_4$-GalNAc and sialic acid onto glycan residues have been found in teleost species and the LH glycans of coho salmon are fucosylated, biantennary structures often terminating in $S0_4$-GalNAc or galactose and sialic acid (Harduin-Lepers et al., 2005; Manzella et al., 1995). Manzella et al. (1995) concluded that the strong conservation of LH carbohydrate moieties throughout vertebrate evolution suggests sulfonated and sialic acid capped glycans play an important physiological role in teleosts, most likely preventing the selective clearance of vertebrate LH and TSH from circulation.

If mammalian-like carbohydrates are important for biological activity in teleosts, then teleost TSH should be generated using a recombinant system capable of producing more complex glycans. Though prokaryotic, yeast, and insect expression systems are relatively easy to maintain and produce high concentrations of proteins, they attach

carbohydrate residues most often terminating in mannose. Mammalian cell lines produce the more complex glycan residues but are more difficult to produce and maintain and have relatively low protein yields (Grossmann et al., 1997; Montreuil et al., 1995; Verma et al., 1998). Recently, SWT-4 insect cell lines have been developed from *Spodoptera frugiperda* (SF)-9 cell lines which are biologically engineered to contain the enzymes capable of producing mammalian type carbohydrates while still producing high protein yields. These SWT-4 cell lines along with SF-9 cell lines have been successfully used to produce canine TSH (Dr. Scott Jaques, personal communication). Any differences in the biological activity of these forms of recombinant canine TSH could be attributed to the carbohydrates, since the protein backbones are identical. Thus, comparing the potencies and clearance of SF-9 and SWT-4 canine TSH in a fish bioassay would aid in the determination of an appropriate recombinant system to produce fish thyrotropins that could be used to evaluate the effects of homologous TSH and create immunoassays.

Recombinant teleost TSH must be purified from the *in vitro* media in which it is produced. Because the complex media used for insect cell lines contain large amounts of glycoproteins to ensure that TSH is properly glycosylated (Jaques and Jarvis, 2006), techniques previously used to purify teleost TSH from pituitary homogenates must be modified. For example, ethanol precipitation used successfully to purify teleost glycoprotein hormones in the past (Swanson et al., 1987; Swanson et al., 1991) would concentrate too many glycoproteins to be a useful technique to purify recombinant teleost TSH from insect cell incubation media. Additionally, traditional pituitary

homogenate purification techniques yield relatively small volumes, allowing for samples to be lyophilized between purification steps (Moriyama et al., 1997; Swanson et al., 1987). Recombinant TSH, in contrast, is produced in large quantities and volumes (Jaques and Jarvis, 2006), making lyophilization during the initial stages of purification difficult. Ion exchange chromatography is a technique that allows for separation of proteins with varying charges from large volumes (Williams and Frasca, 2001). The net charge of TSH is dependent upon its isoelectric point (pI) which ranges from 4.5 to 8.8 (Sergi et al., 1991). Initially increasing the pH of insect cell media to levels just below the pI of TSH would thereby allow TSH and other proteins with equal or higher pI to pass through an anion exchange column (Williams and Frasca, 2001). If the pH of media containing TSH is lowered to a value below its pI, then TSH would adhere to cation resins and allow proteins with higher pI's to be separated from TSH. TSH could then be eluted from the cation column using a buffer with a high concentration of charged ions, such as NaCl. The large volume of salty solution containing TSH could then be subjected to tangential flow chromatography, which allows for salt and water but not TSH or other larger proteins to permeate through a hollow fiber membrane with a molecular weight cut off smaller than TSH (Shinkazh et al., 2010). This would concentrate TSH in the retentate while reducing salinity of the buffer. Once a small volume of concentrated TSH is obtained, it can be subjected to size exclusion chromatography. Size exclusion chromatography resins allow large molecular weight molecules to pass more quickly (Mori and Barth, 1999), thereby separating TSH from any remaining proteins of differing size and eliminating any remaining salts. Relatively

small fractions can be then collected from a size exclusion column, lyophilized, and tested for TSH concentration using an immunoassay or for biological activity using a bioassay.

Superactive human glycoprotein hormones

Characterization of TSH structure-function relationships in mammals have led to the generation of superactive human glycoprotein hormones which could be utilized to determine how conserved the relationships between TSH and the TSH receptor (TSH-R) are in teleost fish. Bovine TSH binds to and activates the human TSH-R to a greater degree than human TSH despite high homology in the amino acid sequences of alpha subunit (74.1%) and beta subunit (88.4%) (Szkudlinski et al., 1996). Four positively charged amino acids within positions 11-20 of the bovine GSUα not found in human GSUα were suggested to cause a greater electrostatic interaction with the hTSH-R (Szkudlinski et al., 1996). Using site-directed mutagenesis, it was demonstrated that substituting four lysine molecules in place of acidic or neutral amino acids in the n-terminal of the human GSUα generated a human TSH analog (TR-1401) equipotent to bTSH *in vitro* (Leitolf et al., 2000). Two negatively charged residues in the hinge region of the human TSH-R (Glu^{297} and Asp^{382}) were recently demonstrated to be necessary and sufficient to cause the increased binding by bTSH and TR-1401 over hTSH (Mueller et al., 2009). Because the interaction of basic amino acids in the n-terminal of the GSUα and the LH receptor is also present in humans (Szkudlinski et al., 1996), it is possible that this mechanism of binding is an ancient and conserved feature of glycoprotein hormones and their receptors, having evolved before the divergence of the LH and TSH-

Rs. Since teleost fish are the most ancient vertebrate from which TSH and its receptors have been cloned (MacKenzie et al., 2009), it would be interesting to determine if basic amino acids in the n-terminal of GSUα increase activation of teleost TSH-Rs.

Because the amino acid substitutions which create the superactive hTSH analog are located in the GSUα which is shared with the GTHs, it is possible that if the same positive charges are inserted into GTHs they would become thyrotropic. Though increased homologous GTH in goldfish does not increase T_4 (MacKenzie et al., 1987) it has been reported that mammalian GTHs have an intrinsic ability to activate fish TSH-Rs (heterothyrotropic activity) (Brown et al., 1985; Byamungu et al., 1990; Fontaine, 1969; MacKenzie, 1982). Since the divergence of teleost fish, it is possible mammalian GTHs independently evolved structural characteristics capable of activating the teleost TSH-R. However, GTHs used for these studies were pituitary-purified and though thyrotropic activity was determined in mammalian TSH bioassays, TSH immunoassays were not used to quantify TSH contamination. More recent studies have demonstrated that amago salmon TSH-Rs are stimulated by pituitary purified luteinizing hormone (LH) and follicle stimulating hormone (FSH) in transiently transfected cells (Oba et al., 2000) but recombinant FSH and LH, which lack TSH contamination, were not able to activate transiently expressed striped bass TSH-Rs (Kumar et al., 2000). For the first time in teleost fish, we will utilize a modern mammalian TSH immunoassay to determine the TSH contamination of mammalian gonadotropins prior to injection and inject recombinant gonadotropins to more accurately determine thyrotropic activity of mammalian GTHs *in vivo*. Additionally, if the human FSH modified to be more

thyrotropic in humans is also more thyrotropic in teleost fish, it would provide further evidence of strong conservation across vertebrates of the hinge region of the TSH-R which interacts with the modified amino acids.

Goldfish TSH bioassay

Goldfish have a history of being successfully used for the bioassay of heterologous TSH. Gorbman (1940) first suggested goldfish were a suitable bioassay species for mammalian thyrotropin due to their low cost, small size, ease of maintenance, and sensitivity to exogenous TSH. The goldfish TSH bioassay was re-established by Ortman and Billig (1966) and was demonstrated to be sensitive enough to monitor thyroid status in human clinical patients (Hutton and Hayter, 1970). Since then, goldfish plasma T_4 has been demonstrated to respond in a dose- and time-dependent fashion to bovine TSH injections (MacKenzie et al., 1987). My goal was to utilize modern mammalian TSH immunoassays to characterize the clearance of mammalian TSH in goldfish as well as the T_4 response after an injection and determine the repeatability of the assay utilizing the same fish. Determining how circulating TSH is regulated in fish would further our understanding of how central mechanisms regulate thyroid hormone release.

A well characterized goldfish bioassay could be used to monitor the biological activity of fractions during the purification of recombinant teleost TSH as well as relate the protein structure of superactive human glycoprotein hormones to their ability to activate their TSH-R. Teleost TSH-Rs expressed in mammalian cell lines have provided contradicting results, with LH or FSH having thyrotropic activity in amago salmon and

African catfish (Oba et al., 2000; Vischer and Bogerd, 2003) but not in the striped bass (Kumar et al., 2000). These divergent findings could be due to multiple species of glycoprotein hormones being used or species specificity of receptor binding. However, endogenous teleost TSH-Rs expressed by mammalian cells in an *in vitro* system may have altered sensitivity to hormones compared to when expressed by thyroid follicular cells *in vivo*. It would be more appropriate to utilize piscine thyroid cell cultures, but the thyroid is dispersed in most fish making it difficult to culture thyroid follicles without other contaminating tissue. The lower jaw of a number of fish have been incubated to determine if there are direct effects of gonadotropin-releasing hormone on T_4 secretion, but the assay was not validated with TSH and it is impossible to determine direct effects on thyroid tissue with so much other tissue present in the incubation media (Chiba et al., 2004; Roy et al., 2000b). The encapsulated thyroid of parrot fish has also been utilized to demonstrate that coho salmon TSH was more potent *in vitro* than bTSH, while bTSH was more potent *in vivo* in coho salmon (Swanson et al., 1987; Swanson et al., 1988). Thus, coho salmon TSH that is biologically active *in vitro* could be inactivated or cleared from circulation too rapidly to be biologically active *in vivo* (Swanson et al., 1988), as has been shown for human TSH produced in insect cell lines (Grossmann et al., 1997). Therefore, thyrotropic potencies of recombinant hormones found *in vitro* should be verified using an *in vivo* bioassay, such as in the goldfish TSH bioassay. Since goldfish and mammalian thyrotropins are available worldwide, establishing the goldfish T_4 responses to multiple species of mammalian TSH would allow for other researchers to utilize it as a standardized teleost bioassay, as has been done with the

McKenzie mouse bioassay (East-Palmer et al., 1995). Goldfish have low circulating levels of thyroid hormone, with T_4 reported to vary by less than 2 ng/ml over 24 hours, independent of food intake (Spieler and Noeske, 1984). Likewise, seasonally goldfish thyroid hormones only vary in magnitude by approximately 7 ng/ml (Sohn et al., 1999a), suggesting their endogenous TSH production may be relatively low. Although these low basal levels make them good candidates for TSH stimulation studies, low amplitude changes in thyroid hormones make them less interesting subjects for studies of dynamic regulation of circulating TSH. It would more desirable to produce recombinant teleost TSH for development of a TSH immunoassay in a teleost species which have regulated seasonal and diurnal thyroid hormone cycles, suggesting measurable changes in circulating TSH. Such a species could provide a valuable laboratory model for examining the regulation of TSH production, secretion, and action in fish.

Red drum

The red drum is an aquacultured perciform fish whose high magnitude daily T_4 cycles are entrained to photoperiod and feeding schedule (Leiner and MacKenzie, 2001). It has been demonstrated that the peak of the daily T_4 cycles of red drum could be diminished by T_3 immersion, leading Leiner and MacKenzie (2003) to propose that TSH, under control of the hypothalamus and subject to negative T_3 feedback, drives the daily T_4 cycle. Wild red drum thyroid hormones show large magnitude seasonal cycles of thyroid hormone with plasma T_4 elevated in spring, T_3 maximal during the summer months, and the magnitude of both increasing annually by over 20 ng/mL (D. MacKenzie, unpublished data). Moreover, red drum grow quickly but can be studied as

juveniles for more than two years in captivity, avoiding complicating effects of reproductive hormones on the thyroid axis found in other fish of similar size, such as goldfish (Cyr and Eales, 1996). Red drum circulating T_4 responds in a dose- and time-dependent fashion to bovine TSH injections (Leiner, 2000), demonstrating the thyroid gland is sensitive to TSH. The red drum is thus a desirable species for which to generate an homologous TSH immunoassay to study the contribution of centrally-regulated TSH secretion to the control of thyroid function in a teleost fish. This will require the development of methods for producing homologous red drum TSH, either through traditional pituitary purification or more recent *in vitro* expression methods, as well as establishment of techniques to evaluate the biological activity of the hormones produced. It is particularly important to establish a TSH bioassay which can be utilized to ensure that homologous TSH generated for antibody production is biologically active. Such a TSH bioassay, to be suitable for use over the prolonged periods to evaluate multiple, small yield preparations of TSH, should be simple sensitive, stable, and inexpensive to conduct.

Significance

The overall goal of this study is to better understand the importance of central regulation of thyroid hormones in teleost fish by developing and characterizing fish bioassays that will aid in the purification and characterization of TSH, furthering our understanding of TSH regulation and receptor activation. I will first establish a red drum bioassay which could be used to validate red drum TSH produced in a recombinant system and then determine how stable their T_4 response to exogenous TSH is. Then I

will reestablish a goldfish bioassay which could be utilized to monitor the production and purification of recombinant TSH produced for mammals or fish.

If TSH regulates thyroid function in teleost fish as in mammals, then structural mechanisms which ensure specificity of binding of TSH to its receptor likely evolved before the divergence of actinopterygians from the vertebrate lineage. However, studies demonstrating the binding of mammalian GTHs to teleost TSH-Rs suggest that considerable evolution of glycoprotein hormone and receptor structure occurred after the evolution of actinopterygians. One goal of these studies is to begin to determine what aspects of the protein structure of mammalian GTHs allow them to bind to the teleost TSH receptor. This will help identify structural characteristics of the TSH molecule important for binding to fish TSH-Rs, and help determine whether TSH structure-function relationships established for mammals are also conserved in teleost fish. Specifically, I will determine if positively charged amino acids at the n-terminal of the GSUα are important for thyrotropic activity of glycoprotein hormones in fish as in mammals (Leitolf et al., 2000).

Because carbohydrates directly increase TSH synthesis, duration in circulation, and receptor activation (Szkudlinski et al., 2002), an examination of the ability of differentially-glycosylated TSHs to activate thyroxine secretion should allow for a better informed choice of expression system for production of recombinant teleost glycoprotein hormones. Comparing the biological activity of canine TSH produced in cell lines that differ in their abilities to glycosylate will allow us to determine if mammalian-type glycosylation enhances TSH biological activity in teleost fish. These studies will also

aid in the purification and characterization of recombinant canine TSH which is being developed for application to the diagnosis of clinical hypothyroidism in companion animals. Determining which expression systems appropriately glycosylate TSH for teleost fish will also contribute to the production of biologically active teleost gonadotropins for use to promote reproductive activity in research, aquaculture, and species conservation studies.

Production of recombinant teleost TSH for homologous TSH immunoassays might also aid in the detection and characterization of environmental contaminants. Experimental alteration of thyroid function can impair nervous system development and behavior, inhibit reproduction, diminish body growth and bone development, alter metabolism, body composition, and nutrient utilization, and impact the function of a variety of other endocrine systems (McNabb, 1992). Chemicals which disrupt thyroid function may thus have significant impact on growth and development of wild animals (Brucker-Davis, 1998; Colborn, 2002; Rolland, 2000). Traditionally, thyroid disruption has been measured using histology or circulating thyroid hormone levels. Histological examination of changes in thyroid structure is an accepted bioassay, but requires long exposure and is not very sensitive (Eales et al., 1999). Furthermore, compensation mechanisms can function to maintain total thyroid hormone levels despite disruption of their synthesis and release, confounding efforts to use thyroid hormones as an index of thyroid disruption. In contrast, increases of TSH which serves as a primary component of these compensation mechanisms, directly reflect alterations in thyroid function and are consequently often utilized in the diagnosis and monitoring of clinical thyroid

disruption, such as hypothyroidism and hyperthyroidism (DeGroot and Jameson, 2001). Immunoassays for the structurally similar and functionally analogous gonadotropins have been successfully applied to monitor reproductive disruption in non-mammalian species, including fish (Guillette, 2001; Thomas and Rahman, 2007). Similar assays for TSH may thus provide sensitive tools for understanding the impact of proposed disruptors on fish thyroid function.

CHAPTER II

SENSITIVITY OF RED DRUM (*Sciaenops ocellatus*) TO EXOGENOUS THYROTROPIN

Introduction

Thyrotropin (thyroid stimulating hormone, TSH) is a pituitary glycoprotein hormone that stimulates thyroid follicles to synthesize and secrete thyroid hormones, primarily thyroxine (T_4). Following the deiodination of T_4 into 3,5,3'-triiodothyronine (T_3) in target cells, T_3 binds to nuclear receptors to regulate transcription of genes which promote development, metabolism, and growth (DeGroot and Jameson, 2001; Norris, 2007). In mammals, it has been demonstrated that injections of TSH elevate free T_4 and free T_3 (Nielsen et al., 2004b), and that plasma TSH shows a circadian rhythm which corresponds with an increase of free T_3 in humans (Russell et al., 2008). This suggests that central activation of TSH-dependent T_4 secretion is a primary driver of thyroid hormone supply to target cells in mammals (DeGroot and Jameson, 2001). In contrast, active deiodination pathways and the inability of exogenously applied T_4 to consistently increase T_3 levels has led to the hypothesis that in teleost fish TSH plays a less important role by simply maintaining a circulating pool of T_4 prohormone from which peripheral mechanisms regulate cellular utilization (Eales and Brown, 1993).

As TSH is the critical central signal activating thyroid hormone synthesis and release, a thorough knowledge of its physiology is essential to an understanding of the

relative importance of the hypothalamo-pituitary axis in the regulation of teleost thyroid function. However, largely due to the lack of purified teleost TSH suitable for biological testing and immunoassay development (MacKenzie et al., 2009), examination of the effects of TSH on the fish thyroid have been mostly limited to studies utilizing mammalian TSH (Brown and Stetson, 1985; Chan and Eales, 1976; Kuhn et al., 1986; MacKenzie, 1982; MacKenzie et al., 1987; Milne and Leatherland, 1978; Swanson and Dickhoff, 1987). These studies have consistently found that exogenous, heterologous thyrotropins are capable of stimulating growth, iodine uptake, and T_4 secretion by the teleost thyroid. Additionally, in coho salmon (*Oncorhynchus kisutch*) and mummichog (*Fundulus heteroclitus*), mammalian TSH has been used to characterize seasonal changes in the sensitivity of the thyroid to TSH stimulation in fish under natural conditions (Brown and Stetson, 1985; Swanson and Dickhoff, 1987). Whereas this demonstrates that exogenous mammalian TSH is useful for examining the regulation of the thyroid axis in fish, due to lack of homologous TSH suitable for biological testing and immunoassay development relatively little is known about the regulation or action of endogenously secreted teleost TSH (MacKenzie et al., 2009). It has been proposed that recombinant systems should be utilized to produce teleost TSH (MacKenzie et al., 2009), which would allow for production of large amounts of protein without contamination of the structurally similar GTHs. This homologous fish TSH could then be used to develop immunoassays suitable for the studies of basal TSH secretion and TSH action needed to help clarify its importance in the physiological regulation of fish thyroid function.

Although thyroid function has been examined in a number of teleost species, only a few appear to serve as suitable models for the examination of TSH function. Ideally, such a species should be large in size (to facilitate blood sampling), easy to obtain and maintain in the laboratory, and have a well characterized pituitary-thyroid axis. Additionally, it is most desirable to study TSH function in a species which appears to display dynamic TSH changes, reflected in dynamic changes in circulating T_4. The red drum (*Sciaenops ocellatus*) is a perciform fish developed as an aquaculture species on the Gulf Coast. In captivity, red drum exhibit dynamic, high magnitude daily T_4 cycles that are sensitive to environmental manipulation and thyroid hormone feedback (Leiner and MacKenzie, 2001), suggesting the cycles are driven by central mechanisms (Leiner and MacKenzie, 2003). In the wild, circulating red drum thyroid hormones show dramatic seasonal changes with plasma T_4 peaking in spring, T_3 reaching a maximum during the summer months, and the magnitude of annual changes of both plasma T_4 and T_3 exceeding 20 ng/mL (D. MacKenzie, unpublished data). These dynamic daily and seasonal changes in T_4 levels suggest that plasma TSH is dynamically regulated, making red drum a good candidate species for more detailed studies of the physiological function of fish TSH. As a first step towards producing a recombinant red rum TSH suitable for development of a red drum TSH immunoassay, my objective was to develop a red drum TSH bioassay. To better understand the time course of TSH activation of T_4 secretion, I utilized for the first time a mammalian TSH immunoassay to determine the duration of circulating TSH after injection in a teleost fish. Through use of this TSH immunoassay, I was able to demonstrate that sensitivity of the red drum

thyroid gland to stimulation by mammalian TSH can be lost during growth under controlled laboratory conditions, even while circulating levels of exogenously-administered mammalian TSH remain elevated.

Materials and methods

Animals

Red drum (0.5-1g) were obtained from Texas Department of Parks and Wildlife Sea Center Texas hatchery in Lake Jackson, Texas and transported to holding facilities at Texas A&M in the Biological Sciences Building East (BSBE) or the Aquacultural Research and Teaching Facility (ARTF). Fish kept in BSBE were held in 400 liter, round fiberglass tanks connected to a 7,500 liter recirculating system. Red drum kept at the ARTF were held in 800 liter, round fiberglass tanks connected to a 15,000 gallon recirculating system. Red drum at both locations were held in artificial 6 ppt seawater at 25 ^{o}C on 12L:12D photoperiod. Fish held in BSBE and the ARTF were fed once a day to apparent satiation with Aquamax Grower 400 (PMI Nutrition International LLC, Brentwood, MO) and Rangen Salmon Grower #4 (Rangen Inc., Buhl, ID) commercial fish feed, respectively. All fish were juveniles and weighed between 20 and 110 g when used for experiments.

Injection protocol

For TSH injection, groups of 7 fish were removed from the tanks, anesthetized by immersion in tricaine methanesulfonate (MS-222) (200mg/L), injected intraperitoneally (IP) with varying doses of bovine TSH (bTSH, Sigma, 2.2 IU/mg) in BSA-saline, and then returned quickly to their respective tanks. Control fish were

injected with BSA-saline. Fish were recaptured at specific times after injection and anesthetized to obtain approximately 250µl of blood from the caudal vasculature into 1 ml heparinized syringes. Blood was centrifuged to separate plasma which was frozen for hormone determination.

Hormone measurement

Specific immunoassays were used to measure blood bTSH, T_4, and T_3 at the Endocrine Diagnostic Laboratory at the Texas Veterinary Medical Diagnostic Laboratory. Total T_4 and T_3 were measured using Coat-A-Count RIA tubes (Siemens). Sensitivity (defined by the lowest point on the linear portion of the standard curve) of the T_4 assay was 2.5 ng/ml; inter- and intra-assay variabilities were 3.8 and 9.1% (calculated as coefficient of variation, CV), respectively (manufacturer's data). Serial dilutions of red drum plasma ran parallel to the standard curve. At 1,000 ng/ml T_3 has 2% cross reactivity in the T_4 RIA (manufacturer's data) and 88 ± 3% (N=4) of T_4 spiked into red drum plasma was recoverable. Sensitivity of the T_3 assay was 0.7 ng/ml; inter- and intra-assay variabilities were 7.6 and 5.8%, respectively (manufacturer's data). At 100 ng/ml T_4 has 0.38% cross reactivity in the T_3 RIA. Blood samples were diluted six fold using phosphate buffered saline before T_3 values were determined.

Plasma bTSH was measured using an IMMULITE 2000 canine TSH immunoassay (Siemens). This assay sensitivity was 0.01 ng/mL (as defined by the lowest dose on the linear portion of the standard curve) and inter- and intra-assay variabilities were 7.6 and 5.5%, respectively. Bovine TSH in red drum plasma diluted parallel to standards. TSH values for control plasma were always below the sensitivity

of the assay. Endogenous TSH in red drum plasma was therefore either at levels below the sensitivity of the immunoassay or did not cross-react within the assay.

Time and dose response of T_4 to TSH stimulation

The objective of these experiments was to determine whether bTSH stimulated a time and dose dependent T_4 elevation *in vivo,* and whether this response could be replicated over time in the same red drum. The single group of fish was used for Experiments 1-4. In the time course experiments, I examined the clearance of bTSH and the T_4 response to bTSH injections in fish held in BSBE for 4 (30 ± 1 g mean wt, Experiment 1) and 22 weeks (105 ± 6 g mean wt, Experiment 4), respectively. In both experiments, fish were weighed, injected with 5 mU/gBW bTSH, plasma was taken at various times between 30 minutes and 11 hours, and the resultant plasma TSH and T_4 were measured. T_3 levels were also determined in Experiment 1. In a subsequent pair of dose response experiments, increasing doses of bTSH (0.078125 to 10 mU/gBW) were injected into fish held in BSBE for 6 (37 ± 2 g mean wt, Experiment 2) or 17 weeks (77g ± 4 mean wt, Experiment 3). Fish were bled 5 hours after injection for measurement of circulating bTSH and T_4.

TSH insensitivity in red drum

After being held in BSBE for at least 17 weeks, red drum became insensitive and no longer responded to TSH injection. To determine whether this insensitivity was due to repeated exposure to bTSH, red drum from the same hatchery cohort as red drum from experiments 1-4 were grown in BSBE or at the ARTF for 22 weeks (133 ± 8 g mean wt, Experiment 5). By this time, the fish maintained in BSBE had been used for 5

previous bTSH injection experiments whereas fish from the ARTF had never been injected. ARTF fish were transported to BSBE, segregated from previously injected fish within the same recirculating system, and acclimated for 3 weeks. Both groups of fish were then given injections of 2.5 or 5.0 mU/gBW bTSH and bled at 0, 2, and 5 hours. To control for the possibility that differences in water quality, diet, or temperature between BSBE and the ARTF might influence the T_4 response to TSH, a new cohort of red drum was maintained in BSBE or at the ARTF for 10 weeks (61 ± 4 g mean wt, Experiment 6). Neither group had been used for previous experiments. Fish at each location were injected on the same day with the same solutions of saline, 0.625, or 2.5 mU/gBW bTSH. Plasma was collected at 5 hours after injection for T_4 measurement. Finally, to determine if diet influenced thyroid sensitivity to bTSH, new groups of red drum were raised in BSBE for 42 days on Rangen commercial diet, then switched to one of three diet treatments: Rangen, Aquamax, or a laboratory-manufactured diet (40% protein, 8% lipid, anchovy meal-based diet, (Moon and Gatlin, 1994). Tanks of fish were collectively weighed each week. On day 105 (64 ± 4 g mean wt, Experiment 7) fish were injected with saline, 0.15625, 0.625, or 2.5 mU/gBW bTSH and bled after 5 hours for T_4 and T_3 measurement.

Statistics

Multiple comparisons for statistical significance were performed using the Mann–Whitney U-test with a $P \leq 0.05$. The half-time clearance rate for immunoreactive TSH was determined by graphing on a semi-log axis the percent disappearance of TSH from circulation where the peak plasma TSH value was assigned to be 100%.

Results

Time and dose response of T_4 to TSH stimulation

In Experiment 1, bTSH stimulated a significant elevation of immunoreactive TSH in plasma within 30 minutes. Circulating TSH peaked at 2 hours, declined significantly by 5 hours, and remained above controls at 11 hours (Figure 2.1). The half-time clearance of TSH in red drum was determined to be 2.0 hours. Plasma T_4 levels rose coincidentally with TSH, but showed a much more prolonged duration, peaking at 5 hours and remaining maximally elevated throughout the remainder of the experiment (Figure 2.1). Plasma T_3 in bTSH-injected fish was significantly elevated over saline injected animals at 1 and 2 hours after injection before returning to control levels. There was a second significant elevation of T_3 at 8 and 11 hours in bTSH treated fish (Figure 2.1). Based on the results of these time-course experiments, 5 hours after injection was chosen as a standard sampling time for dose-response studies. Injection of bTSH in Experiment 2 produced a dose-dependent elevation of T_4 between 0.3125 and 2.5 mU/gBW (Figure 2.2). In fish that had remained longer in BSBE, however, insensitivity was observed as bTSH injections failed to stimulate a dose or time dependent increase of T_4 despite no difference in the half-time clearance of TSH (Experiments 1 and 4, Figures 2.1 and 2.2).

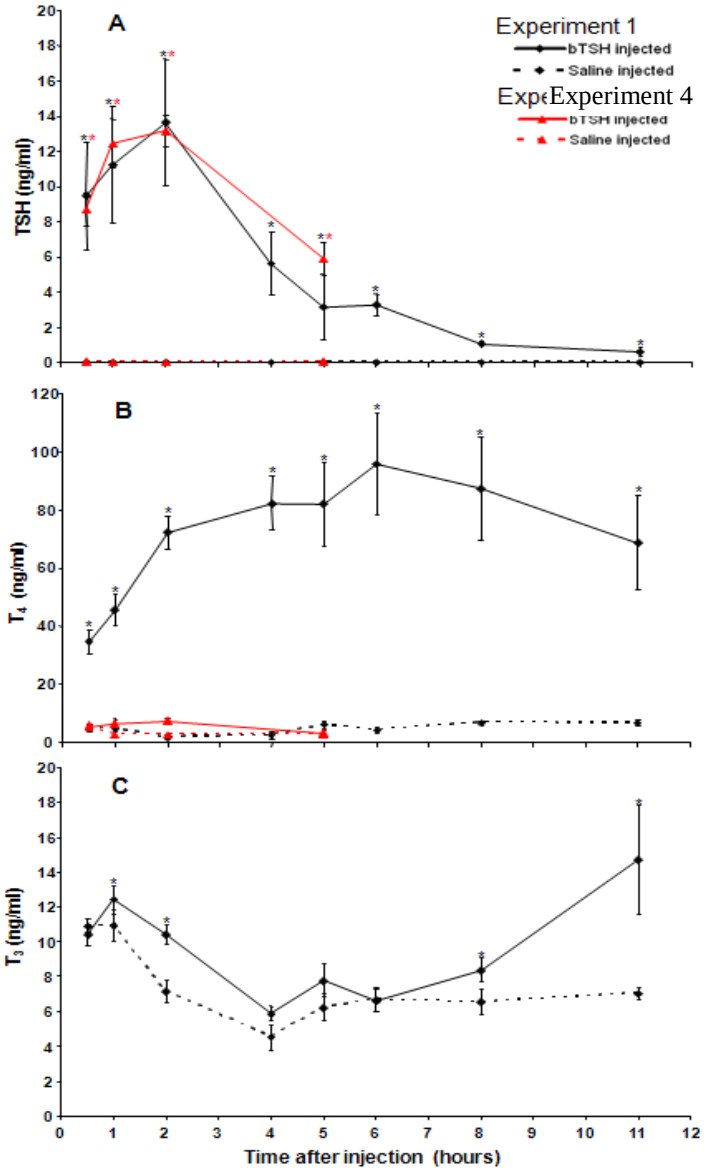

Figure 2.1. Time course of TSH and thyroid hormones post bTSH injection. Circulating TSH (A), T_4 (B), and T_3 (C), in response to 5mU/gBW bTSH in red drum 4 weeks (◆, Experiment 1) and 22 weeks (▲, Experiment 4) after being moved into BSBE. Solid lines connect means of injected animals and dashed, control animals. Vertical bars represent standard error of the mean.
*Significant difference ($p<0.05$) between bTSH injected and control fish at same date, n=7

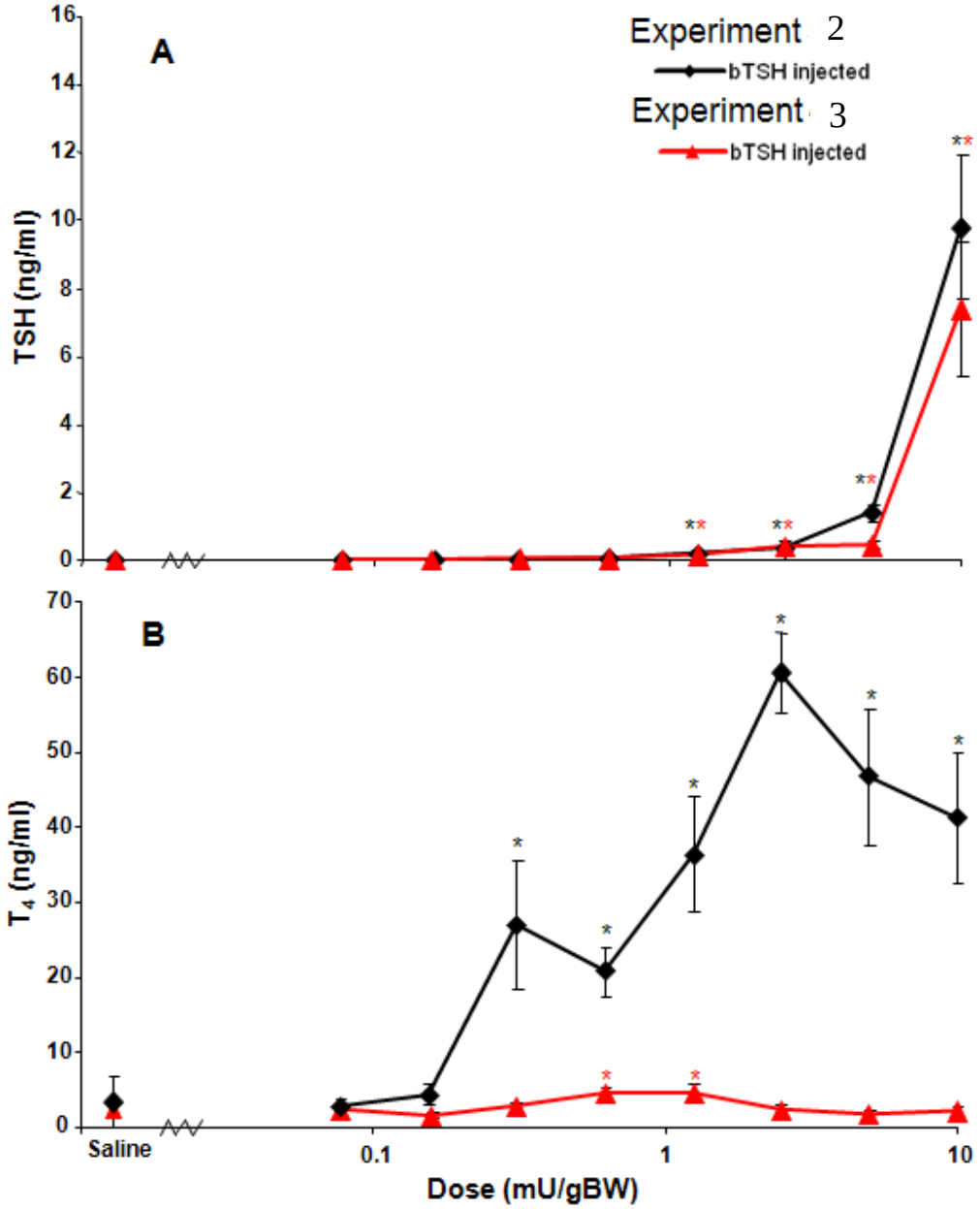

Figure 2.2. Dose response of TSH and T_4 5 hours after bTSH injection. Circulating TSH (A) and T_4 (B) response to an injection of doses ranging from 0 to 10mU/gBW bTSH in young fish (◆ Experiment 2) and old fish (▲, Experiment 3). Solid lines connect means of injected animals and dashed, control animals. Vertical bars represent standard error of the mean.
*Significant difference ($p<0.05$) between bTSH injected and control fish at same date, n=7

TSH insensitivity in red drum

Red drum used repeatedly for Experiments 1-4 became unresponsive to bTSH injections (Figures 2.1 and 2.2). Likewise, red drum from the ARTF, which had not been previously injected, were insensitive to bTSH after being maintained in BSBE for 3 weeks in Experiment 5 (Figure 2.3b). In Experiment 5, circulating TSH levels for both uninjected and previously injected fish reached equivalent values to Experiment 1 results (Figure 2.3a and Figure 2.1). In Experiment 6, red drum held at the ARTF did show a significant T_4 response to bTSH injections (Figure 2.4), whereas equivalent fish of the same size and age held in BSBE were unresponsive to bTSH (Figure 2.4). In Experiment 7, a new cohort of red drum held in BSBE for 15 weeks and fed Rangen and Aquamax diets for 63 days had positive growth rates (Figure 2.5) and were sensitive to bTSH injections (Figure 2.6). This T_4 response to bTSH was unexpected because fish maintained in BSBE for 10 weeks in experiment 5 had become unresponsive. Fish fed the lab-made diet showed reduced growth rates (Figure 2.5) and a T_4 response to bTSH injections that was significantly lower than Rangen or Aquamax fed fish but still greater than 0 or 5 hour controls (Figure 2.6). It was determined after the experiment that the lab-made diet was manufactured using lipids that had oxidized. This was reflected in basal plasma T_3 levels in Time 0 fish, which were significantly lower in red drum fed the lipid-oxidized diet (4.95 ± .3 ng/ml) than in Rangen (10.2 ± 1.11 ng/ml) and Aquamax (11.2 ± .9 ng/ml) fed fish.

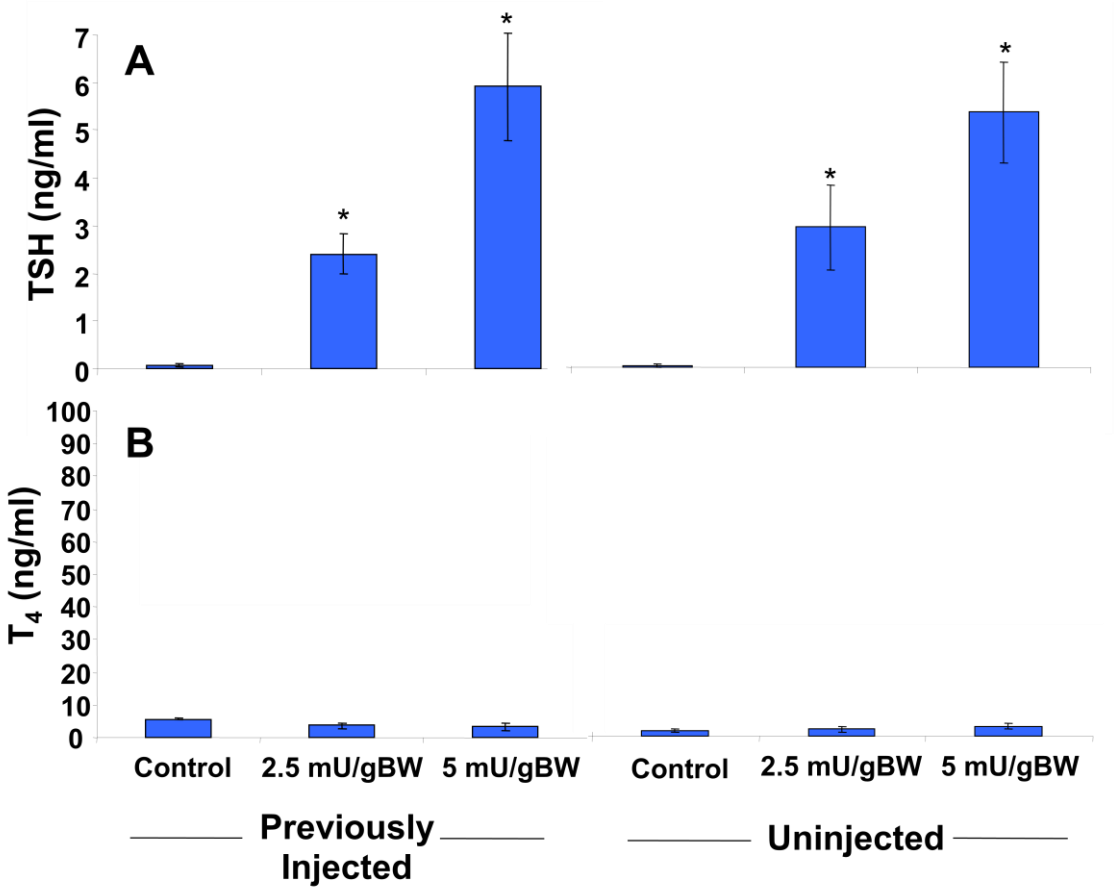

Figure 2.3. The effects of previous injection on plasma TSH and T_4 response to bTSH. Circulating TSH at two hours (A) and T4 at 5 hours (B) in response to bTSH injections in red drum that had been previously injected with bTSH or had never been injected (Experiment 5). Both previously injected fish and the uninjected fish were treated with single ip injection of 0, 2.5, or 5mU/gBW bTSH.
*Significant difference ($p<0.05$) between bTSH injected and control fish n=7

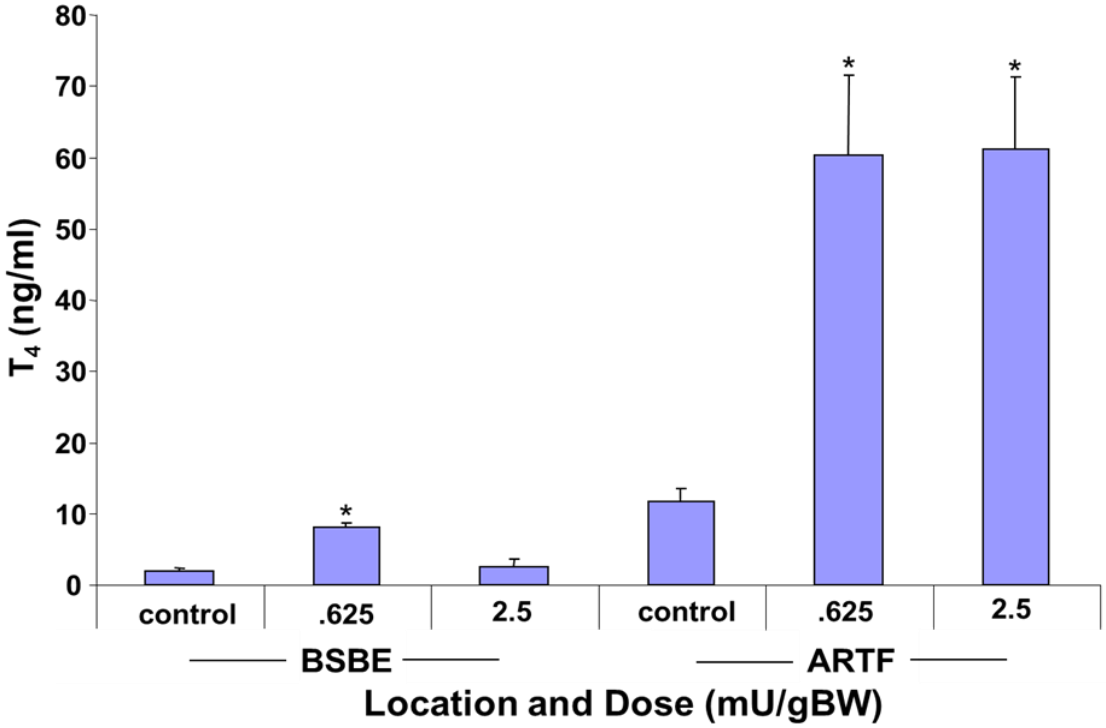

Figure 2.4. The effect of location on T_4 response to bTSH. Circulating T4 five hours after an injection to 0, 2.5, or 5 mU/gBW bTSH in fish from a single cohort held either at BSBE or at the ARTF (Experiment 6).
*Significant difference ($p<0.05$) between bTSH injected and control fish n=7

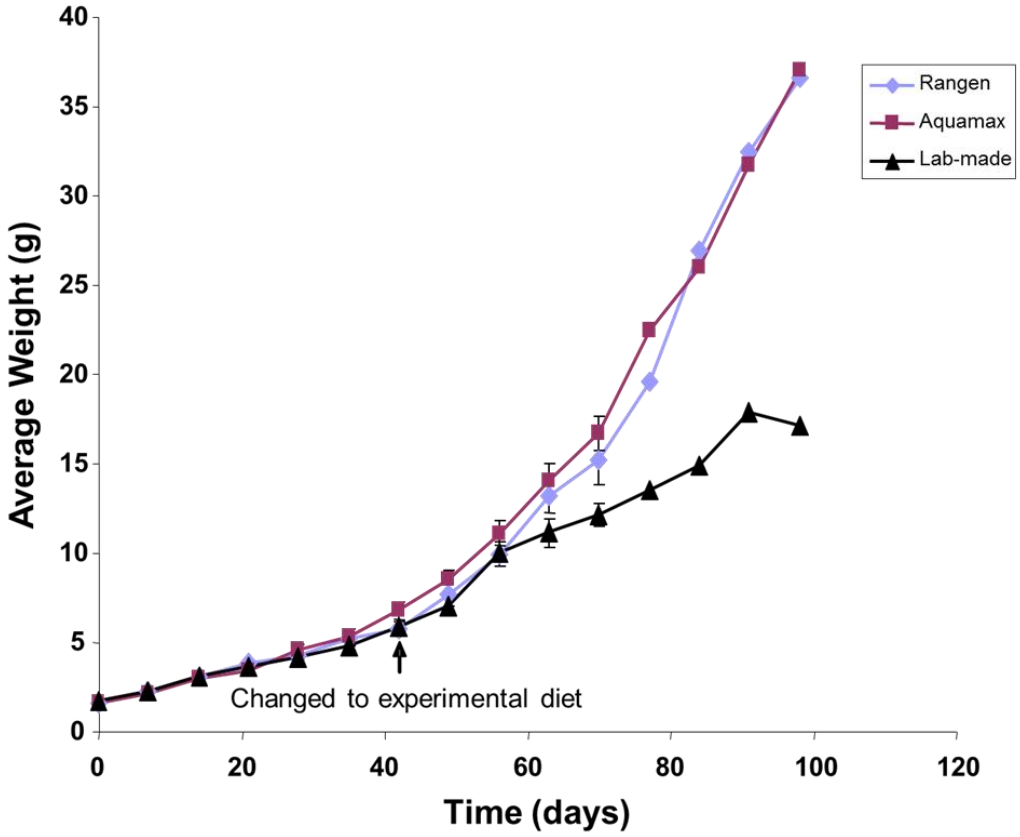

Figure 2.5. Growth curve of red drum on varying diets. Growth curve of red drum raised for 42 days on a commercial diet (Rangen), then switched to one of three diet treatments: Rangen (◆), Aquamax (■), or a lab-manufactured diet (▲) (Experiment 7).

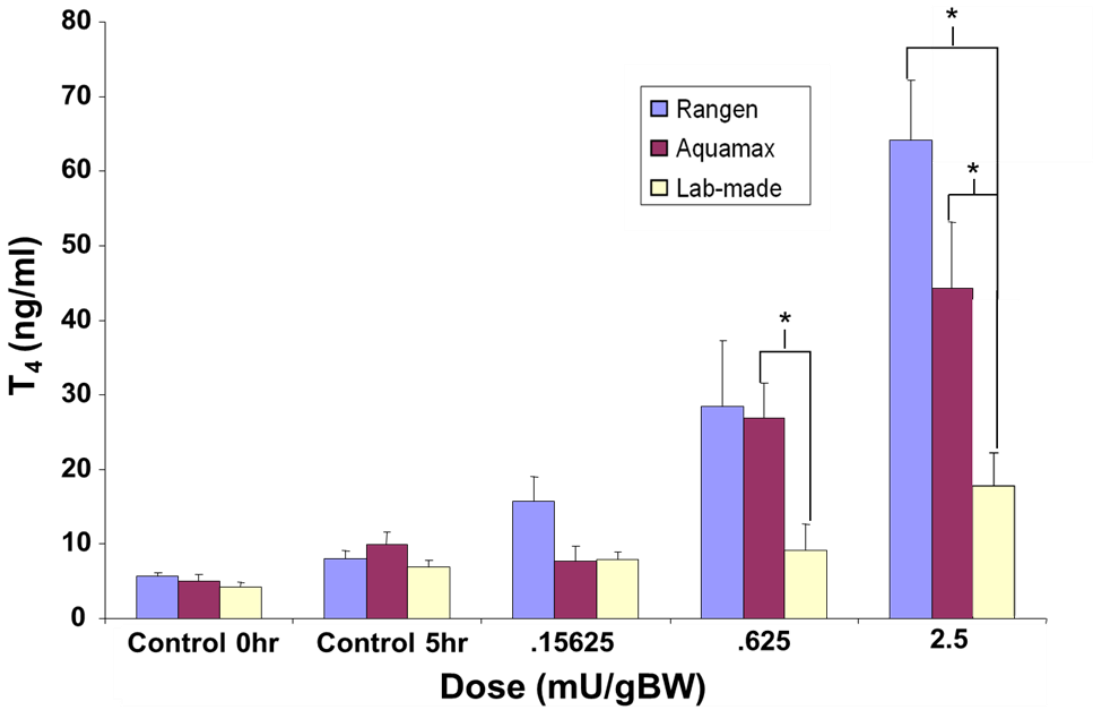

Figure 2.6. The effect of diet on T_4 response to bTSH. Circulating T_4 response at five hours to an IP injection of bTSH at doses ranging from 0.15625 to 2.5 mU/gBW in fish fed Rangen, Aquamax, or a lab-made diet (Experiment 7).
*Significant difference ($p<0.05$) between fish fed different diets at the same dose, n=7

Discussion

My objective in this study was to describe the *in vivo* T_4 release response in red rum to an exogenous TSH with established biological activity in mammals. This bioassay could later be utilized to characterize the thyrotropic potency of recombinant red drum TSH of unknown purity during its production and purification. In initial experiments, the range of doses of bTSH which produced a linear T_4 response and the duration of the T_4 response to TSH injection were similar in red drum to what has been observed in other fish species (Chan and Eales, 1976; MacKenzie, 1982; MacKenzie et al., 1987; Swanson and Dickhoff, 1987). The magnitude of the T_4 response to TSH was in most cases higher than has been observed to the same doses in other vertebrate species (Darras and Kühn, 1983; East-Palmer et al., 1995; MacKenzie, 1981; Nielsen et al., 2004a). For the first time in a non-mammalian vertebrate, I utilized a TSH immunoassay to characterize the time course of circulating TSH in response to an injection of heterologous TSH. Plasma TSH peaks in red drum at two hours, after which is cleared with a half time of approximately two hours. Only one equivalent study exists of the simultaneous measurement of circulating levels of exogenously-administered TSH and the T_4 response it elicited. This study in humans found that exogenous TSH is cleared after two days while the resultant T_4 remains elevated for nearly a week (Nielsen et al., 2004a). Similarly, T_4 levels were still at maximum 11 hours post-injection in red drum, despite nearly 90% of the peak level of immunoreactive TSH being cleared by 8 hours. Though it takes less time to clear TSH from red drum plasma, the more rapid clearance of TSH relative to T_4 in red drum is consistent with the findings in humans.

The longer duration of T_4 in circulation is likely a factor of both the continued entrance of T_4 from the thyroid and the slower clearance rate of T_4 due to the presence of blood thyroid hormone binding proteins (Bartalena, 1990; Nielsen et al., 2004a; Ridgway et al., 1974; Surks et al., 1973; Thotakura et al., 1991). Plasma T_3 was inconsistently elevated in bTSH injected fish and increases in T_3 could not be replicated in subsequent experiments (data not shown), despite consistently elevated plasma T_4. The inability of elevated TSH or T_4 to consistently increase circulating T_3 in fish has been well documented (Eales and Brown, 1993), suggesting that TSH does not drive production of the biologically active thyroid hormone in red drum. However, exogenous TSH and daily cycles of endogenous TSH are correlated with elevated free T_3 in humans (Nielsen et al., 2004b; Russell et al., 2008) and it is not known if free T_3 and free T_4 increase after injection of TSH in fish. Plasma levels of free T_3 should be examined in red drum after injection of TSH to determine if TSH is enhancing T_3 availability for cellular uptake and utilization despite inconsistent increases in total T_3. Whereas these results indicate that T_4 is a good bioassay of TSH, they also demonstrate the independence of circulating total T_3 from T_4 in red drum, as previously described by Eales and Brown (1993).

Surprisingly, despite consistent and repeatable TSH responses in Experiments 1 and 3, the T_4 response to bTSH injections was abolished as fish were held over time in BSBE. New cohorts of red drum brought into BSBE consistently produced linear T_4 responses to exogenous TSH before becoming insensitive. In the only other studies to show that fish were unresponsive to exogenous TSH (Grau et al., 1985; Swanson and Dickhoff, 1987), it was suggested that the fish may seasonally inactivate the thyroid

gland or increase TSH clearance. The insensitivity of the red drum to TSH is not likely due to changes in season, as they were kept on a constant photoperiod and at a constant temperature. Alternatively, red drum sensitive to TSH always weighed less than 45g, suggesting sensitivity to TSH in red drum decreases with an increase in size. This is opposite to what was found in brook trout by Chan and Eales (1976), who proposed that smaller fish may clear TSH more rapidly. Measurement of circulating TSH post-injection in red drum indicated that the diminished T_4 response was most likely due to insensitivity to the injected TSH rather than an increase in TSH clearance. I therefore hypothesized that sensitivity to exogenous TSH may simply decrease with an increase in size of fish. However, I demonstrated red drum of the same age and size as insensitive BSBE fish were sensitive to exogenous TSH if kept at the ARTF. Though the ARTF fish were shown to be sensitive to bTSH, they had never been previously injected with bTSH. It has also been shown that repeated injections of bTSH in brook trout can decrease their sensitivity to bTSH, possibly due to antibody formation against the heterologous TSH (Chan and Eales, 1976). It was later established that humans can acquire immunity to exogenous bTSH during clinical treatment (Krishnamurthy, 1978). I therefore hypothesized that the insensitivity of older red drum might be due repeated exposure to bTSH. However, when I tested red drum held in BSBE for 22 weeks that had never been previously injected with TSH, they too were insensitive to bTSH. The insensitivity thus did not appear to be due to prior bTSH exposure, but appeared to be related to holding in BSBE.

To determine if the development of insensitivity was related to nutrition, fish in BSBE were fed the same diet used at the ARTF. In mammals, TSH secretion decreases in response to fasting, dieting, or low-carbohydrate intake, leading to a decrease in circulating thyroid hormones (Danforth and Burger, 1989). Similar studies have demonstrated that thyroid hormones decline with decreasing food rations in teleost fish (Finnson and Eales, 1999; Gaylord et al., 2001; Leatherland and Farbridge, 1992; MacKenzie et al., 1998), including the red drum (MacKenzie et al., 1993). Not only quantity but quality of feed can influence production and removal of thyroid hormones in teleosts (Eales, 1988). Therefore, I hypothesized that the diet source could be responsible for altered sensitivity of the thyroid. A new batch of young red drum fed Rangen (another commercial diet) and Aquamax diets for 63 days in BSBE showed equivalent linear T_4 responses to increasing doses of bTSH. Thus, being held in BSBE for 15 weeks is not sufficient to cause insensitivity. However, a third group of fish fed a lab-made diet formulated to ensure optimal growth of red drum did show a reduced T_4 response. It was later determined that this lab-made diet was manufactured using lipids which had oxidized, which likely explains their reduced growth (Serrano et al., 1992). Moreover, the basal T_3 levels in fish fed the lab-made diet were half as high as fish fed the commercial diets. Low plasma T_3 suggests animals fed diets deficient in lipids were hypothyroid. Similarly, red drum fed reduced rations of diets had decreased levels of plasma T_3 but not T_4 (MacKenzie et al., 1993). Thus Aquamax or Rangen diets, but not a lipid poor diet, were sufficient to maintain thyroid sensitivity of fish held in BSBE for 15 weeks. However, previously injected fish maintained in BSBE for 10 weeks in

Experiment 6 were demonstrated to have lost sensitivity to bTSH. Additionally, uninjected fish from Experiment 6 were only in BSBE for 3 weeks before they were shown to be insensitive. Experiment 6 fish were brought into BSBE when they were larger and were nearly 22g heavier than Experiment 7 fish when challenged with bTSH. This suggests that though being in BSBE is necessary to induce a loss of response to bTSH, it also appears to be more dependent upon size than duration within BSBE.

Though I have yet to experimentally determine what causes insensitivity, it is possible that insensitivity to TSH in BSBE red drum is an indication of hypothyroidism induced by an anthropogenic influence in BSBE that is absent at the ARTF. Chemicals which disrupt thyroid function can have significant impact on growth and development of wild animals (Brucker-Davis, 1998; Colborn, 2002; Rolland, 2000), which has led to a call for development of techniques suitable for evaluating the effects of endocrine disrupting chemicals (EDCs) on thyroid function in wildlife species (DeVito et al., 1999). Traditionally, thyroid disruption has been measured using histology or circulating thyroid hormone levels. Histological examination of changes in thyroid cells is an accepted bioassay, but requires long exposure and is not very sensitive (Eales et al., 1999). Furthermore, compensation mechanisms can function to maintain total thyroid hormone levels despite disruption of their synthesis and release, confounding efforts to use thyroid hormones as an index of thyroid disruption. We have demonstrated that a TSH challenge can be utilized to show altered thyroid sensitivity before changes in basal levels of thyroid hormone are present. This suggests that a TSH challenge test may be a

useful means for evaluating the impact of anthropogenic disruptors on thyroid function in teleost fish.

The only other studies demonstrating thyroid insensitivity to mammalian TSH used coho salmon (*Oncorhynchus kisutch*) and killifish (*Fundulus heteroclitus*), which were found to develop insensitivity during winter or summer, respectively (Grau et al., 1985; Swanson and Dickhoff, 1987). Moreover, laboratory studies in these two species demonstrated the insensitivity was due to changes in photoperiod and not temperature (Grau et al., 1985; Swanson and Dickhoff, 1987). Although the interior lights at the ARTF are held on a 12:12 photoperiod, I have observed that low amounts of sunlight leak into the buildings at the ARTF, possibly exposing fish to seasonal changes in photoperiod. Thus, exposure to subtle circannual cues at the ARTF may have functioned to endow seasonality to the ARTF animals which may have helped to maintain thyroid sensitivity. TSH release in red drum is thought to be controlled by a central oscillator that is sensitive to length of photoperiod, producing larger amplitude daily T_4 cycles in response to longer photoperiods and increased feedings (Leiner and MacKenzie, 2003). Thus, it seems plausible that length of photoperiod could impact TSH secretion on a seasonal basis in red drum as well and a constant photoperiod may remove sensory information necessary to maintain that secretion. If seasonality is important for increasing circulating thyroid hormones through TSH secretion, then maintaining a constant photoperiod may have detrimental effects on growth and nutrient utilization in aquacultured species (MacKenzie et al., 1998). Therefore, I propose that future studies

include introduction of seasonal photoperiodic cues to determine if seasonality can sensitize red drum thyroids to exogenous TSH.

The ability of TSH to regulate seasonal cycles of thyroid hormones, which in turn serve as a mechanism to integrate photoperiodic and nutritional status to drive seasonal developmental and reproductive cycles, provides a potential explanation for the evolution of central control of the thyroid axis. It is well accepted that the thyroid gland evolved from the protochordate endostyle (Eales, 1997) and functions in extant agnathans in the absence of pituitary TSH control (Sower et al., 2009; Uchida et al., 2010). A longstanding question has been why central control of the thyroid evolved (MacKenzie et al., 2009; Sower et al., 2009). The putative ancestral pituitary glycoprotein to TSH found in the sea lamprey binds a receptor found in gonads and on thyroid follicular cells (Freamat and Sower, 2008), suggesting an evolutionary link between TSH and reproduction. In elasmobranchs, thyroid hormones cycle in relation to reproductive development and seasonal migration (Sage, 1973). Similarly, thyroid hormones cycle with reproductive state and are necessary for maturation of the gonads in seasonally reproducing teleost fish, further implicating TSH as an integrative signal to convey seasonality to peripheral tissues (Sage, 1973). Thus, I hypothesize that central control of the thyroid evolved within lower vertebrates to enable thyroid hormone to drive seasonal cycles in physiological processes that are dependent upon sufficient nutrient intake, such as reproduction, migration, and smoltification. TSH from the pars tuberalis, a lobe of the adenohypophysis which is found only in tetrapods, has been found to activate hypothalamic pathways involved in the thyroid hormone-dependent

photoperiodic regulation of seasonal reproduction in birds and mammals (Hazlerigg and Loudon, 2008). This suggests that as chronically elevated thyroid hormones became important for promoting elevated metabolism in endotherms, (Norris, 2007), retrograde flow of TSH from the pars tuberalis to the hypothalamus (Nakao et al., 2008) evolved to maintain TSH's more ancient function in promoting seasonal cyclicity.

In conclusion, although I have not determined why red drum in BSBE have reduced sensitivity to exogenous TSH, it may be due to an anthropogenic compound disrupting the thyroid or due to the lack of seasonal changes in photoperiod. I have confirmed that thyroid is insensitive in red drum and the lack of T_4 response is not due to increased clearance of TSH or the failure of exogenously-applied TSH to enter the circulation. Moreover, this insensitivity is dependent on both the environment and the size of the fish. For the moment this insensitivity problem, although of interest as a potential avenue for exploration of mechanisms of thyroid disruption in captive fish, precludes the use of red drum as a bioassay species for the production and purification of recombinant teleost TSH.

CHAPTER III

CHARACTERIZATION OF T_4 RESPONSE TO HETEROLOGOUS THYROTROPIN IN GOLDFISH (*Carassius auratus*)

Introduction

Thyrotropin (thyroid stimulating hormone, TSH) is a pituitary glycoprotein hormone comprised of a hormone-specific β subunit coupled to an α subunit (glycoprotein subunit α, GSUα). TSH stimulates the secretion of thyroid hormone, primarily thyroxine (T_4), from the thyroid gland (Szkudlinski et al., 2002). T_4 is enzymatically converted to 3,5,3'-triodothyronine (T_3) by deiodinases within peripheral tissues. Intracellular T_3 then binds to nuclear receptors to regulate growth, reproduction, development, and metabolism through gene transcription, or travels to other tissues by reentering the circulation (Norris, 2007). T_4 and T_3 not bound by blood transport proteins (free T_4 and free T_3) are available for uptake by cells (Eales and Brown, 1993). In mammals, it has been demonstrated that injections of TSH elevate free T_4 and free T_3 (Nielsen et al., 2004b), and that plasma TSH shows a circadian rhythm which corresponds with an increase of free T_3 in humans (Russell et al., 2008). This suggests that central activation of TSH-dependent thyroid secretion is a primary driver of thyroid hormone supply to target cells in mammals (DeGroot and Jameson, 2001). There is evidence that TSH is present in the pituitary of elasmobranchs and actinopterygians, but little information exists on its physiology or control in these taxa (MacKenzie et al.,

2009). Active deiodination pathways and the inability of exogenously applied T_4 to consistently increase T_3 levels has led to the hypothesis that in teleost fish TSH plays a less important role by simply maintaining a circulating pool of T_4 prohormone from which peripheral mechanisms regulate cellular utilization (Eales and Brown, 1993). Therefore, more studies are needed in teleost fish to determine if TSH is as important in driving thyroid hormone utilization as in mammals.

Homologous TSH and immunoassays are valuable tools needed to examine the regulation of endogenous teleost TSH (MacKenzie et al., 2009). Only one immunoassay, a coho salmon (*Oncorhynchus kisutch*) TSH αβ - α subtractive radioimmunoassay (RIA), has been developed to measure piscine TSH (Moriyama et al., 1997). The RIA was utilized to demonstrate thyroid hormone negative feedback and hypothalamic control of TSH secretion in coho salmon (Larsen et al., 1998). Piscine TSH immunoassay can thus be used effectively to study regulation of TSH secretion and aid in determining the relationship between plasma TSH release and thyroid hormone levels. However, the assay was created as a subtractive RIA due to the difficulty encountered in obtaining large enough quantities of purified TSH to generate a TSH antibody. Purification of teleost TSH for use in homologous immunoassays has proven difficult, likely due in part to low concentrations of TSH in the pituitary gland compared to the structurally-similar gonadotropins (GTHs) (Ng et al., 1982). Consequently, such a large number of fish pituitaries are needed to purify sufficient quantities of TSH that it can only be purified from the most abundant, commercially important species.

Therefore, novel approaches are needed for obtaining homologous teleost TSH from fish species which are of endocrinological but not commercial interest.

Biologically active piscine glycoprotein hormones have been produced using *in vitro* expression systems and were utilized to create piscine gonadotropin (GTH) immunoassays (Aizen et al., 2007b; Levavi-Sivan et al., 2009), suggesting *in vitro* expression of teleost TSH is a viable alternative to pituitary purification. Expression of teleost GTH receptors in mammalian cell lines has been used to confirm receptor binding and activation of the recombinant piscine GTHs. However, glycoprotein hormones, including fish GTHs, produced by insect cell lines with high activity *in vitro* have been demonstrated to have low *in vivo* activity, likely due to increased clearance of improperly glycosylated hormone (Kazeto et al., 2008; Ko et al., 2007). This demonstrates that *in vitro* assays must be used in conjunction with *in vivo* bioassays to ensure biological activity of recombinant glycoprotein hormones.

I propose that stimulation of T_4 release in goldfish (*Carassius auratus*) is an ideal *in vivo* bioassay for teleost fish TSH and could be used to evaluate how alteration of glycoprotein hormone structure affects thyrotropic activity. Goldfish have been successfully used in the past for the bioassay of exogenous TSH due to their low cost, small size, ease of maintenance, and sensitivity of response to TSH from fish, amphibians, birds, and mammals (Gorbman, 1940; Hutton and Hayter, 1970; Ortman and Billig, 1966). Goldfish plasma T_4 has been demonstrated to respond in a dose and time-dependent fashion to injections of bovine TSH (bTSH) (MacKenzie et al., 1987), as in other teleost species (Chan and Eales, 1976; Kuhn et al., 1986; MacKenzie, 1982;

MacKenzie et al., 1987; Milne and Leatherland, 1978; Swanson and Dickhoff, 1987). Therefore, my goal was to re-establish the goldfish bioassay to aid in the production and purification of teleost thyrotropin. I further characterized the TSH bioassay in goldfish by measuring for the first time in a teleost fish the circulating levels of exogenous TSH following TSH injection. Once the assay was characterized, I was able to use recombinant human glycoprotein hormone analogs to demonstrate that presence of positive charges at the n-terminal of GSUα is important for thyrotropic activity of both mammalian thyrotropins and gonadotropins in goldfish.

Materials and methods

Animals

Goldfish were purchased from Ozark Fisheries (Stoutland, MO) and held in the Biological Sciences Building East (BSBE) at Texas A&M or at the Texas A&M Aquaculture Research and Teaching Facility (ARTF). Fish kept in BSBE were held in 100 gallon, round fiberglass tanks connected to a 2,000 gallon recirculating system. Goldfish kept at the ARTF were held in 200 gallon, round fiberglass tanks connected to a 4,000 gallon recirculating system. Goldfish at both locations were held at 25 ^{o}C on 12L:12D photoperiod to control for temperature and day length effects on the thyroid axis. Fish were fed twice daily to apparent satiation with TetraMin Tropical Flakes (Tetra Holding, VA) and weighed between 10 and 50 g when used for experiments.

Injection protocol

Fish were randomly sorted into groups of 7 in 5-gallon, grid-bottom buckets which were suspended in their original tanks the night before experiments. Shortly after

lights on goldfish were removed from the tanks, anesthetized with tricaine methanesulfonate (MS-222) (200 mg/L), injected intraperitoneally (IP) with hormone suspended in BSA saline (100 µL/20 gBW), and then returned quickly to their respective buckets. Control fish were injected with BSA saline (0.1% BSA, 0.9% NaCl, Sigma-Aldrich, St. Louis, MO). They were recaptured at specific times after injection, anesthetized to collect approximately 250µl of blood from the caudal vasculature into 1 ml heparinized syringes, and returned to their tanks. Blood was centrifuged to separate plasma which was frozen for hormone determination. Goldfish were allowed at least two weeks to recover between experiments.

Hormone determination

Specific immunoassays were used to determine blood TSH and T_4 at the Endocrine Diagnostic Laboratory at the Texas Veterinary Medical Diagnostic Laboratory. Total T_4 was measured using Coat-A-Count RIA tubes (Siemens). Sensitivity of the T_4 assay was 2.5 ng/ml (as defined by the lowest point on the linear portion of the standard curve) and inter- and intra-assay variabilities (calculated as coefficient of variation) were 3.8 and 9.1%, respectively (manufacturer's data). Serially diluted goldfish plasma diluted parallel to the standard curve. At 1,000 ng/ml T_3 has 2% cross reactivity in the T_4 RIA (manufacturer's data). When T_4 was spiked into goldfish plasma, 90 ± 2% (N=4) was recovered.

Plasma TSH was measured using an IMMULITE 2000 canine TSH immunoassay (Siemens). This assay sensitivity was 0.01 ng/mL (as defined by the lowest point on the linear portion of the standard curve) and inter- and intra-assay

variabilities were 7.6 and 5.5%, respectively. Bovine TSH in goldfish plasma dilutes parallel to standards. Endogenous TSH was undetectable in control treated goldfish using this assay.

Characterization of the goldfish bioassay

The dose and time dependent T_4 and TSH response to bTSH injection was first characterized in goldfish using Sigma bovine TSH (Sigma-Aldrich, St. Louis, MO; 2.2 IU/mg) or NIADDK-bTSH-13 (National Hormone and Pituitary Program, 30.0 IU/mg) by modified methods of MacKenzie et al. (1987). The same fish were used for consecutive experiments. To confirm a dose-dependent response of T_4 to bTSH injection, bTSH was injected at doses ranging from 0.03 to 20 mU/gram body weight (mu/gBW), blood collected at 5 hours, and plasma TSH and T_4 levels determined. To examine the time course of response to TSH injection, plasma TSH and T_4 were measured at times ranging from 2 to 48 hours after injection of a low (0.22 mU/gBW), medium (0.66 mU/gBW), and high dose (2 mU/gBW) of bTSH in separate experiments, each with their own BSA-saline control. The 5-hour T_4 response to standard doses of TSH was compared across 35 experiments over 3 years to evaluate the stability of the dose response characteristics.

T_4 response to mammalian glycoprotein hormones

The T_4 response to increasing doses of various mammalian hormones was determined at 5 hours post-injection using the same methods as described above for bTSH. The thyrotropic activity was determined for pituitary purified bovine LH (0.3 - 10 ug/gBW of bLH-B-5, United States Department of Agriculture, MD) and bovine FSH

(0.2 – 7.0 ug/gBW of bLH-B-5 and bFSH-B-1, United States Department of Agriculture, MD) by injecting them at increasing doses and comparing their T_4 response to the bTSH standard in separate experiments. After being diluted to the highest concentration used for the injection, the TSH contamination in the bLH and bFSH solutions was determined by immunoassay. The apparent immunoreactive contamination for LH was 2.1 ug immunoreactive TSH/mg and FSH was only 0.06 ug immunoreactive TSH/mg. The T_4 response to the following recombinant hormones were also assayed: recombinant hTSH (1.2 – 100 ng/gBW of hTSH, 94/674, National Institute for Biological Standards and Control, Hertfordshire, UK), hTSH analog (1.2 – 100 ng/gBW of TR-1401, Q13K+E14K+P16K+Q20K, Trophogen, Inc., MD), recombinant human FSH analog (0.09 – 2.4 µg/gBW of TR-4401, Q13R+E14R+P16R+Q20R, Trophogen, Inc., MD), and recombinant human FSH (1.2 – 33.3 µg/gBW of hFSH, 92/642, National Institute for Biological Standards and Control, Hertfordshire, UK). Due to a limited amount of hormone, only a single group of goldfish were injected with hCG (5.0 µg/gBW of CR127, NPA/NIDDK, MD). Each assay included a control group injected with BSA-saline and a standard curve of at least three doses of bTSH.

Statistics

Multiple comparisons for statistical significance were performed using the Mann–Whitney U-test with a $P \leq 0.05$. TSH activity in goldfish relative to bTSH was determined by first creating a log-linear regression line for bTSH where T_4 was the dependent variable and mU was the dependent variable. The T_4 responses of other hormones within the same assay which were significantly above controls were then inserted into the regression formula to assign a mU activity which was then divided by the weight of hormone injected. All doses that produced a significant response for a given hormone were averaged and the standard error of the mean calculated. The half time clearance rate for immunoreactive TSH was determined by graphing the percent disappearance of TSH from circulation on a semi-log axis where the peak plasma TSH value was assigned to be 100%. A half-time clearance rate for each dose of TSH was determined, averaged, and the standard error of the mean determined.

Results

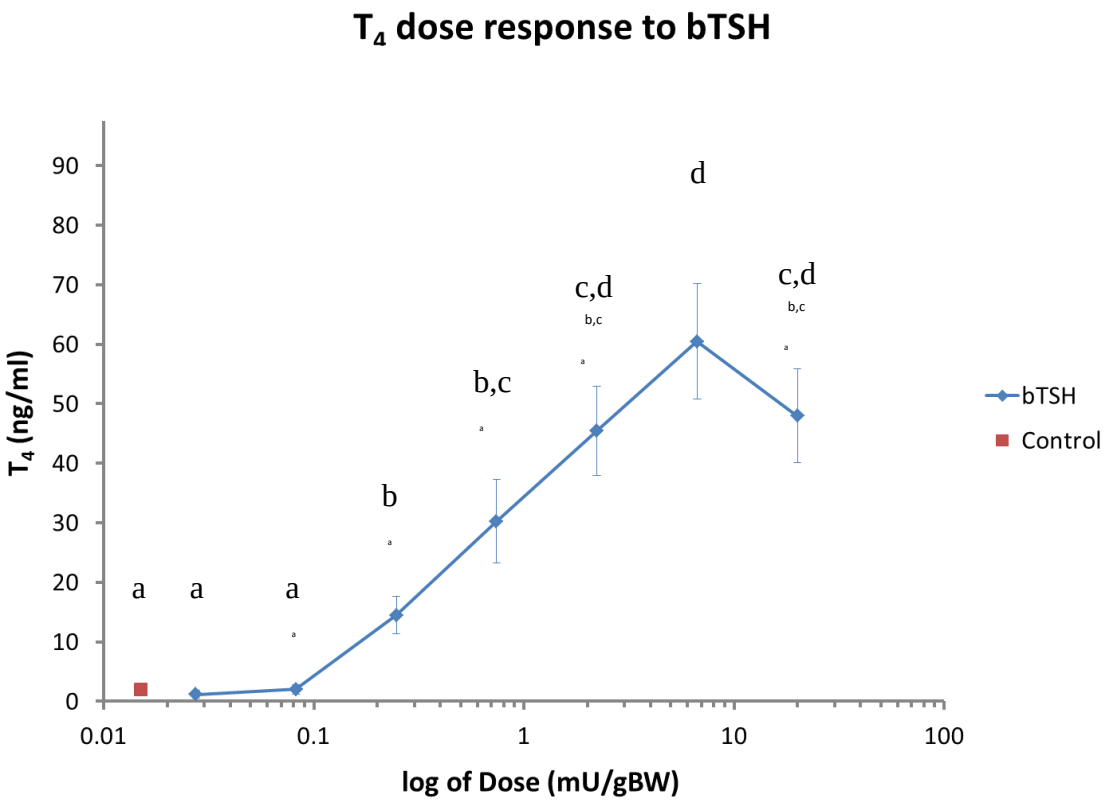

Figure 3.1. T_4 dose response to bTSH. Plasma T_4 response 5 hours after injection of doses ranging from 0 to 20 mU/gBW bTSH. Vertical bars represent standard error of the mean.
Means sharing a letter are not significantly different ($p \geq 0.05$)

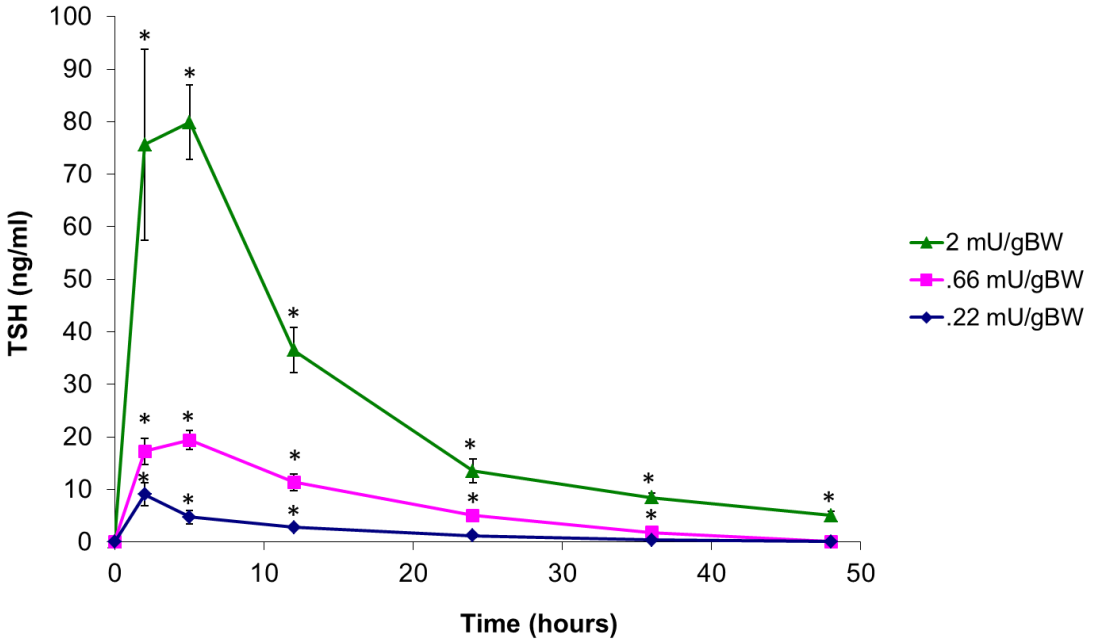

Figure 3.2. Time course of TSH response to varying doses of bTSH. Circulating TSH in response to 0.22, 0.66, or 2.0 mU/gBW bTSH in goldfish. The time course of TSH response for each dose was carried out in a separate experiment. The matched control for each experiment is not shown because all control animals had TSH values below the sensitivity of the assay. Vertical bars represent standard error of the mean.
*Significant difference ($p<0.05$) between bTSH injected and the minimum sensitivity of the TSH immunoassay

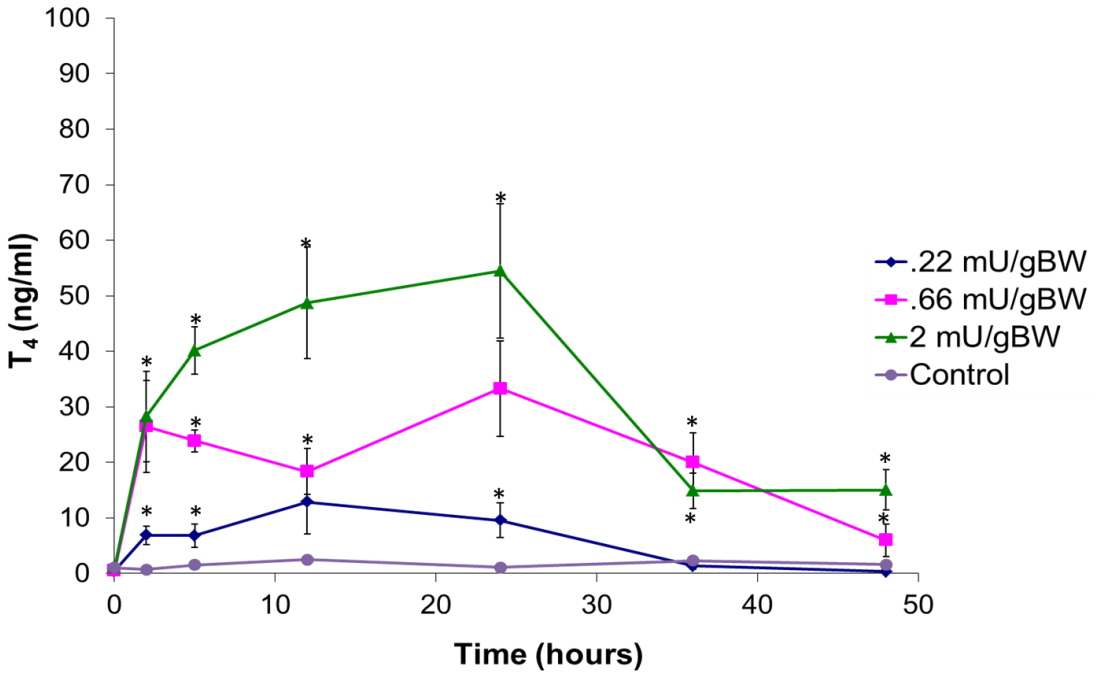

Figure 3.3. Time course of T_4 response to varying doses of bTSH. Circulating T_4 in response to 0.22, 0.66, or 2.0 mU/gBW bTSH in goldfish. The time course of T_4 response for each dose was carried out in separate experiments. As control T_4 levels for each experiment were low and overlapping, only the control from the 2 mU/gBW experiment is shown. Vertical bars represent standard error of the mean.
*Significant difference ($p<0.05$) between bTSH injected and the minimum sensitivity of the TSH immunoassay

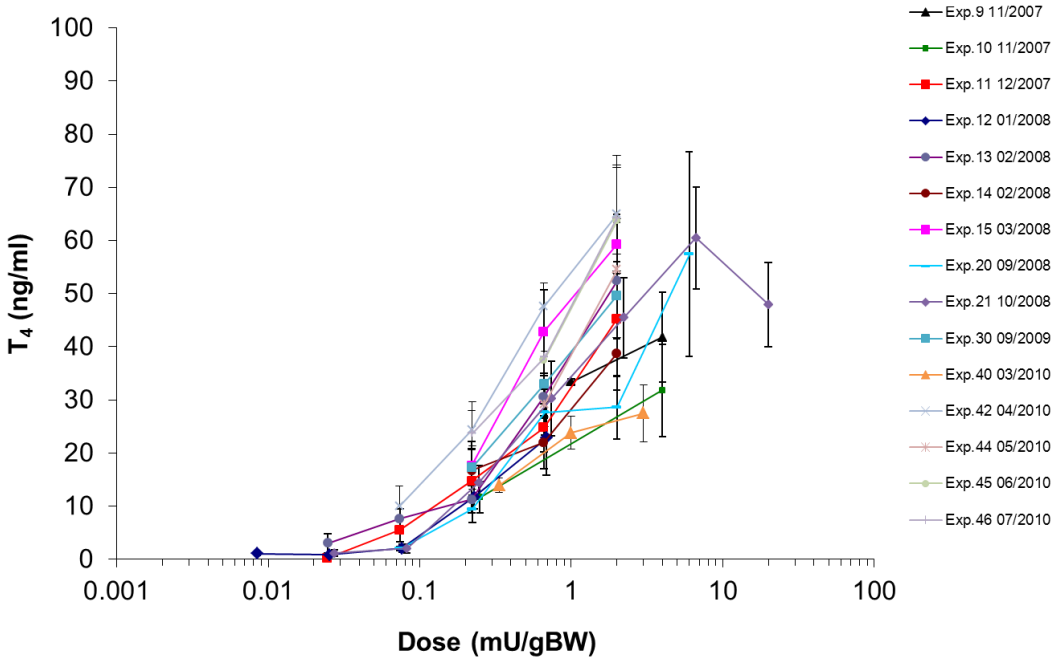

Figure 3.4. T_4 response to varying doses of bTSH. Plasma T_4 responses 5 hours after injection of increasing doses of bTSH over 35 experiments (9^{th} experiment through the 46^{th}) and more than 3 years. Each line represents a different experiment. Vertical bars represent standard error of the mean.

Characterization of the goldfish bioassay

At 5 hours post injection, a dose-dependent T_4 response to bTSH was observed between 0.08 and 2 mU/gBW (Figure 3.1). Therefore, 0.22, 0.66, and 2.0 mU/gBW bTSH were chosen as standard doses to produce a linear T_4 response for inter-assay comparisons. In the time course experiments, plasma TSH peaked by 2 hours and returned to control levels at all but the highest dose by 48 hours (Figure 3.2). Therefore, blood was sampled at 2 hours in all future experiments for TSH measurement. The half-time clearance rate of TSH in goldfish was 8.9 ± .53 hours. The T_4 responses were significantly elevated over controls by 2 hours but only the low dose group had returned to control levels after 48 hours (Figure 3.3). Subsequent dose response experiments used the 5 hour sampling time because the T_4 response to all doses of bTSH could be best discriminated at that time. Though the maximum response varied slightly among goldfish bioassays, the T_4 response to the standard doses of bTSH was consistently in the linear range for more than 3 years and 35 experiments, despite repeated sampling of the same fish (Figure 3.4).

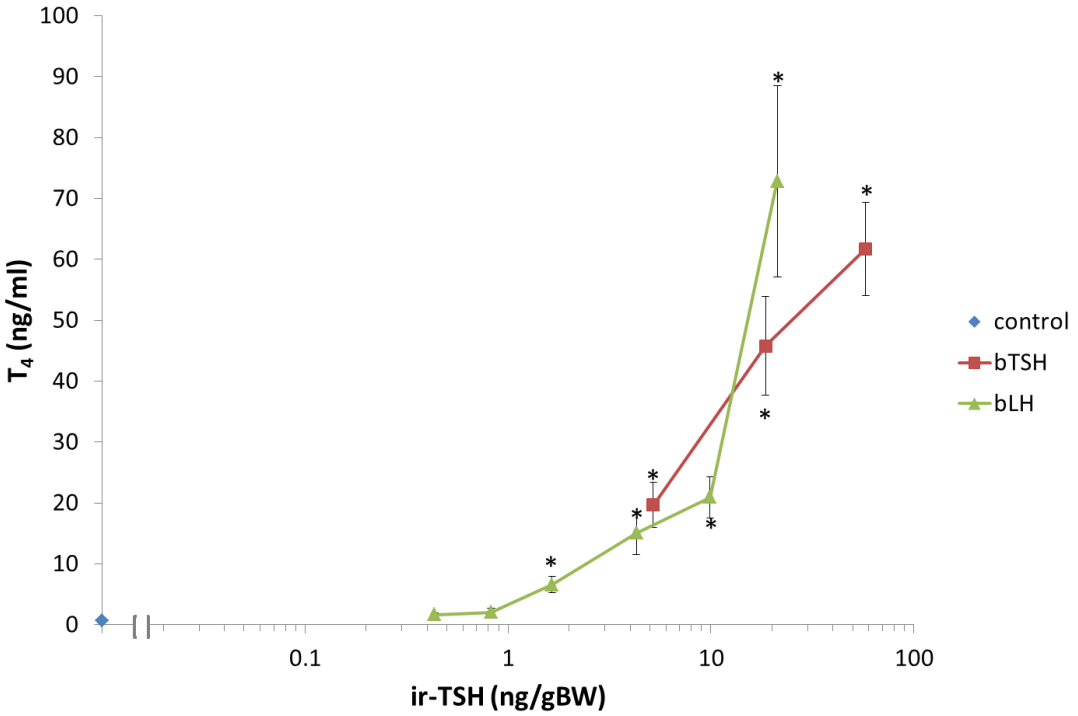

Figure 3.5. Heterothyrotropic activity of bLH. Plasma T_4 responses 5 hours after injection of increasing doses of bTSH or bLH (0.3 - 10 ug/gBW). As the x-axis represents concentration of TSH measured by immunoassay, the LH groups are plotted by TSH contamination in the LH solution and not the quantity of LH injected. Vertical bars represent standard error of the mean.
*Significant difference ($p<0.05$) between injected and control fish (n=7)

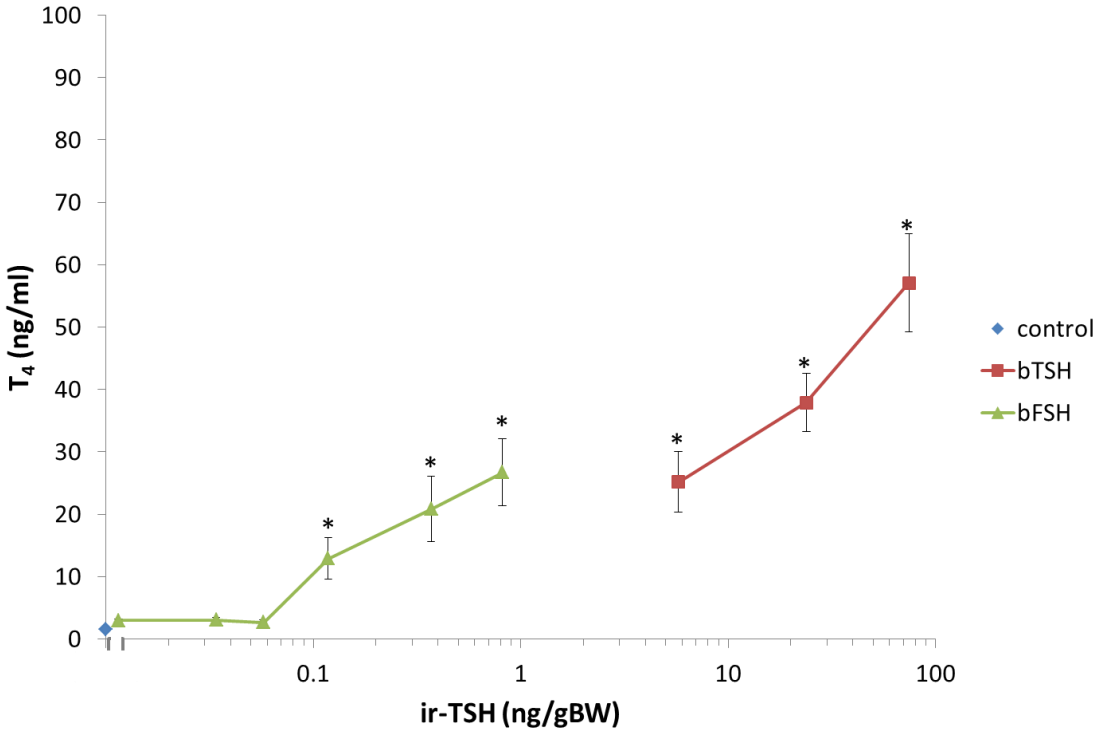

Figure 3.6. Heterothyrotropic activity of bFSH. Plasma T_4 responses 5 hours after injection of increasing doses of bTSH or bFSH (0.2 – 7.0 ug/gBW). As the x-axis represents concentration of TSH measured by immunoassay, the FSH groups are plotted by TSH contamination in the FSH solution and not the quantity of FSH injected. Vertical bars represent standard error of the mean.
*Significant difference ($p<0.05$) between injected and control fish (n=7)

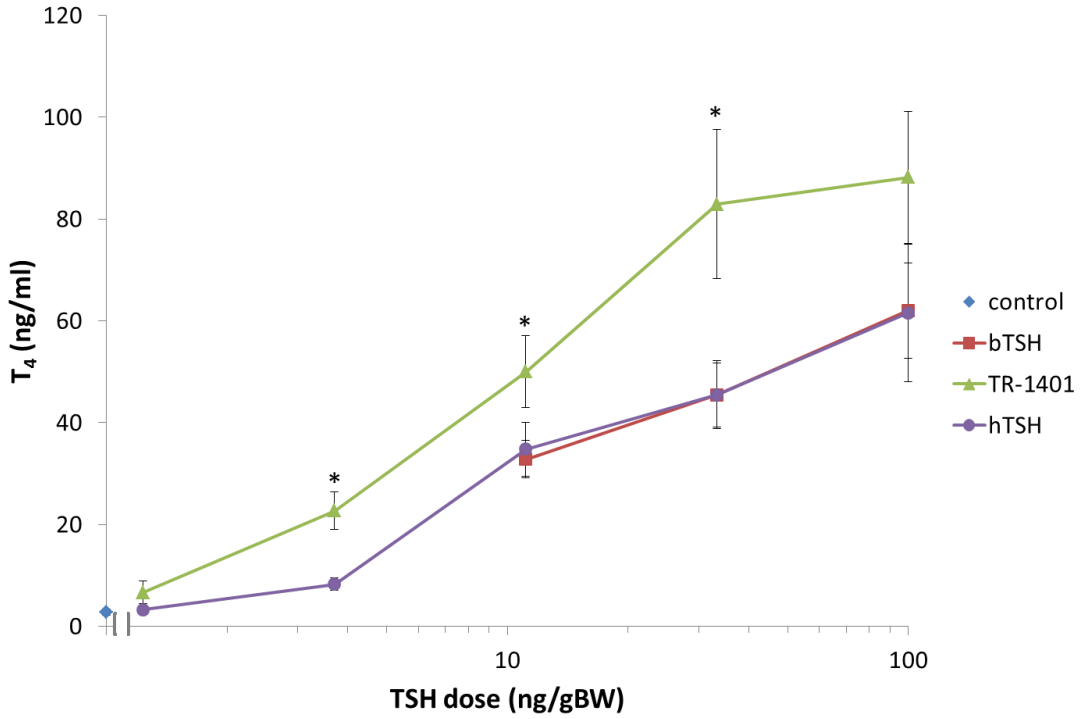

Figure 3.7. Dose dependent T_4 response to hTSH and TR-1401. Plasma T_4 responses 5 hours after injection of increasing doses of bTSH, recombinant human TSH, or recombinant human TSH analog with 4 amino acids near the n-terminal of GSUα substituted to be lysine (TR-1401). The x-axis represents mass of injected hormones. Vertical bars represent standard error of the mean.
*T_4 response of TR-1401 is significantly higher ($p<0.05$) than control, hTSH, and bTSH injected groups (n=7)

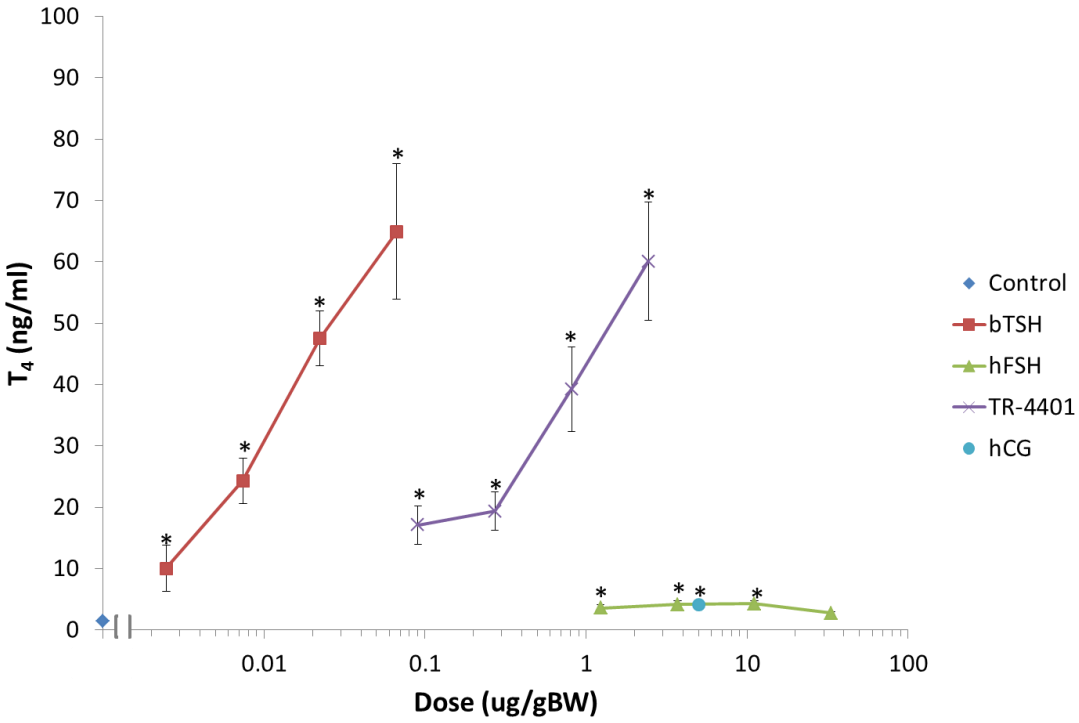

Figure 3.8. Heterothyrotropic activity of hFSH, TR-4401, and hCG. Plasma T_4 responses 5 hours after injection of increasing doses of bTSH, recombinant human FSH, recombinant human FSH analog with 4 amino acids near the n-terminal of GSUα substituted to be lysine (TR-4401), or a single dose of hCG. The x-axis represents mass of injected hormones. Vertical bars represent standard error of the mean. *Significant difference ($p<0.05$) between injected and control fish (n=7)

Table 3.1. Relative potency of glycoprotein hormones in the goldfish bioassay.

Glycoprotein hormone	**Abbreviation**	**Reported TSH activity (mU/ug)**	**TSH activity in GF relative to bTSH (mU/ug) ± std error**	**Top dose (ug) given to 20g fish**
Bovine TSH (NIH p-bTSH-13)	bTSH	30	30	
Recombinant hTSH Analog (TR-1401)	TR-1401		58.6 ± 14.5	1.25
Recombinant Human TSH (r-hTSH NIBSC 94/674)	hTSH	6.7	9.8 ± 1.2	0.06667
Bovine FSH (USDA p-bFSh-B-1)	bFSH	< .01	1.23 ± .096	140
Recombinant hFSH Analog (TR-4401)	TR-4401		0.56 ± .03	48.9
Recombinant Human FSH (UK 92/642)	hFSH		< 0.1	0.6667
hCG (CR127)	hCG		< 0.1	100
Bovine LH (USDA -bLH-B-5)	bLH	.02-.04	<.01	200

T_4 response to mammalian glycoprotein hormones

Though both bLH and bFSH produced a dose-dependent increase in T_4, only the response in the bFSH injected groups were greater than could be accounted for by TSH contamination (Figures 3.5 and 3.6 and Table 3.1). bTSH and hTSH produced almost identical T_4 responses, which were significantly lower in magnitude than TR-1401 (Figure 3.7 and Table 3.1). Although administered at approximately 50 fold higher doses than bTSH, TR-4401 gave a dose-dependent T_4 response, unlike hFSH (Figure 3.8 and Table 3.1). The one, very high dose of hCG given to goldfish only marginally increased T_4 levels above controls (Figure 3.8 and Table 3.1).

Discussion

In this study we characterized the goldfish T_4 response as a bioassay for exogenous TSH. Both the magnitude of T_4 response and the range of concentrations of TSH needed to produce a linear T_4 response were found to be equivalent to what has been reported previously in goldfish (MacKenzie et al., 1987) and other vertebrates (East-Palmer et al., 1995; Grau et al., 1985; MacKenzie, 1981; MacKenzie et al., 1978; Swanson and Dickhoff, 1987). The exception is trout, which do not produce as high magnitude a T_4 response to exogenous TSH (Chan and Eales, 1976; Milne and Leatherland, 1978), suggesting their thyroid gland may not be as sensitive to exogenous TSH as other vertebrates. This study is the first to utilize a mammalian TSH immunoassay to determine plasma TSH levels resulting from exogenous TSH injection in a non-mammalian vertebrate. In goldfish, plasma TSH was at its maximum between 2 and 5 hours after injection of bTSH and nearly 90% was removed from the circulation

by 48 hours. The resulting T_4 remained in the circulation for at least 48 hours in goldfish, similar to other fish (Chan and Eales, 1976; MacKenzie, 1982; Swanson and Dickhoff, 1987). The clearance of TSH prior to the resultant T_4 has also been demonstrated in mammals (Nielsen et al., 2004a; Szkudlinski et al., 1995b). The broad, 3 hour peak of circulating TSH in goldfish was likely due to both TSH release to the blood from the peritoneal cavity and TSH removal from the circulation. Most impressively, goldfish produced a consistent dose-dependent T_4 response to bTSH over 35 experiments and for more than 3 years, demonstrating the same fish can be used repetitively. The reuse of animals allows for a reduction in animal numbers and cost while ensuring reproducibility. Thus, goldfish T_4 response is a sensitive and stable *in vivo* bioassay for exogenous TSH. Moreover, the predictable and clear separation of circulating TSH over time in response to different doses of exogenous TSH demonstrates that goldfish can be utilized to examine the clearance in addition to the potency of exogenous TSH.

I utilized human TSH analogs as an *in vivo* tool to determine if positively charged residues at the n-terminal of the GSUα increase TSH potency in goldfish, as in mammals, thereby allowing us to evaluate the structural constraints on hormone-receptor interactions in vertebrates. It is known that bTSH has a higher binding affinity and 6-10 times greater intrinsic ability to activate the TSH receptor (TSH-R) in humans than hTSH (Szkudlinski et al., 1996). Substituting four neutral or negatively charged amino acids on an exposed loop at the n-terminal of the human GSUα for positively charged amino acids, as occurs naturally in bTSH, created a hTSH analog (TR-1401) nearly 5

times more potent than bTSH *in vitro* (Szkudlinski et al., 1996). Two negatively charged amino acids in the hinge region of the TSH-R have been shown to be necessary for the increased binding of TR-1401 (Mueller et al., 2009). It was therefore suggested that the increased potency of TR-1401 was due to enhanced electrostatic forces binding the n-terminal of GSUα to the hinge region of TSH-R (Mueller et al., 2009). The presence of positively charged amino acids at positions 13, 14, 20, and 22 (relative to human TSH sequence) of goldfish GSUα (Kobayashi et al., 1997) led me to hypothesize that the positively charged amino acids for activation of the TSH-R may be conserved. Moreover, though the goldfish TSH-R is not yet cloned, the two negatively charged amino acids thought to interact with the positively charged amino acids of the GSUα n-terminal are conserved in fish TSH-Rs that have been sequenced (Kobayashi et al., 1997; Kumar et al., 2000; Oba et al., 2000; Rocha et al., 2007; Vischer and Bogerd, 2003). I found that contrary to its diminished potency in humans, hTSH was equipotent to bTSH in the goldfish bioassay. Differences between pituitary purified and recombinant hormones, such as purity and glycosylation, make it difficult to ascribe differences in *in vivo* potencies of bTSH and recombinant hTSHs to changes in protein structure. For instance, bTSH has sulfonated glycans, which increases its clearance from circulation while recombinant hTSH contains sialic acid capped glycans that decreases its clearance from circulation (Green and Baenziger, 1988; Szkudlinski et al., 1993). However, we also found that, as in humans, TR-1401was significantly more biologically active than hTSH in goldfish, demonstrating the interaction between the positive amino acids in the

n-terminal of the GSUα and the TSH-R is conserved across a broad range of vertebrate taxa.

Since the positively-charged amino acids that increase thyrotropic activity are in the shared mammalian GSUα subunit, it is possible that these positively charged amino acids could also cause mammalian GTHs to activate the thyrotropin receptor in fish. Prior studies found that several species of mammalian GTHs can stimulate the thyroid in fish (Byamungu et al., 1990; Fontaine, 1969; MacKenzie, 1982; Milne and Leatherland, 1980). I utilized bFSH and bLH to determine if GTHs which contain positively charged amino acids at the n-terminal of their GSUα are heterothyrotropic in goldfish. Prior to injection, TSH immunoreactivity was determined for the bLH and bFSH preparations to ensure the goldfish T_4 response to mammalian gonadotropins was due to their heterothyrotropic activity and not TSH contamination. Both bLH and bFSH stimulated a T_4-release response in goldfish, suggesting that the goldfish thyroid might be responsive to heterothyrotropic activity of these hormones. The TSH immunoassay enabled me to determine that the thyrotropic activity of the bLH was could be entirely ascribed to TSH contamination. However, none of the bFSH thyrotropic activity could be ascribed to its TSH contamination, suggesting that bFSH can intrinsically bind and activate the fish TSH-R. This suggests that the mammalian bioassays used to quantify TSH contamination of LH preparations in previous studies may have underestimated biologically active TSH (Byamungu et al., 1990; Fontaine, 1969; MacKenzie, 1982). It is also possible that although LH is not heterothyrotropic in goldfish, it is in other species. Previous studies have also found mammalian FSHs to have heterothyrotropic

activity (Byamungu et al., 1990; Fontaine, 1969; MacKenzie, 1982; Milne and Leatherland, 1980), suggesting that structural modifications in the mammalian FSH molecule have enhanced its ability to bind to or activate the goldfish TSH-R. The seatbelt region of glycoprotein hormone β subunits is the most important region for conferring specificity of binding in mammals (Moyle et al., 2005). Evidence suggests that the c-terminal of the seatbelt region of FSHβ and TSHβ interact with the c-terminal region of the leucine-rich repeat domain (LRD) of their receptors (Moyle et al., 2005). Folding models of coho salmon FSH predict that the teleost FSH seatbelt has a dramatically different configuration than in mammals due to binding of alternate cysteine residues (Moyle et al., 2005). It is possible that this shifted the LRD region important for binding of teleost FSH seatbelt away from the LRD region important for interaction of the TSH seatbelt. Thus the teleost TSH-R may not have been constrained to evolve mechanisms to ensure FSH does not bind to the c-terminal region of the LRD as would be expected in mammalian receptors.

I also utilized recombinant hFSH and TR-4401 to determine the importance of positively charged amino acids at the n-terminal of GSUα. Though there are no reports on the thyrotropic activity of TR-4401 in mammals, the same GSUα inserted into hCG increases biological activity for the LH receptor *in vitro* (Szkudlinski et al., 1996). Since these two hormones are produced *in vitro* they contain no TSH contamination, so any heterothyrotropic bioactivity could be attributed to their intrinsic activation of the goldfish TSH-R. Moreover, since hFSH and TR-4401 only differ by 4 amino acids in the 11-20 region of GSUα, any differences in activity could be ascribed to the

differences in these amino acids. While bFSH activated of the amago salmon TSH-R (Oba et al., 2000) *in vitro* and the goldfish *in vivo,* human FSH, which lacks the important positively charged amino acids on the GSUα, was found to be incapable of activating the striped bass TSH-R *in vitro* (Kumar et al., 2000). I therefore hypothesized that hFSH would not stimulate T_4 release in the goldfish, but that the substitution of positively charged amino acids in the GSUα of TR-4401 would recover its heterothyrotropic activity. As expected, recombinant hFSH did not produce a dose-dependent increase in biological activity, despite using a concentration as high as 33.3 ug/gBW. However, TR-4401 did produce a dose-dependent T_4 response (0.56 ± .03 mU/µg). This dramatic increase in biological activity confirms the importance of the interaction of the goldfish TSH-R and positive charges on the exposed GSUα loop. This further demonstrates the utility of using structurally modified mammalian glycoprotein hormones to examine the conservation of receptor binding mechanisms. However, since bLH does not have heterothyrotropic activity but has the same GSUα as bFSH, it is clear that these positively charged amino acids are not solely responsible for the observed heterothyrotropic activity. Therefore, the protein structure of GTH β subunits must also contribute to the heterothyrotropic activity of the gonadotropin dimers in the goldfish.

The β subunit of hCG is a paralog of LHβ (Ludwig et al., 2002), but has been shown to activate human and teleost TSH-Rs *in vitro* (Goto-Kazeto et al., 2009; Kumar et al., 2000; Tomer et al., 1992; Vischer and Bogerd, 2003) and act as a heterothyrotropin at physiological levels in pregnant women (Haddow et al., 2004). I therefore hypothesized that hCG would be heterothyrotropic in goldfish. However, hCG

only marginally increased T_4 in goldfish at 5 ug/gBW (< 0.1 mU/µg), a relatively high dose. This suggests hCG is not heterothyrotropic in goldfish and that its ability to activate the teleost TSH-R is species specific. Chimeric models utilizing TSH and hCG suggest that much of the heterothyrotropic activity of hCG can be localized to amino acids in the seatbelt region (Farid and Szkudlinski, 2004). Since LH and hCG do not have heterothyrotropic activity in goldfish, it is possible that the central region of the goldfish TSH-R LRD, which is the putative binding site of the LH seatbelt, has acquired the specificity for binding (Moyle et al., 2005). However, the interactions of GTHs and the TSH-R are most certainly not limited to the seatbelt and LRD, but could be due to interactions between the hormone and additional exposed extracellular and transmembrane domains of the receptor.

In conclusion, the goldfish TSH bioassay is a sensitive and stable bioassay for mammalian thyrotropins and should therefore be useful for monitoring the purification of teleost TSH. This study conclusively demonstrated that select mammalian GTHs can be heterothyrotropic in a teleost fish. Heterothyrotropic activity of mammalian glycoprotein hormones can be increased in goldfish by the addition of four positively charged amino acids in the exposed loop on the n-terminal region of the GSUα. Since the basic amino acids at positions 11-20 of GSUα increases the binding and activation of the LH receptor in humans (Szkudlinski et al., 1996), it is possible that this mechanism of binding is an ancient and conserved feature of glycoprotein hormones and their receptors, having evolved before the divergence of the LH and TSH-Rs. If this is the case, then the positively charged amino acids on GSUα should increase binding and

activation of teleost fish GTH receptors. Thus, it might be possible to produce superactive teleost GTH analogs, as has been done for humans (Szkudlinski et al., 1996), that would be suitable for application in aquaculture.

CHAPTER IV

THE ROLE OF CARBOHYDRATES IN PLASMA CLEARANCE AND BIOLOGICAL ACTIVITY OF THYROTROPIN IN GOLDFISH (*Carassius auratus*)

Introduction

Thyrotropin (thyroid stimulating hormone, TSH) is a glycoprotein hormone released from the pituitary that stimulates the biosynthesis and secretion of thyroid hormone from the thyroid gland in vertebrates. TSH is comprised of a hormone-specific β subunit (TSHβ) non-covalently coupled to a glycoprotein subunit α (GSUα) which is shared with the two other pituitary hormones, the gonadotropins (GTH) (Szkudlinski et al., 2002). Relatively little is known about the regulation or action of endogenously secreted teleost TSH, largely due to lack of purified TSH suitable for biological testing and immunoassay development (MacKenzie et al., 2009). It was therefore proposed that teleost TSH be produced in recombinant expression systems (MacKenzie et al., 2009), which would allow for production of large amounts of protein without contamination of the structurally similar GTHs. A major consideration in choosing the most effective *in vitro* expression system is their ability to glycosylate the protein dimer in a manner that ensures biological activity (Szkudlinski et al., 1993).

In mammals, glycosylation of TSH is important for formation of tertiary structure, decreases its clearance rate *in vivo*, and increases receptor binding and

activation *in vitro* (Szkudlinski et al., 2002). Two asparagine-linked, biantennary oligosaccharide structures are attached to GSUα and one to TSHβ. The oligosacharides have multiple isoforms which are alternatively attached to TSH dependent upon hypothalamic TRH and affect the biological activity of TSH in mammals (Weintraub et al., 1989). The terminal sequences of these carbohydrates are most often N-acetylgalactosamine (GalNAc), sulfonated N-acetylgalactosamine ($S0_4$-GalNAc), or galactose and sialic acid (Green and Baenziger, 1988). TSH containing oligosaccharides terminating in GalNAc is quickly cleared by the liver (Szkudlinski et al., 1995a). Sulfonated carbohydrate moieties on TSH increase receptor activation *in vitro* but are similarly recognized by GalNAc sulfate-specific receptors in the liver and cleared rapidly from circulation (Szkudlinski et al., 1993; Szkudlinski et al., 1995a). The rapid clearance of sulfonated TSH is thought to aid in the generation of TSH pulses in plasma, which allows for maximal stimulation of the thyroid without down-regulation of TSH receptor (TSH-R) (Fiete et al., 1991; Szkudlinski et al., 1995a). TSH with glycans that terminate in sialic acid escape removal by the liver and instead are cleared more slowly by the kidneys, thereby increasing the duration of *in vivo* biological activity and T_4 production (Szkudlinski et al., 1993; Szkudlinski et al., 1995a). TSH produced for use in humans was therefore expressed in a Chinese hamster ovary (CHO) cell line that attached complex, biantennary oligosaccharides terminating in sialic acid (Szkudlinski et al., 1993).

Compared to mammals, relatively little is known about carbohydrates on endogenously secreted teleost glycoprotein hormones. However, several lines of

evidence suggest that glycosylation is similar in fish and mammals. Molecular cloning of teleost TSH from a number of species has demonstrated conservation of N-glycosylation sites throughout vertebrates (MacKenzie et al., 2009). Lectin affinity studies utilizing teleost TSH suggested some carbohydrates common in mammalian type glycosylation may be conserved in teleost fish (Ng et al., 1982; Sarasquete et al., 1997). Furthermore, enzymes capable of transferring sialic acid and sulfonated carbohydrates onto the ends of TSH glycans have been characterized from the brain of *Takifugu rubripes* and pituitary of coho salmon, respectively (Lehmann et al., 2008; Manzella et al., 1995). In the only study to examine fish glycoprotein hormone oligosaccharides, coho salmon LH was demonstrated to contain complex biantennary carbohydrates (Manzella et al., 1995). Manzella et al. (1995) concluded that the strong conservation of sulfonated LH carbohydrate moieties throughout vertebrate evolution suggests sulfonated glycans play an important role in teleosts, most likely the selective clearance of vertebrate glycoprotein hormones from circulation. Thus, evidence suggests that teleost TSH has mammalian-type glycosylation but its importance for clearance or receptor binding and activation in teleost fish is not known.

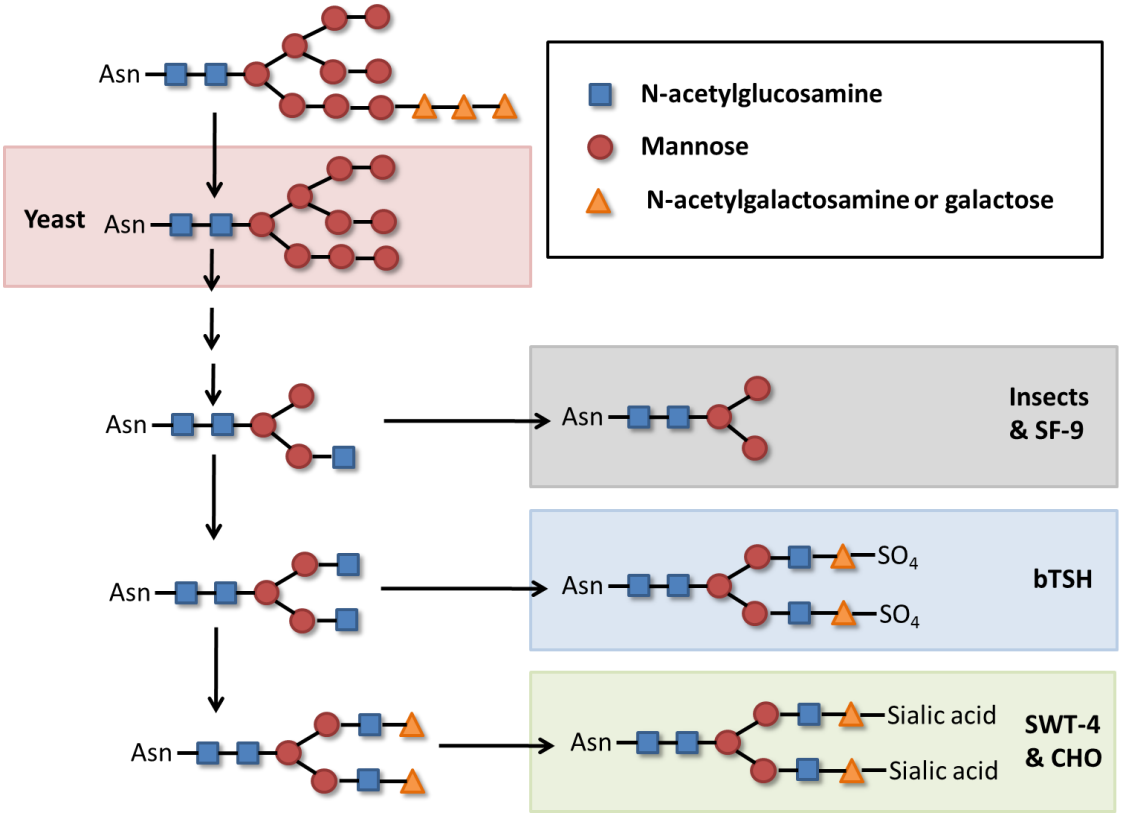

Figure 4.1. Representative enzymatic pathway of N-linked carbohydrate formation for various species and cell lines (Harrison and Jarvis, 2006; Kukuruzinska et al., 1987; Montreuil et al., 1995). Each arrow represents an enzymatic conversion.

A number of expression systems have been utilized to produce fish GTHs which are known to vary in their glycosylation activity (Figure 4.1). African catfish LH and FSH produced by soil amoeba (*Dictyostelium discoideum*) (Vischer et al., 2003) bound to what kind of receptors transiently transfected into mammalian cells and stimulated steroid production in testes *in vitro*. Though prokaryotic systems, such as amoeba, produce large protein yields relatively quickly, they do not have the ability to glycosylate (Verma et al., 1998). Zebra fish, Japanese eel, and tilapia GTHs have been produced in methylotropic yeast (*Pichia pastoris*). This yeast cell line was demonstrated to produce biologically active GTHs *in vitro* and glycosylate tilapia LH and Japanese eel FSH (Aizen et al., 2007a; Kamei et al., 2003; Kasuto and Levavi-Sivan, 2005; Yu et al., 2008). Though Aizen et al. (2007) demonstrated the tilapia FSH and LH expressed by yeast could be utilized to generate antibodies for immunoassay development and inhibiting activity of endogenously secreted GTHs, no *in vivo* activity of the hormones themselves were reported (Verma et al., 1998). *Spodoptera frugiperda* (SF) 21 insect cell lines (Huang et al., 1991), SF-9 insect cell lines (Cui et al., 2007), S2 *Drosophilia* insect cell lines (Kazeto et al., 2008; Zmora et al., 2007), and silkworm larvae (*Bombyx mori*) (Ko et al., 2007; Kobayashi et al., 2003) have all been used to generate fish GTHs with high *in vitro* biological activity but with mixed results regarding *in vivo* activity. Insect cell lines retain the ability to produce large yields of protein like yeast and prokaryotic systems while producing the more complex paucimannose residues. Moreover, insect cell transfection mechanisms cannot infect human cells, making them safer to use than mammalian systems (Verma et al., 1998). However, oligosaccharides

of recombinant glycoproteins terminating in mannose are recognized by mannose binding lectins (MBLs) and cleared as part of the innate immune response of mammals (Lam et al., 2007). MBLs have been cloned from the common carp (Nakao et al., 2006) and multiple polymorphs have been cloned from the zebra fish (Jackson et al., 2007), suggesting recombinant glycoprotein hormones produced by insect and yeast cell lines would be recognized and selectively cleared by the innate immune system of teleosts. It is possible this is the reason for low *in vivo* biological activity reported for goldfish (Hayakawa et al., 2008; Hayakawa et al., 2009) and Japanese eel (Kazeto et al., 2008) recombinant GTHs produced by insect expression systems. The biological activity of piscine GTHs that were produced using CHO cell lines (Choi et al., 2003; Yu et al., 2008) have not been reported. Therefore, though there is some evidence to expect fish TSH will need to be produced in a cell line capable of producing mammalian-type glycosylation to ensure *in vivo* biological activity there have been no directed studies to examine the relative biological activity of differentially glycosylated glycoprotein hormones in fish.

An alternative to producing teleost TSH in mammalian cell lines would be to utilize SF-9 insect cell lines which have been genetically altered to express mammalian glycosylation enzymes while still producing high yields of protein (Harrison and Jarvis, 2006). One of these modified SF-9 cell lines, SWT-4 was recently utilized to produce recombinant canine TSH (rcTSH) for a TSH challenge test to aid in the diagnosis of hypothyroidism in dogs (Dr. Scott Jaques, personal communication). Comparing the *in vivo* biological activity of rcTSH produced in SF-9 versus SWT-4 in fish would

demonstrate how insect and mammalian type glycosylation effects TSH biological activity. If SWT-4 rcTSH is biologically active, then fish glycoprotein hormones could also be produced using this modified cell line. Additionally, canine TSH immunoassays are available to determine how glycosylation affects TSH clearance from fish plasma thereby providing evidence to help determine whether altered biological activity is due to decreased receptor binding and activation or reduced duration of biological activity.

In order to understand the contributions of glycosylation to biological activity of TSH in fish, a bioassay is needed which can quantify the *in vivo* potency of TSH and characterize its removal from the circulation. Goldfish (*Carassius auratus*) can be used as a sensitive and stable bioassay of mammalian TSH and have been shown to differentially respond to even minor modifications in protein sequence (Chapter III; (Gorbman, 1940; MacKenzie et al., 1987). My objective was to first determine if removal of TSH oligosaccharides decreases biological activity and increases clearance, as in mammals. I then examined whether sulfonated carbohydrates are selectively cleared in goldfish, as some mammalian expression systems attach $S0_4$-GalNAc onto the terminal ends of glycoprotein hormones and may therefore not be suitable for producing recombinant teleost TSH. Finally, I utilized SF-9 and SWT-4 produced rcTSH to study the effects of varying isoforms of carbohydrates on biological activity in the goldfish bioassay to establish if insect-type glycosylation increases clearance and therefore decreases biological activity, as suggested by insect cell produced fish GTHs. These data indicate that glycans play a less important role in increasing the duration of circulating TSH in teleost fish than in mammals but that glycosylation is necessary for

stimulating a T_4 release response from the thyroid gland. Moreover, canine TSH produced in insect cell lines has low and variable biological activity, even when the cells had enzymes capable of producing mammalian-type glycosylation.

Materials and methods

Animals

Goldfish were purchased from Ozark Fisheries (Stoutland, MO) and held in the Biological Sciences Building East (BSBE) at Texas A&M in 100 gallon, round fiberglass tanks connected to a 2,000 gallon recirculating system. They were held at 25 ^{o}C on 12L:12D photoperiod. Fish were fed twice daily to apparent satiation with TetraMin Tropical Flakes (Tetra Holding, VA) and weighed between 10 and 50 g when used for experiments.

Injection protocol

Fish were randomly sorted into groups of 7 in 5-gallon, grid-bottom buckets which were suspended in their original tanks the night before experiments. Shortly after lights on goldfish were removed from the tanks, anesthetized with tricaine methanesulfonate (MS-222) (200 mg/L), injected intraperitoneally (IP) with hormone suspended in BSA saline (100 μL/20 gBW), and then returned quickly to their respective buckets. Control fish were injected with BSA saline (0.1% BSA, 0.9% NaCl, Sigma-Aldrich, St. Louis, MO), unless otherwise specified. Fish were recaptured at specific times after injection, anesthetized to obtain approximately 250μl of blood from the caudal vasculature into 1 ml heparinized syringes, and returned to their tanks. Blood

was centrifuged to separate plasma which was frozen for hormone determination. Goldfish were allowed at least two weeks to recover between experiments.

Hormone measurement

Specific immunoassays were used to determine blood TSH and T_4 at the Endocrine Diagnostic Laboratory at the Texas Veterinary Medical Diagnostic Laboratory. Total T_4 was measured using Coat-A-Count RIA tubes (Siemens). For the T_4 assay, sensitivity was 2.5 ng/ml (defined as the lowest point on the linear portion of the standard curve) and inter- and intra-assay variabilities were 8.2 and 9.1% (CV), respectively (manufacturer's data). Serially diluted goldfish plasma diluted parallel to the standard curve. At 1,000 ng/ml T_3 has 2% cross reactivity in the T_4 RIA (manufacturer's data) and 90 ± 2% (N=4) of T_4 spiked into goldfish plasma was recovered.

Plasma TSH was measured using an IMMULITE 2000 canine TSH immunoassay (Siemens). For the TSH assay, sensitivity was 0.01 ng/mL (as defined by the lowest point on the linear portion of the standard curve) and inter- and intra-assay variabilities were 7.6 and 5.5% (CV), respectively. Bovine TSH in goldfish plasma diluted parallel to standards. Endogenous TSH was undetectable in control treated goldfish in this assay.

The clearance and biological activity of deglycosylated bTSH

The importance of glycosylation to TSH clearance and bioactivity was examined by comparing the time course of circulating TSH and the T_4 response after an injection of 9.7 immunoreactive (ir)-ng/µl deglycosylated (dg)-bTSH or native bTSH, which has

been previously shown to be an effective dose of native bTSH in goldfish (Chapter III). dg-bTSH was obtained by chemically deglycosylating bovine TSH (Sigma-Aldrich, St. Louis, MO; 2.2 IU/mg) using the GlycoProfile IV Chemical Deglycosylation Kit (Sigma-Aldrich) per manufacturer's instructions. Briefly, 1 mg of bTSH was treated with trifluoromethanesulfonic acid (TFMS) utilizing anisole as a free-radical scavenger. A Spetra/Por Float-A-Lyzer with a 20 KD molecular weight cut off (Spectrum Labs, Rancho Dominguez, CA) was used to dialyze the resulting solution of deglycosylated bTSH (dg-bTSH) and pyridinium salt of TFMS. The dialysis tubing was allowed to soak in 2 liters of phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich) overnight. dg-bTSH was then lyophilized and rehydrated in PBS for characterization by SDS-PAGE and Western blot. To aid in determination of whether TFMS disassociated bTSH, bTSH subunits were disassociated in 10M urea at 40 ^{o}C for 1 hour followed by dilution with an equal volume 0.3M glycine (Birken et al., 1990). 2-mercaptoethanol (BME) was added to the urea-glycine solution (yielding 0.1% BME) and warmed to 56 ^{o}C for 1 hour to ensure separation of GSUα and TSHβ of bTSH. 250 ng of immunoreactive bTSH and dg-bTSH was then loaded onto 4-20% polyacrylamide the gels (Mini-Protean TGX Precast Gel, Biorad) along with separated bTSH, bovine serum albumin as a negative control for the western (BSA, Sigma-Aldrich), and molecular weight markers (Prestained SDS-PAGE standards, Biorad, Hercules, CA). The gel was run under non-reducing conditions (Thotakura and Bahl, 1987) and protein was visualized by silver stain (Biorad). A duplicate gel was run at the same time and the proteins were transferred onto a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic

Transfer Cell (Biorad) instead of being visualized by silver staining. Unbound sites on the blot were saturated with 1.5 µg/ml casein and then the blot was incubated with polyclonal canine TSH antibodies diluted in PBS. Unbound antibody was removed by rinsing with the casein solution and antibody remaining bound to TSH was visualized using a peroxidase-conjugated secondary antibody kit (Vectastain ABC-AmP kit, Vector Laboratories, Burlingame, CA).

Clearance of bTSH in fish

This experiment was undertaken to determine if bLH or BSA could saturate the mechanism used to clear bTSH from circulation as has been shown in rodents (Szkudlinski et al., 1995a). The effect of 10 µg/µl of bovine luteinizing hormone (bLH) on clearance of 10 ir-ng/µl bTSH from circulation was examined in goldfish at 11-34 hours after injection when circulating bTSH has been previously shown to be declining (Chapter III). BSA (10 µg/µl) was injected with and without bTSH (10 ir-ng/µl) to ensure that differences in clearance of bTSH and bTSH + bLH was due to sulfonated glycans and not any other isoforms of carbohydrates present on BSA.

Biological activity of recombinant canine TSH

The soluble fraction of homogenized canine pituitaries (gift of Dr. Scott Jaques, Texas Veterinary Medical Diagnostics Laboratory, College Station, TX) was first injected in goldfish to establish that they responded to canine TSH. Concentration of canine TSH in the pituitary extract was 3.3 ir-ng/µl, as measured by TSH immunoassay. The T_4 response to the pituitary extract and bTSH (injected at 1.1, 3.3, and 10 ir-ng/µl) was determined 5 hours after injection in goldfish. The contribution of carbohydrate

composition to biological activity and TSH clearance was determined in goldfish by utilizing canine TSH produced by cell lines with different glycosylation capabilities (Figure 3.1). Recombinant canine TSHs were gifts from Dr. Don Jarvis (University of Wyoming, Laramie, Wyoming) and Dr. Scott Jaques. SWT-4 and SF-9 produced rcTSH was shipped frozen in modified serum-free Grace's medium to the Texas Veterinary Medical Diagnostics Laboratory (TVMDL) for purification. Purification followed modified procedures of (Cole et al., 1993) in collaboration with Dr. Scott Jaques. The media was brought to a pH of 10.5 to precipitate cellular debris, centrifuged, and the supernatant collected. The supernatant was then subjected to anion exchange chromatography in an IEX sepharose fast flow column with HiTrap SP HP positively charged resin at 10 mL/hour at room temperature and the eluent collected. The eluent's pH was gradually lowered to 5.0 with acetic acid to allow strong binding of TSH without salt formation when subjected to cation exchange chromatography in an IEX sepharose fast flow column with HiTrap Q HP negatively charged resin (10 mL/hour and room temperature). The column was then washed with ddH_2O to further remove contaminants before 1M NaCl was used to elute TSH from the column. A Pellicon tangential flow column (Millipore, <20 PSI) was used to concentrate the eluted protein and remove salts. Afterwards, 5 mL fractions was collected following HR-200 size exclusion chromatography. Fractions high in immunoreactive canine TSH were combined and lyophilized. I examined the importance of mammalian or insect type glycosylation on the potency of canine TSH in goldfish by comparing the T_4 response to SF-9 produced rcTSH (3.5-113 ir-ng/µl) and SWT-4 produced rcTSH (5-100 ir-ng/µl) relative to bTSH

(0.1-21.5 ir-ng/µl) after 5 hours in separate experiments. Then the time course of plasma TSH and T_4 response to 50 ir-ng/µl of SF9 and SWT-4 rcTSH in addition to 10 ir-ng/µl bTSH was examined in goldfish to determine how insect-type glycosylation effects TSH clearance and biological activity.

Results

The clearance and biological activity of deglycosylated bTSH

The silver stained gel (Figure 4.2a) shows TFMS treatment causes bTSH subunits to disassociate, as dg-bTSH has two bands of the same size as dissociated bTSH and no apparent band of the same size as native bTSH (Figure 4.2a). The Western blot (Figure 4.2b) demonstrated that intact protein was also present in dg-bTSH and slightly reduced in size, as would be expected if carbohydrates were removed. Because the Western blot (Figure 4.2b) demonstrated subunits were not immunoreactive when disassociated, injection doses determined by immunoassay were for intact dg-bTSH. bTSH and dg-bTSH reached equivalent maximum values in goldfish plasma 2 hours after injection but dg-bTSH was cleared more quickly than bTSH (Figure 4.3). Despite bTSH producing a time-dependent increase in T_4 (Figure 4.4) of equivalent magnitude to that previously described in Chapter III, there was no T_4 response to dg-bTSH in goldfish.

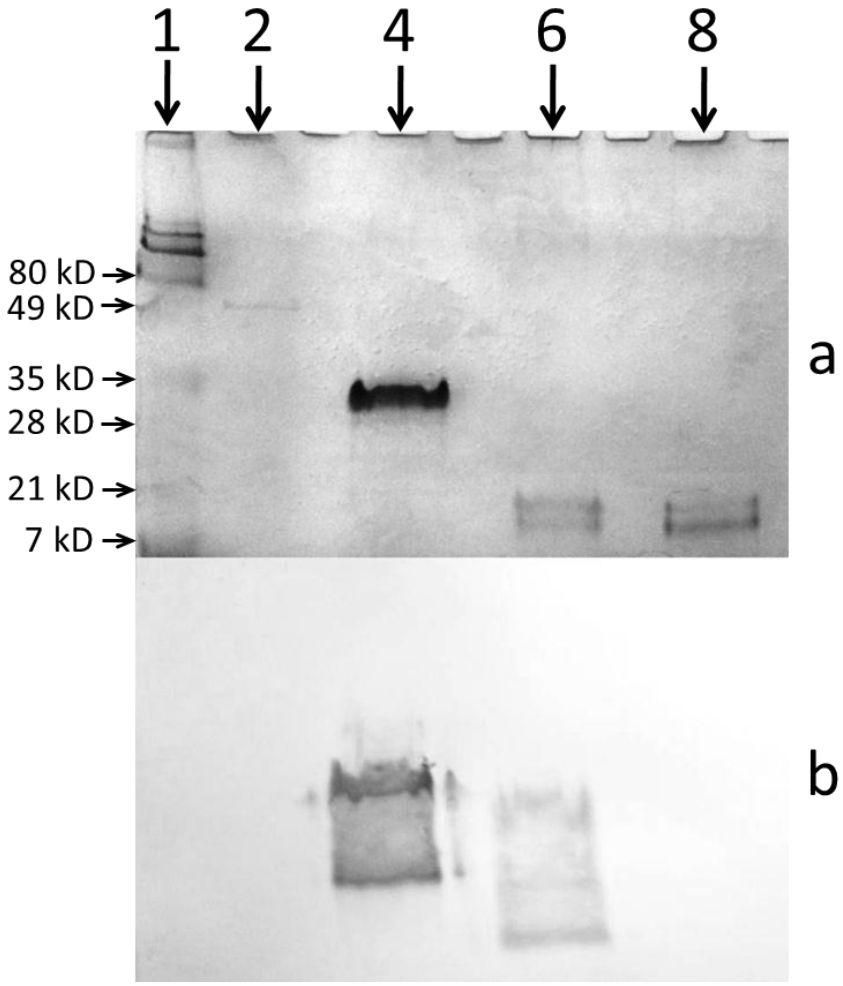

Figure 4.2. SDS-PAGE (a) and Western blotting (b) analysis of BSA (lane 2), bTSH (lane 4), dg-bTSH (lane 6), and disassociated bTSH (lane 8). Molecular weight markers are in lane 1. The samples were electrophoresed in a 4-20% gel in non-denaturing conditions. The proteins were visualized using silver stain (a) or blotted onto a nitrocellulose membrane and labeled using a peroxidase-conjugated antibodies (b).

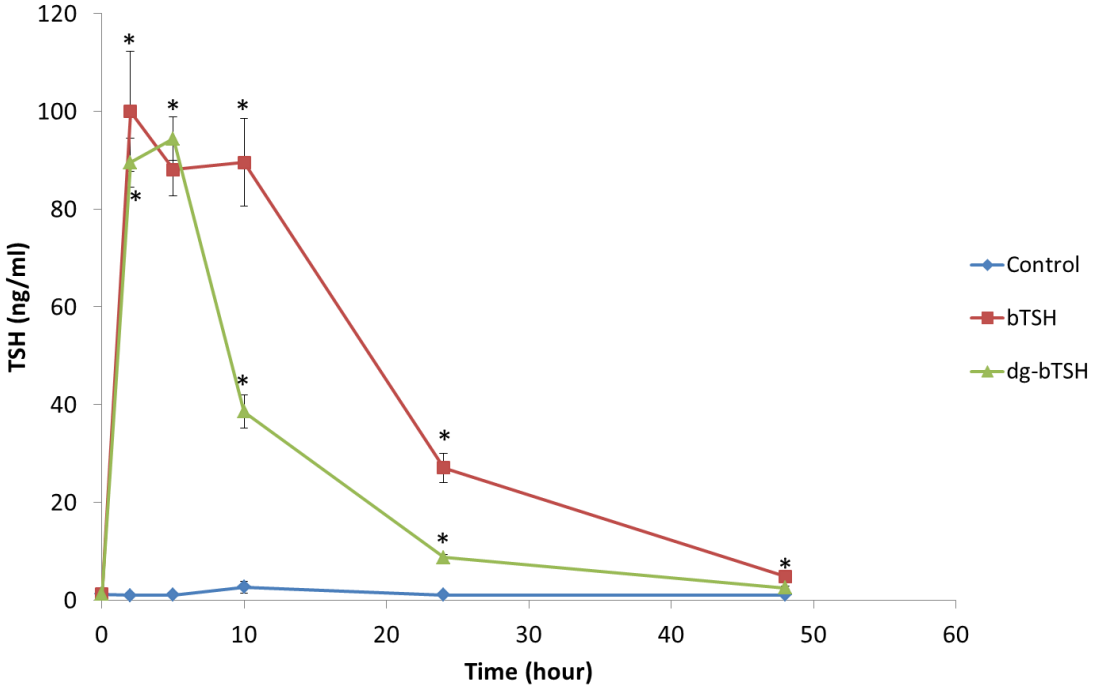

Figure 4.3. The effects of deglycosylation on clearance of TSH. Circulating TSH in response to saline, 9.7 ng/ul bTSH, or 9.7 ng/ul dg-bTSH in goldfish. Vertical bars represent standard error of the mean.
*Significantly different ($p<0.05$) than the minimum sensitivity of the TSH immunoassay

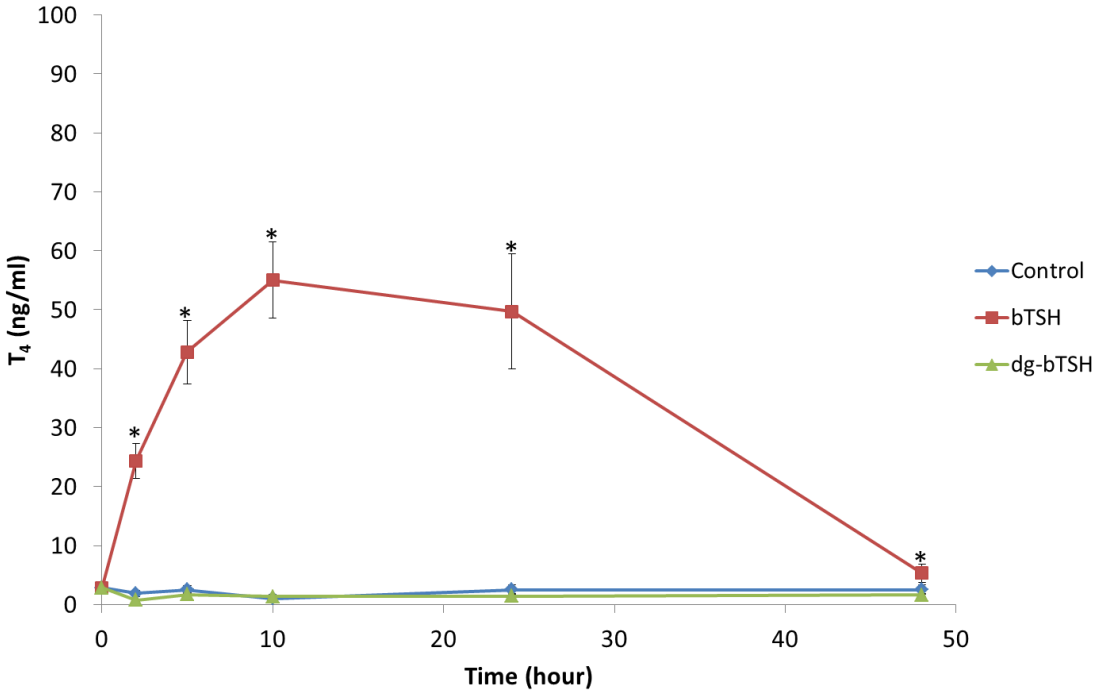

Figure 4.4. The effects of TSH deglycosylation on T_4 stimulation. Circulating T_4 in response to saline, 9.7 ng/ul bTSH, or 9.7 ng/ul dg-bTSH in goldfish. Vertical bars represent standard error of the mean.
*Significantly different ($p<0.05$) than control

Clearance of sulfonated bTSH in fish

The removal of bTSH from plasma was not affected by co-injection of LH (Figure 4.5). However, plasma bTSH was significantly lower at 11 hours after co-injection with high doses of BSA (Figure 4.5).

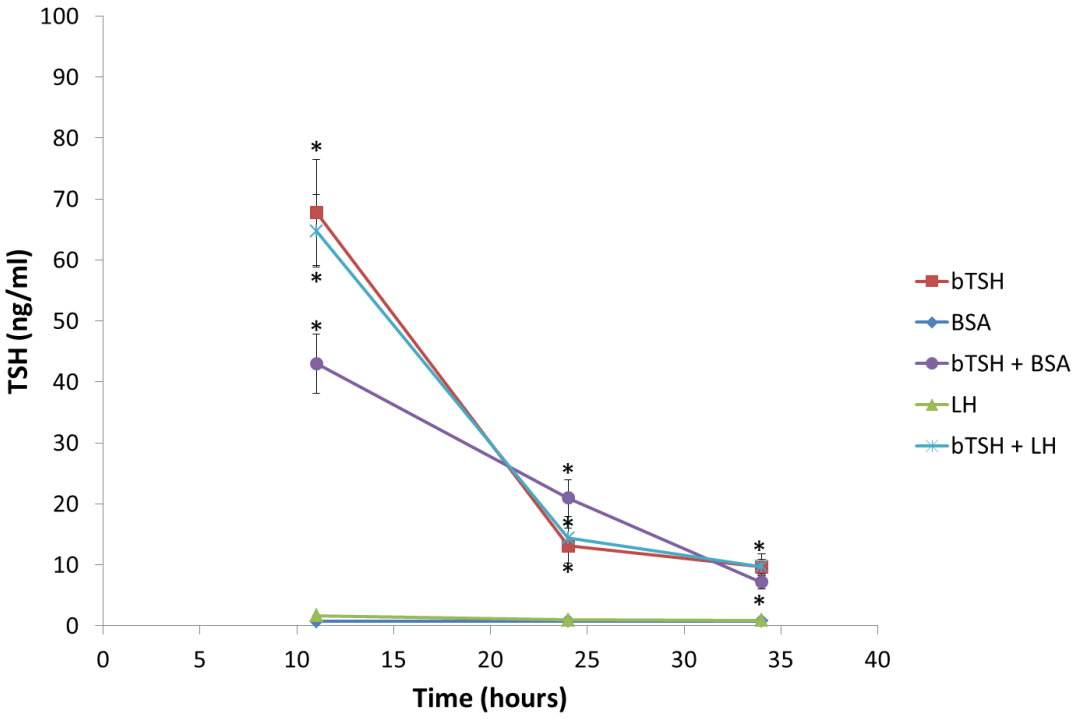

Figure 4.5. Removal of TSH from goldfish plasma over time. Circulating TSH in response to bTSH (10 µg/µl), BSA (10 ng/µl), bTSH+BSA, bLH (10 ng/µl), or bTSH+bLH. Vertical bars represent standard error of the mean.
*Significantly different ($p<0.05$) than the minimum sensitivity of the TSH immunoassay.

Biological activity of recombinant canine TSH

Canine pituitary extract at a single dose was equipotent to bTSH (Figure 4.6). rcTSH produced by a SWT-4 cell line had a minimum effective dose of 17.5 ng/µl and was less biologically active than bTSH (Figure 4.7). However, SWT-4 produced canine TSH was more potent than SF-9 produced rcTSH which had a minimum effective dose of 56.5 ng/ µl (Figure 4.8). Both rcTSHs were significantly lower than bTSH by 5 hours, despite being injected at much higher concentrations (Figure 4.9). Surprisingly, SF-9 expressed rcTSH produced only a slight but statistically significant increase in T_4 at 2 and 5 hours after injection, while SWT-4 rcTSH did not produce any significant increase in T_4 (Figure 4.10).

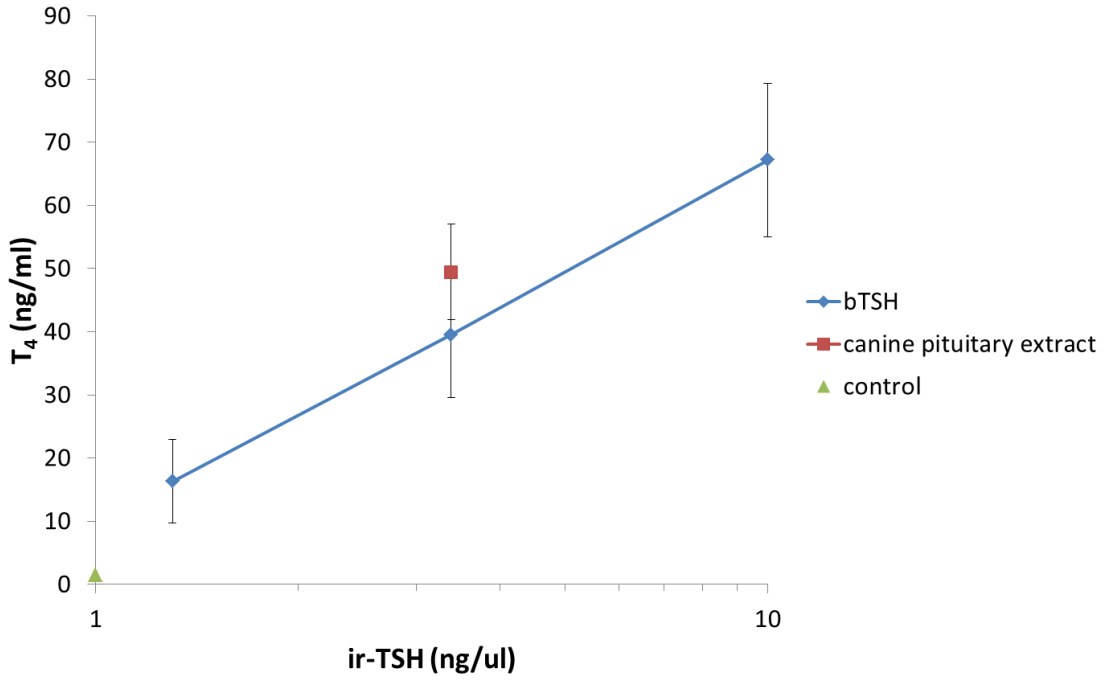

Figure 4.6. The T_4 response to canine pituitary extract. Plasma T_4 response 5 hours after injection of bTSH (1.1, 3.3, or 10 ir-ng/µl) or canine pituitary extract which had 3.3 ir-ng/µl of TSH. Vertical bars represent standard error of the mean. There was no significant difference ($p>0.05$) between canine pituitary extract and bTSH injected groups.

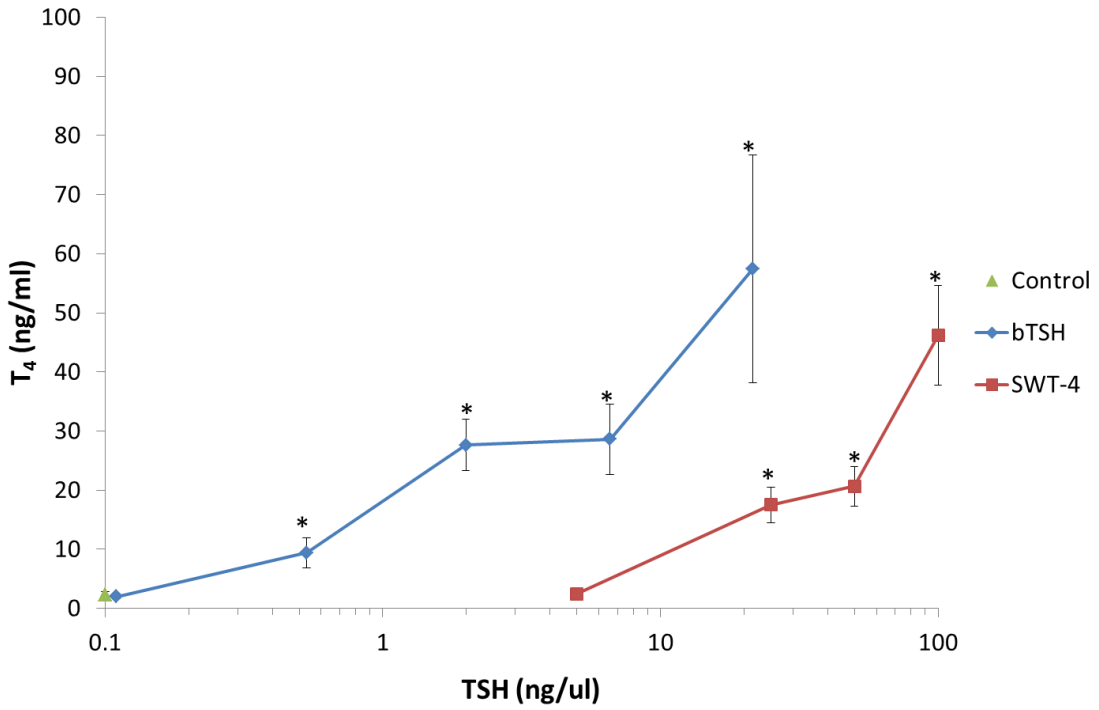

Figure 4.7. T_4 response to rcTSH produced by SWT-4 cells. Plasma T_4 response 5 hours after injection of bTSH (0.1-21.5 ir-ng/µl) or SWT-4 produced rcTSH (5-100 ir-ng/µl). Vertical bars represent standard error of the mean.

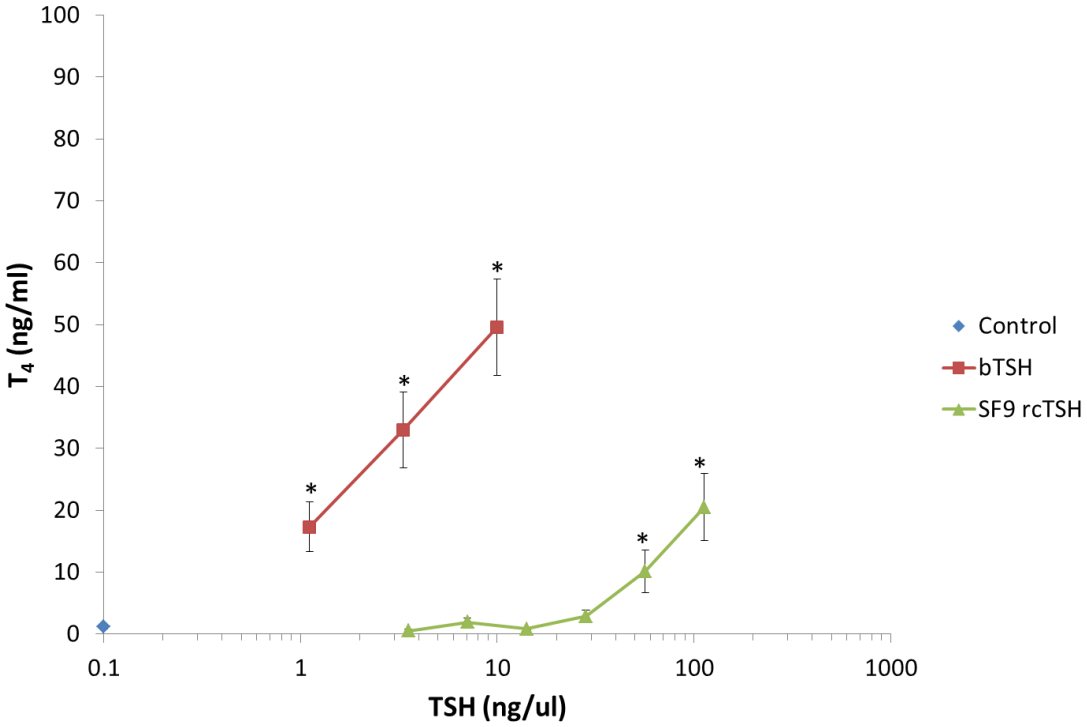

Figure 4.8. T_4 response to rcTSH produced by SF-9 cells. Plasma T_4 response 5 hours after injection of bTSH (0.1-21.5 ir-ng/µl) or SF-9 produced rcTSH (3.5-113 ir-ng/µl). Vertical bars represent standard error of the mean.

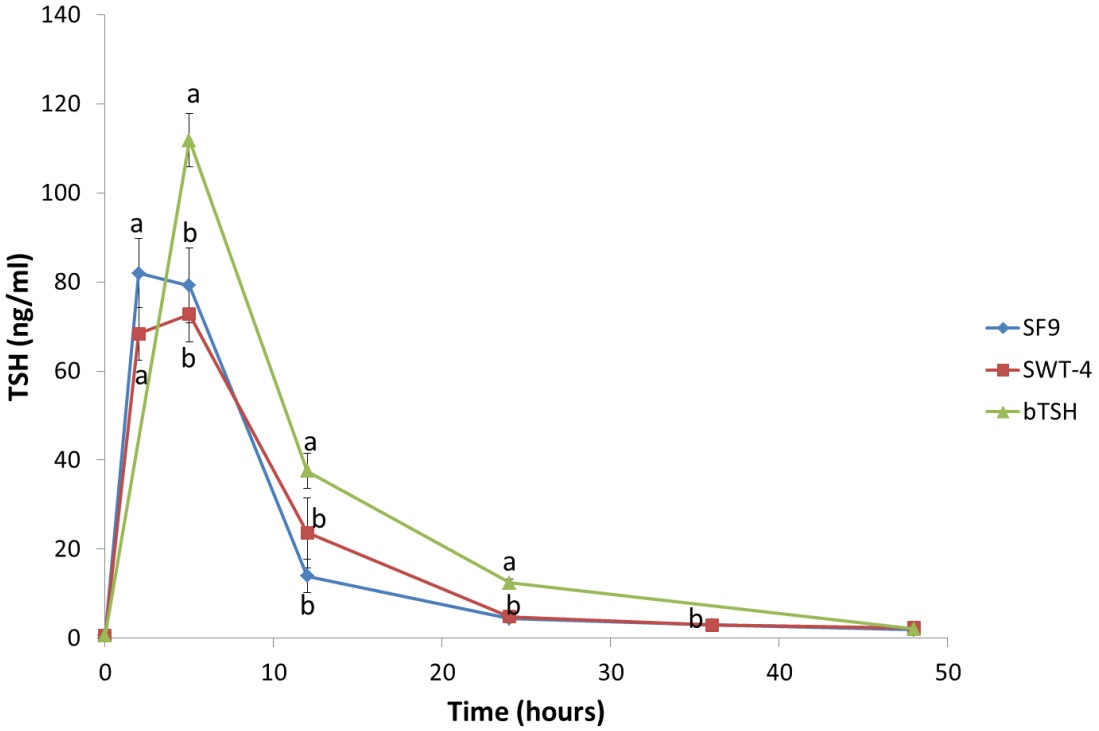

Figure 4.9. Goldfish plasma TSH over time after TSH injection. Circulating TSH in response to 10 ir-ng/ul bTSH, 50 ir-ng/ul SWT-4 produced rcTSH, or 50 ir-ng/ul SF-9 produced rcTSH. Vertical bars represent standard error of the mean.
Means sharing a letter at each time are not significantly different ($p \geq 0.05$).

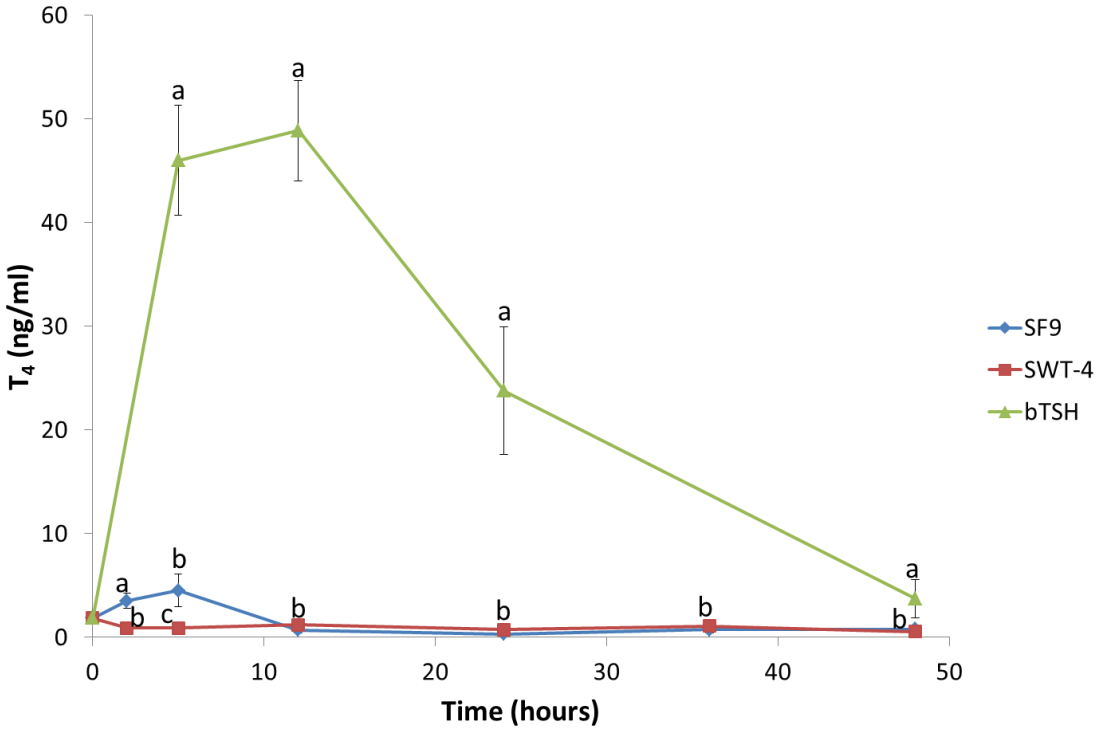

Figure 4.10. Goldfish plasma T_4 over time after TSH injection. Circulating T_4 in response to 10 ir-ng/ul bTSH, 50 ir-ng/ul SWT-4 produced rcTSH, or 50 ir-ng/ul SF-9 produced rcTSH. Vertical bars represent standard error of the mean.
Means sharing a letter at each time are not significantly different ($p \geq 0.05$).

Discussion

This study demonstrates that the goldfish can be utilized as a biological assay to determine the relative potency of recombinant or native mammalian thyrotropins that vary in their carbohydrate composition. Moreover, utilization of a mammalian TSH immunoassay allowed for the determination of the importance of glycosylation for glycoprotein hormone clearance for the first time in a teleost fish. Though TFMS was demonstrated to be effective at removing bTSH carbohydrates to determine if glycans are necessary for biological activity, the chemical deglycosylation process may also have separated a significant portion of TSH into subunits. After injected dg-bTSH was adjusted to equivalent concentration to native bTSH to compensate for loss of protein dimers, we found that there was only a slight increase in the clearance of circulating dg-bTSH. There was adequate time and concentration of circulating dg-bTSH to stimulate the goldfish thyroid but no T_4 response was elicited. In mammals, glycan removal from TSH reduces but does not eliminate receptor activation and causes a pronounced increase in clearance (Szkudlinski et al., 2002). Thus, it appears that in teleost fish, the presence of carbohydrates is more critical for receptor binding and activation and less important for clearance of circulating TSH than in mammals. However, not only the presence but also the composition of oligosaccharides could determine its clearance in fish, as has been shown in mammals (Szkudlinski et al., 2002).

It has been hypothesized that teleost TSH and LH carbohydrates are sulfonated to aid in their rapid removal from circulation as in mammals (Manzella et al., 1995). Mammals selectively clear bTSH using $S0_4$-GalNAc-specific receptors in the liver

which can be saturated by bovine LH (bLH) (Fiete et al., 1991; Green and Baenziger, 1988; Szkudlinski et al., 1995a). We found that even at 1000 fold higher concentration than bTSH, bLH did not reduce clearance of TSH from circulation. The nearly exact match of plasma TSH levels over time between TSH and TSH + LH coinjected groups suggests the majority of bTSH is cleared by a non-saturable mechanism, such as kidney filtration (Markkanen and Rajaniemi, 1979). Surprisingly, our negative control, BSA, did affect circulating bTSH. BSA had no effect on the clearance of bTSH in rats (Szkudlinski et al., 1995a). Since bTSH + BSA treated fish had lower plasma TSH at the earliest time sampled, it is not known whether BSA increases the clearance of TSH or its ability to enter circulation from the interperitoneal cavity, where it was injected. To more precisely understand the mechanisms of TSH clearance, techniques should be used to better characterize the pharmacokinetics of TSH clearance in teleost fish, such as monitoring plasma and tissue distribution of [^{125}I]TSH (Szkudlinski et al., 1995a) and identify putative TSH clearance mechanisms, including attasialoglycoprotein or $S0_4$-GalNAc-specific receptors from the liver. My results, nonetheless, suggest that receptor mediated removal of TSH possessing sulfonated oligosaccharides is not active in goldfish or is present at such low levels of activity that it is readily saturated. It is also possible that the ability to recognize carbohydrates for selected removal of endogenous or exogenous glycoprotein hormones from circulation evolved after teleost fish.

Insect cell lines have been used to produce recombinant teleost GTHs that have low *in vivo* activity despite acceptable *in vitro* activity, which suggests that fish can recognize and clear glycoproteins containing paucimannose glycans (Hayakawa et al.,

2008; Hayakawa et al., 2009; Kazeto et al., 2008). We therefore utilized SF-9 and SWT-4 produced rcTSH to determine how insect and mammalian glycosylation affects biological activity in goldfish. The increased potency of pituitary canine TSH over both forms of rcTSH was surprising, given that SWT-4 produced rcTSH was biologically active in rats and dogs (Dr. Scott Jaques, personal communication) and could have been due to high levels of FSH also in the homogenate which may likely be heterothyrotropic in goldfish (Chapter III). However, it could also be due to incomplete glycosylation by insect cell lines. Equine luteinizing hormone (LH) produced in SWT-1 cell lines, another SF-9 modified cell line which has enzymes capable of mammalian type glycosylation, did not contain sialic acid (Legardinier et al., 2005). This led the authors to conclude modified SF-9 cells may not be able to properly glycosylate all mammalian glycoproteins. It is therefore possible that the SWT-4 cell line has the enzymes necessary for mammalian glycosylation but the enzymes were not capable of properly glycosylating the canine TSH.

Improper glycosylation might also explain why there was little to no T_4 response in goldfish to rcTSH produced in either insect cell line, despite adequate levels of circulating TSH in the time course experiment. More than five times more immunoreactive rcTSH was injected than bTSH in the time course experiment and neither rcTSH produced plasma TSH values as great as bTSH. This strongly suggests that large portions of recombinant canine TSH produced by both types of insect cell line are cleared very rapidly or never escape the interperitoneal cavity. Interestingly, SF-9 produced rcTSH is not removed more rapidly than SWT-4 produced rcTSH from

circulation, suggesting fish cannot distinguish between mammalian and insect-type glycosylation. As previously stated, it is also plausible that SWT-4 is producing insect and not mammalian-type carbohydrates on canine TSH and fish can selectively clear glycoproteins with paucimannose residues. As the methods for production and purification of SWT-4 produced canine TSH are still undergoing development, it is possible that the canine TSH was not optimally pure and had interfering contamination. More studies are needed to clarify why both SF-9 and SWT-4 produced dose dependent T_4 responses after 5 hours when at equal or lower concentrations than was injected for the time course experiment. Nonetheless, the experiments with recombinant canine TSH clearly demonstrate the goldfish TSH bioassay is sensitive enough to monitor biological activity of recombinant TSH with variable carbohydrate isoforms.

In conclusion, the inability of rcTSH produced in cells capable of mammalian-type glycosylation to increase its duration in plasma, the apparent lack or low expression of $S0_4$-GalNAc-specific receptors, and the only slight increase in the rate of dg-bTSH removal suggests oligosaccharide-dependent removal of glycoprotein hormones from the circulation is less important in fish than in mammals. Moreover, because the precise regulation of clearance is thought to aid in the generation of TSH pulses in mammals (Fiete et al., 1991; Szkudlinski et al., 1995a), discrete pulses of TSH may not be as important in fish. However, these findings could be artifacts of using heterologous TSH and need to be confirmed using homologous TSH. Generation of recombinant, homologous fish TSH would also aid in production of immunoassays which could measure endogenous secretion to determine if there is pulsatile release of teleost TSH.

These studies strongly suggest that fish TSH and GTHs be produced using recombinant systems capable of mammalian and not insect or yeast-type glycosylation to ensure *in vivo* biological activity.

CHAPTER V

CONCLUSION

Relatively little is known about the regulation or action of endogenously secreted teleost TSH, largely due to lack of purified TSH suitable for biological testing and immunoassay development (MacKenzie et al., 2009). It was therefore proposed that teleost TSH be produced in recombinant expression systems (MacKenzie et al., 2009). An objective of these studies was to develop and characterize a fish bioassay to aid in the purification and characterization of recombinant teleost TSH. The low cost and ease of maintaining goldfish, combined with their world-wide availability and small size make them an excellent laboratory species. These studies demonstrate that the goldfish T_4 response can be utilized as a sensitive and repeatable bioassay for exogenous TSH. Including my studies, TSH preparations from 11 species representing every vertebrate class have now been shown to be biologically active in the goldfish (Gorbman, 1940; MacKenzie et al., 1987; Ortman and Billig, 1966). Furthermore, goldfish were utilized in these studies to aid in the purification and characterization of recombinant canine TSH being developed for a clinical TSH challenge test to diagnose hypothyroidism in companion animals. Goldfish were also shown to respond to recombinant human TSH produced in a human cell line *in vitro*. Thus, I propose that goldfish should be adopted as a standard, universal *in vivo* bioassay to aid in the characterization or purification of vertebrate TSH produced *in vitro*, including recombinant teleost TSH.

Whereas a number of authors have examined the T_4 response to exogenous TSH in fish (Chan and Eales, 1976; Kuhn et al., 1986; MacKenzie, 1982; MacKenzie et al., 1987; Milne and Leatherland, 1978; Swanson and Dickhoff, 1987), my study was the first to measure circulating TSH following injection. I determined that the half-life of immunoreactive TSH was 2.0 hours in red drum and 8.9 hours in goldfish. As TSH clearance has been studied in only two other species, rats and humans (Nielsen et al., 2004a; Szkudlinski et al., 1995b), it is difficult to draw conclusions about the physiological significance of these differing clearance rates. The relationship between clearance rate and thyrotropic activity of exogenous TSH needs to be examined in other vertebrate species to determine how changes in clearance rate may be related to the magnitude and duration of T_4 response. Using this mammalian immunoassay, I was able for the first time to demonstrate that carbohydrates increase the survival of a glycoprotein hormone in fish circulation as in mammals (Szkudlinski et al., 2002). I was also able to utilize to determine that recombinant hormones produced in insect cell expression systems are cleared more rapidly than bTSH. This suggests recombinant teleost glycoprotein hormones should be produced in cell lines capable of mammalian-type glycosylation, unlike most of the piscine gonadotropins which have been produced using yeast or insect cell lines (Aizen et al., 2007a; Cui et al., 2007; Huang et al., 1991; Kamei et al., 2003; Kasuto and Levavi-Sivan, 2005; Kazeto et al., 2008; Ko et al., 2007; Kobayashi et al., 2003; Verma et al., 1998; Yu et al., 2008; Zmora et al., 2007). Finally, I was able to use the mammalian TSH immunoassay to establish that the diminished T_4 response to exogenous TSH in red drum was due to the thyroid becoming unresponsive

to TSH stimulation and not due to alteration in the TSH clearance rate. Thus, TSH immunoassays promise to be valuable tools for studies of the activity and clearance of exogenous TSH in fish.

It has been proposed that TSH evolved by duplication of an ancestral pituitary glycoprotein hormone gene after agnathans but before divergence of actinopterygians from the vertebrate lineage (Sower et al., 2009). If TSH has acquired a central role in stimulating thyroid hormone secretion that has been conserved from actinopterygians to mammals then you might expect conservation of specific molecular mechanisms governing the specificity of TSH interaction with its receptor. I utilized superactive human analogs to determine that four positively charged amino acids at the n-terminal of GSUα are important for activation of the goldfish TSH-R as in mammals (Szkudlinski et al., 1996). Since the positively charged amino acids at the n-terminal of GSUα can also increase the binding of GTHs in mammals (Szkudlinski et al., 1996), it is possible that the importance of these amino acids for receptor binding evolved prior to the divergence of TSH and GTHs. Lampreys belong to the ancient vertebrate taxon Agnatha, and appear to have both GTH receptor-like and TSH receptor-like glycoprotein hormone receptors but only a single glycoprotein hormone presumed to be the precursor of GTHs and TSH (Sower et al., 2009). If the TSH receptor-specific function of the positively charged amino acids in the 10-21 region of GSUα evolved prior to the divergence of TSH and GTHs, then positive amino acids in that region of the lamprey GSUα should increase activation of their TSH-like receptor. Unfortunately, an agnathan GSUα has not been described (Sower et al., 2009). Future studies on the functional evolution of TSH –

TSH-R relationships should focus on determining if the positively charged amino acids in the 10-21 region of GSUα are important for binding in animals that evolved prior to actinopterygians, such as agnathans.

Very few studies have examined the regulation of thyroid sensitivity to circulating TSH in fish. Changes in a species' sensitivity to exogenous TSH over time in a controlled laboratory environment with constant photoperiod is a novel finding, suggesting there may be a fundamental difference in the regulation of the goldfish and red drum thyroid axes. Since red drum at the ARTF remained sensitive to TSH, the ability of goldfish but not red drum to retain sensitivity to TSH in BSBE is not due to differences in reproductive maturity or age. Photoperiodically-induced insensitivity or endocrine disruption of the thyroid axis remain reasonable hypotheses as to why thyroid sensitivity is lost in red drum (Chapter II). It is possible that the red drum thyroid axis is much more sensitive to seasonal changes in photoperiod in captivity than in goldfish. This may have important practical implications for red drum aquaculture, suggesting, for example, that thyroid function may be more effectively maintained in captive animals through photoperiod manipulation. Alternatively, if an endocrine disrupting chemical (EDC) were responsible for desensitizing the red drum thyroid, then it would be expected that goldfish kept in the same water and maintained using the same equipment would also be exposed. However, I cannot rule out differences in sensitivity to an EDC. It is possible that goldfish have better compensation mechanisms to maintain thyroid status when exposed to EDCs. Future studies are needed to determine whether insensitive red drum have reduced TSH-R expression in thyroid follicular cells and may

therefore maintain thyroid hormone secretion more independently of central TSH stimulation. If red drum regulate the sensitivity of their thyroid by altering TSH-R expression independent of circulating TSH, then this suggests peripheral mechanisms play an important role in regulating T_4 release from the thyroid. Thus understanding the physiological mechanism underlying the reduced T_4 response to exogenous TSH in red drum while helping to determine the cause of insensitivity, may also further our understanding of the importance of central TSH control in the regulation of circulating thyroid hormones in teleost fish.

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