PURIFICATION AND CHARACTERIZATION OF A RECOMBINANT GLYCOPROTEIN, CANINE THYROID STIMULATING HORMONE, AS A PRELUDE TO THE DEVELOPMENT OF THE REPRODUCTIVE GLYCOPROTEINS

A Dissertation

by

MALCOLM LEIHULU DELOVIO, JR.

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

August 2012

Major Subject: Physiology of Reproduction

Purification and Characterization of a Recombinant Glycoprotein, Canine Thyroid Stimulating Hormone, as a Prelude to the Development of the Reproductive Glycoproteins

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ABSTRACT

Purification and Characterization of a Recombinant Glycoprotein, Canine Thyroid Stimulating Hormone, as a Prelude to the Development of the Reproductive Glycoproteins. (August 2012)

Malcolm Leihulu Delovio, Jr., B.S., Texas A&M University

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Dr. David Forrest

A baculovirus (*Spodoptera frugiperda*) system was designed to express recombinant canine thyroid stimulating hormone (rcTSH). The efficacy of rcTSH was measured against pituitary derived bovine thyroid stimulating hormone (bTSH) through a series of *in vitro* and *in vivo* experiments.

Initial purification of rcTSH was performed in order to characterize the hormone for further analyses. Ion exchange columns and tangential flow membranes were chosen based upon the traits of the rcTSH molecule. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels visualized by Coomassie blue, silver stain, and western blot demonstrated the effectiveness of the purification process.

Primary cell, static tissue, and perifusion tissue cultures were employed to investigate *in vitro* thyroid cell/tissue response to rcTSH and bTSH. Canine thyroid cells were liberated from tissue samples, cultured, then exposed to TSH treatments in which media was subsequently harvested and measured for cyclic adenosine monophosphate (cAMP), a second messenger in the TSH downstream signaling cascade.

The cAMP concentrations measured were sporadic and not consistent with expected concentrations for treatments or controls. For the static tissue culture, slices of bovine thyroid tissue were incubated and exposed to a series of media-only wash steps as well as treatment steps using varying concentrations of rcTSH. Unfortunately, the experiment was compromised resulting in the slow release of thyroxine (T4) for all samples due to tissue death. Perifusion experiments conducted on bovine thyroid tissue compared the release of T4 due to bTSH and rcTSH stimulation in a dynamic system. Unable to perform statistical analysis due to small sample sizes, graphical representation demonstrated stimulatory effects by bTSH and rcTSH when compared to control.

Biological assays were used to compare the *in vivo* efficacy of rcTSH to bTSH which included 3 species (goldfish, rat, and canine). Results from mammalian experiments when subjected to analysis of variance (ANOVA) resulted in the rejection of the null hypothesis of equal means ($P<0.05$) between control, bTSH, and rcTSH treatments. Further analysis by Tukey's W procedure demonstrated the stimulatory actions of rcTSH and bTSH to be similar ($P>0.05$) to each other while greater ($P<0.05$) than control at the 4 and 6 hour post injection time.

ACKNOWLEDGEMENTS

"A pupil is not above his teacher; but everyone, after he has been fully trained, will be like his teacher." Luke 6:40

Our Holy Father has blessed me with a wonderful and supportive family that has given me all the reasons to finish what I started. My wife, you have been a rock through this and deserve to have your name on the diploma alongside mine. My daughter, you brought me joy and would rescue me through times of frustration. My parents, you instilled in me the motivation and determination to better myself and to have confidence in my abilities.

I have also been blessed to have a committee of members who would not see me fail and would mold me into the student, teacher, and scientist I have become as a result of their tutelage and guidance. Dr. Jaques, you have been my employer, committee co-chair, and friend. We have seen both our families and the endocrinology lab grow and flourish. We are not done yet!

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In September 1927, researchers discovered a substance produced by the anterior pituitary which stimulated thyroid activity inducing metamorphosis in larval salamanders (Uhlenhuth and Schwartzbach, 1927). The substance was thyroid stimulating hormone (TSH), a glycoprotein produced and released by the thyrotropes of the anterior pituitary. Secretion of TSH is stimulated when thyrotropin releasing hormone (TRH) produced by the medial neurons of the paraventricular nuclei are released and traverse the hypothalamic-hypophyseal portal system and bind to its receptors on the pituitary thyrotropes. In the human species, TSH will travel via venous drainage from the anterior pituitary to the cavernous sinus leading to the inferior and superior petrosal sinus and enter systemic circulation through the superior bulb of the jugular vein. Binding will eventually occur to TSH receptors of follicular cells located in the thyroid gland after returning from systemic circulation through one of the three following thyroid supply arteries: superior thyroid artery (common carotid), inferior thyroid artery (subclavian), or small thyroid ima artery (brachiocephalic at the aortic arch) (Greenspan and Baxter, 1994). This, in turn, will stimulate the production and release of triiodothyronine (T3) and T4 from the thyroid gland which are necessary for numerous cell activities (i.e. metabolism, protein expression).

This dissertation follows the style of Journal of Animal Science.

The TSH molecule is a heterodimer produced by the thyrotropes of the anterior pituitary (adenohypophysis) comprised of an alpha (α) and a beta (β) subunit which are non-covalently bound to one another. The α subunit is conserved among TSH and three other glycoprotein hormones within the same species: luteinizing hormone (LH), follicle stimulating hormone (FSH) and chorionic gonadotropin (CG). While FSH and LH are produced by the gonadotropes of the anterior pituitary, CG production occurs in the placenta during pregnancy. It is the variations in the β subunit that confer specificity of the aforementioned glycoprotein hormones (Keel and Grotjan, 1989). In the canine, the common α subunit consists of 96 amino acids with five intramolecular disulfide bonds and two N-linked oligosaccharide chains extending from asparagine (Asn) residues at positions 56 and 82. The TSH-β subunit is a slightly larger peptide chain with 118 amino acid residues, six intramolecular disulfide bonds, and one N-linked carbohydrate chain extending from an Asn residue at position 23 (Yang et al., 2000a,b). While non-covalent bonds such as Van Der Waals, hydrogen, ionic and hydrophobic interactions are largely responsible for TSH-α and TSH-β dimerization, a "seatbelt" region is formed by two disulfide bonds between three cysteine amino acids of the TSH-β subunit which wraps around and stabilizes the α subunit (Grossman et al., 1997).

Thyroid stimulating hormone is termed an N-linked glycoprotein due to the long chains of carbohydrates that are attached to the N-groups of specific asparagine residues, two on the α subunit and one on the β subunit. Glycosylation is the process of enzymatically attaching and altering a chain of carbohydrates, such as sugars, onto a

protein or other molecule. These Asn, N-linked oligosaccharides have been shown to affect TSH by: increasing plasma half-life, increasing bio- and immuno-activity, promoting subunit dimerization, and enhancing biosynthesis and secretion (Szkudlinski et al., 1995). In eukaryotes, N-linked glycosylation is performed in the endoplasmic reticulum (ER) and Golgi apparatus as a post translational modification to proteins. Oligosaccharides containing 3 fucose, 9 mannose, and 2 N-acetylglucosamine molecules are attached to the N-group of an asparagine residue by the enzyme oligosaccharyltranferase (Mohorko et al., 2011). In the Golgi apparatus, fucose and six mannose molecules will be enzymatically cleaved by α-glucosidase and α-mannosidase, respectively. This is followed by the enzymatic additions of fucose, mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, and/or sialic acid (N-acetylneuraminic acid). The following table 1 lists the various carbohydrates and the enzymes used for removal or attachment to a developing chain:

Table 1. N-linked Glycan Structures. These are comprised of various carbohydrates which are enzymatically added or removed from the developing chain.

Carbohydrate	Removal Enzyme	Attaching Enzyme
N-acetylglucosamine	N-acetylglucosaminidase	N-acetylglucosaminyltransferase
Mannose	α-mannosidase	Mannosyltransfease
Fucose	α-glucosidase	Fucosyltransferase
Galactose	α-galactosidase	Galactosyltransferase
N-acetylgalactosamine	N-acetylgalactosaminidase	N-acetylgalactosyltransferase
Sialic acid	Sialidase	Sialyltransferase

The carbohydrate molecules and the order of their addition to the truncated chain is species driven. For example, figure 1 illustrates that insects and mammals have similar glycosylation patterns with insect cells producing paucimannose N-glycans and mammalian cells producing complex N-glycans. The paucimannose structure consists of 2 N-acetylglucosamine and 3 mannose molecules while a complex N-glycan uses the same framework as the paucimannose but adds to the structure with any number and combination of the previously mentioned carbohydrates (Jarvis, 2003). This is an important concept when designing a baculovirus system to express mammalian proteins. The ability to achieve mammalian glycosylation is not inherent to the insect cells so the insect cells must be programmed to produce the enzymes necessary for mammalian glycosylation. Also present must be the substrates onto which the enzymes will act in order to propagate the carbohydrate chain in the same manner as would a mammalian cell.

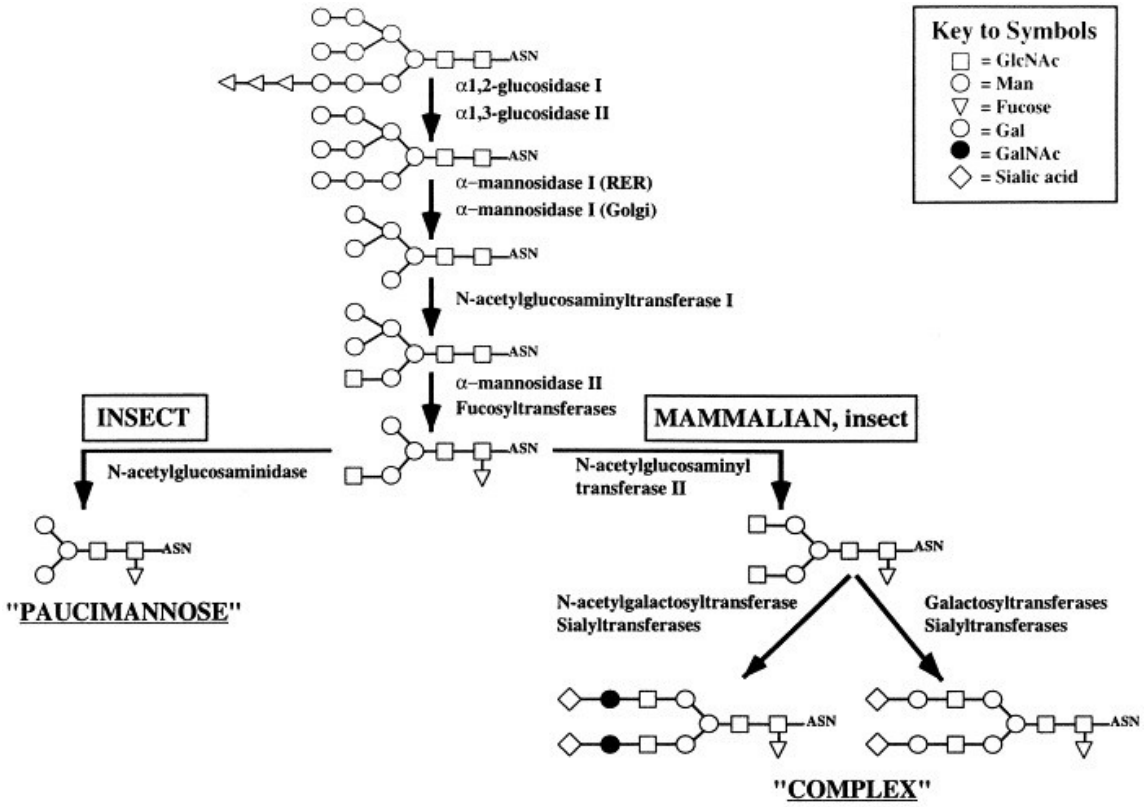

Figure 1. Insect and Mammalian Glycosylation Pathways. A common pathway is shared between insect and mammalian glycosylation. While mammalian cells will regularly produce complex carbohydrate chains, insect cells generate a more truncated version of the mammalian glycan but demonstrate the ability to form more complex structures (Jarvis, 2003).

Stimulation of TSH production and subsequent release is affected by TRH, which consists of three amino acids, binding to the thyrotropin releasing hormone receptors (TRH-R) on the thyrotropes of the adenohypophysis. There exist two different forms of TRH-R, TRH-R1 and TRH-R2. Both forms are found outside the anterior pituitary while only the TRH-R1 form inhabits thyrotropes (Neumann et al., 2010). As a G protein-coupled receptor (GPCR), TRH-R initiates outside of the cell (N-terminus) then forms seven transmembrane, α-helix loops and terminates inside the cell (C-terminus). This enables extracellular molecules to cause an intracellular effect by binding to the N-terminus promoting a conformational change of the receptor protein initiating a cascade of events. A GPCR will mediate the signal of a bound hormone by the cAMP or the diacylglycerol/inositol triphosphate (DAG/IP3) pathway. Studies have shown TRH stimulates TSH secretion by the DAG/IP3 pathway with little support for the cAMP cascade (Mariotti, 2011). Both mechanisms share the same initial steps where hormone binding and subsequent receptor conformational change promotes the replacement of guanosine diphosphate (GDP) on the inactive α subunit of the G protein with guanosine triphosphate (GTP). This, in turn, leads to the activation of the G protein and dissociation of the βγ subunits from the α subunit. The G protein βγ dimer will activate the membrane bound phospholipase enzyme resulting in the breakdown of membrane phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP_2) into DAG and IP3. The DAG molecule will activate protein kinase C (PKC) leading to TSH gene transcription. IP3 will promote intercellular Ca^{2+} concentrations to rise via stimulating

the smooth endoplasmic reticulum to release its Ca^{2+} stores and by opening Ca^{2+} channels in the cell membrane allowing for an efflux of Ca^{2+} from the extracellular fluid. An increase in intercellular Ca^{2+} concentrations stimulate the transcription of the TSH gene by activating PKC through direct binding to the enzyme or indirectly by binding first to calmodulin which will then activate PKC (Mariotti, 2011). The intercellular Ca^{2+} increase also stimulates exocytosis of the secretory vesicles containing the mature TSH molecules.

The thyroid stimulating hormone receptor (TSH-R) belongs to the glycoprotein hormone receptor (GPHR) family due to the N-linked glycosylation of this hormone receptor. GPHRs are a subset of the larger leucine-rich repeats containing GPCRs (LGR) family which are also a subset of the GPCR superfamily (Farid and Skudlinski, 2004). As with other GPCRs, TSH-R contains seven transmembrane, α-helix loops with an intracellular C-terminus and an extracellular N-terminus.

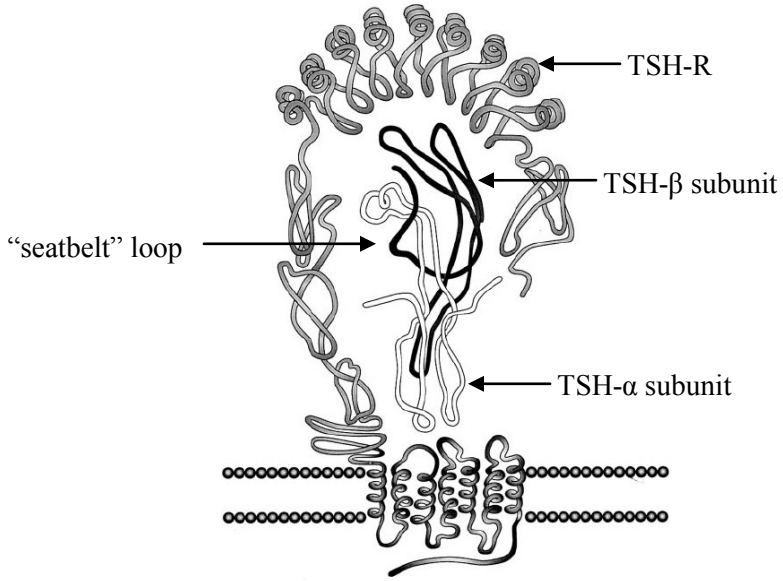

Figure 2. Interaction Between Thyroid Stimulating Hormone and Receptor. The interaction between the large extracellular domain of TSH-R (grey) and the TSH molecule (Szkudlinski et al., 2002). The TSH-α subunit is shown in white and the TSH-β subunit is shown in black. Note the "seatbelt" loop formed on the terminal segment (upper left) of the TSH-β subunit.

As shown above in figure 2, the TSH-R contains a large extracellular domain with nine leucine-rich repeats seen forming an arch around the TSH molecule. Unlike TRH-R, ligand binding to the TSH-R will favor activation of the cAMP cascade to propagate the effects of hormone binding rather than the DAG/IP3 mechanism (Metcalfe et al., 1998; Dremier et al., 2007). Similar to the DAG/IP3 pathway, activation through ligand binding of TSH-R will promote the substitution of GDP with GTP activating the G protein; however, the G protein will activate adenylate cyclase which transforms adenosine triphosphate (ATP) to cAMP. Once converted, cAMP activates protein kinases needed to drive the formation of T3 and T4 by the follicular cells of the thyroid gland.

The canine thyroid is a bi-lobed, secretive gland containing numerous follicles. The follicles are spherical in shape formed by a single layer of simple cuboidal epithelial cells. The center of this sphere forms a reservoir where colloid material is stored. Colloid represents a mixture of substances (i.e. thyroglobulin, iodine) utilized by the follicular cells to manufacture thyroid hormones. The primary substrates utilized for the production of T3 and T4 are: iodide, thyroglobulin, tyrosine, thyroid peroxidase, and various lysosomal proteases. Thyroglobulin, a large glycoprotein produced by the thyroid follicular cells, serves as a backbone for multiple tyrosine residue attachments. The thyroglobulin/tyrosine structure is packaged into a vesicle by the rough endoplasmic reticulum and undergoes exocytosis into the colloid. Under stimulation by TSH binding, the thyroid follicular cell will actively transport iodide from circulation via an energy requiring sodium-iodide pump, across the cell, and into the follicular reservoir. Upon

deposition into the colloid, thyroid peroxidase oxidizes the iodide molecules forming very reactive intermediates which will rapidly iodinate the tyrosine residues attached to the thyroglobulin protein. Following iodination, the tyrosine residues will couple forming T3 or T4 depending on the iodinated state of the individual tyrosine residues. Monoiodotyrosine (MIT) is formed when a tyrosine residue carries one iodide while diiodotyrosine (DIT) is a fully iodinated tyrosine residue with two iodide molecules. Two DIT moieties form T4 and one MIT coupled to one DIT will make T3. Iodination and coupling occurs in close proximity to the colloid-follicular cell border allowing for endocytosis of colloid material, including thyroglobulin, back into the follicular cell. Lysosomes will fuse with the colloid vesicle where lysosomal enzymes will break down thyroglobulin liberating MIT, DIT, T3, and T4. The T3 and T4 hormones will enter circulation while MIT and DIT will be deiodinated and recycled for future thyroid hormone production (Greenspan and Baxter, 1994).

Once in circulation, T3 and T4 will find protection from circulating enzymes that might break down the thyroid molecules by binding to carrier proteins such as albumin, thyroxin binding globulin (TBG), or transthyretin (TTR) (Bartalena, 1990). These proteins will also increase the plasma solubility of thyroid hormones while in circulation as T3 and T4 are lipid soluble with the ability to pass through plasma and nuclear membranes of cells. The half-life of T4 can range from 5 to 7 days while T3 is only 1 day. This could explain why T4 is released from the thyroid in much greater amounts than T3 (20 to 1). Though the majority of thyroid hormones found in circulation are attached to binding proteins, small amounts are available in a "free" form. The "free"

hormones are not bound by the constraints of a large a carrier molecule and may pass in and out of circulation and cells. Once inside the cell, deiodination by 5' deiodinase converts T4 into T3 which is the ligand for nuclear thyroid receptors (TR). This intracellular conversion implies that T3 is the hormonal effector with the role of T4 being one of a prohormone. It must be noted that nuclear TRs are nondiscriminatory as to whether T3 originates from circulation or from T4 deiodination. The T3-TR complex will bind to a thyroid hormone responsive element which will alter RNA polymerase II activity on a gene responsive to T3 (Greenspan and Baxter, 1994). Released T3 and unmodified T4 will enter circulation again to be metabolized by the liver or serve as negative feedback mechanisms at the hypothalamus and anterior pituitary inhibiting TRH and TSH secretion, respectively.

Many intermediate mechanisms exist separating the recognition of low, systemic thyroid hormone concentrations and the replenishing of T3 and T4 by the thyroid gland. Defects in these mechanisms could lead to a state of hypothyroidism. In the canine, hypothyroidism is an extremely common endocrine disease that is usually acquired rather than congenital. Primary hypothyroidism, reduced output of thyroid hormones directly due to lack of thyroid gland function, is the main cause of the disease. Secondary and tertiary hypothyroidism is rare, but documented as defects in pituitary and hypothalamic output, respectively (Mooney, 2011). Euthyroid illness will also depress T3 and T4 concentrations even though the thyroid gland is fully functional. For example, Cushing's Disease occurs when the adrenal glands produce an excess of cortisol. An overabundance of serum cortisol can suppress thyroid output; however,

thyroid function is likely to return upon resolution of the hyperadrenocorticism (Kenefick and Neiger, 2008).

With the major proportion of canine hypothyroidism caused by destruction of the thyroid gland tissues, two histological causes exist for this form of the disease: lymphocytic thyroiditis and idiopathic follicular atrophy (Graham et al., 2007). Lymphocytic thyroiditis, an autoimmune disease, occurs as lymphocytes invade the thyroid gland effectuating destruction to the thyroid follicles. This condition can be determined through the detection of thyroglobulin autoantibodies (TgAA). The onset of lymphocytic thyroiditis is marked by the presence of TgAA and still normal T3, T4 and TSH concentrations. As the disease slowly progresses, the subclinical phase is represented by positive TgAA titers, normal T3 and T4, but TSH starts to rise as thyroid tissue is destroyed and the remaining tissue strains to maintain thyroid hormone production. When the clinical phase is reached, TgAA concentrations are still elevated and over 75% of thyroid tissue has been destroyed resulting in low plasma T3 and T4 concentrations causing an increase in TSH production by the thyrotropes of the anterior pituitary. When TgAA concentrations finally recede, little thyroid tissue is left and the individual is left in a permanent state of hypothyroidism marked by increased TSH and low T3 and T4. Idiopathic follicular atrophy occurs when thyroid tissue is destroyed and replaced by adipose or fibrous, connective tissue. The exact cause of idiopathic follicular atrophy remains unclear; however, the end result mirrors that of lymphocytic thyroiditis (Graham, 2009).

Due to the high incidence of hypothyroidism in the canine, definitive diagnostics are important to assist the clinician in determining the state of the thyroid gland in his or her patients. Clinical signs of hypothyroidism include, but are not limited to: lethargy, weight gain, alopecia, hyperpigmentation, seizures, cold intolerance, and reproductive issues such as peri-parturient mortality in pups (Panciera et al., 2007). Fortunately, diagnostic labs offer many thyroid hormone assays such as T3, reverse T3, Free T3, T4, Free T4, Free T4 by dialysis, and TgAA. Unfortunately, the laboratory results sometimes do not reflect the clinical findings of hypothyroidism due to interactions by thyroid-like molecules (i.e. TgAA) interacting with the assays giving a false-negative diagnosis. Non-thyroidal illness or thyroid inhibiting drugs (i.e. propylthiouracil (PTU) (Richards and Ingbar, 1959)) could depress thyroid function providing a false-positive diagnosis of hypothyroidism on an otherwise healthy thyroid gland. In the past, to combat the possibility of a false diagnosis, the TSH response test was employed and considered the "Gold Standard" for diagnosing primary hypothyroidism. The protocol for administering this test included drawing a baseline blood sample, injecting 0.2 iu/kg of bTSH intravenously (IV), and drawing a 6 hour post blood sample (Panciera, 1999). Both blood samples would then be assayed for T4 concentrations to determine if the thyroidal output was an appropriate response to what was considered normal for the bTSH injection. Concerns arose as some patients reacted adversely (anaphylaxis) to the chemical grade bTSH as a pharmaceutical grade bTSH was not available (Panciera, 1999). An additional consideration when using bTSH is the injection of a hormone produced by the neural tissue of a one species into another. This could introduce

diseases such as rabies or bovine spongiform encephalopathy into a companion animal. Since bTSH is not approved for use by the U.S. Food and Drug Administration (FDA), its use as a diagnostic tool is not appropriate.

To circumvent the need to harvest and purify tissue samples from individuals in order to isolate a specific protein to use as a source of an exogenous hormone, various methods have been employed to manipulate a "host" cell to produce a recombinant version of that specific protein. The relative ease of designing and assembling a strand of deoxyribonucleic acid (DNA) and utilizing natural, cellular processes provide the tools for the reprogramming of cells to perform a given task. The DNA encoding the recombinant protein must be inserted into the host cell where the transcribing and translating mechanisms of the host cell are appropriated in order to produce the mRNA and the assembled protein, respectively. The insertion is achieved by means inherent to the host cell. For example, if using bacteria such as *Escherichia coli (E-coli)* as an expression vector, a compatible plasmid is opened to receive the recombinant protein DNA. The plasmid vector is then transformed into the *E-coli* cells where the recombinant protein gene will be inserted into the host cell DNA and transcribed along with the other genes on the plasmid vector (Baneyx, 1999).

Selection of the host cell is dependent on the size, complexity (i.e. multiple chains, folding, glycosylation, disulfide bonds, phosphorylation), amount of expression, and function of the protein. Selection of the transfer vector will be determined by its ability to infect the host cell. Choices of protein expression systems include: chemical synthesis, cell-free, yeast, bacterial, plants, insect cells, and mammalian cells.

Production time and fiscal constraints may also factor into the choice of a protein expression system.

Since the early 1980s, baculovirus systems have been used to express recombinant proteins. Researchers hoped this system would produce proteins in high quantities similar to bacterial expression systems while performing typical mammalian modifications such as phosphorylation and glycosylation (Jarvis, 2009). The hypothesis that a baculovirus would provide high concentrations of protein expression was based upon the protein, polyhedrin. As a baculovirus propagates, it produces two types of virion: a cell to cell type that buds from the infected cell to attack another cell in the host and an occlusion type that is protected within a protein matrix (polyhedrin) and can survive the death of the host (van Oers, 2011). After ingestion by another insect, the protective polyhedrin capsule and matrix will be digested allowing the hibernating virus to infect the cells of the gut lining. Fortunately, utilizing a baculovirus and insect cells to express mammalian proteins dispels the concerns of using a virus during the proteogenic process as a baculovirus' effects are limited to insect hosts. If the gene sequence for polyhedrin is replaced by a gene of interest (GOI), then the same promoters used to produce large amounts of polyhedrin may be employed to produce similar quantities of recombinant protein encoded by the GOI.

Initially, scientists utilized the *Autographa californica* nuclear polyhedrosis virus (AcNPV) to infect cells from the Lepidoptera order, or more specifically the *Spodoptera frugiperda* (fall armyworm, Spf), with the GOI which coded for human beta interferon (hIFN-β). The Spf was chosen due to its cells' ability to produce and store polyhedrin to

amounts where 25% or more of the total protein mass in the infected cell is the polyhedrin protein (Smith et al., 1983). At the time, extensive research had been performed on AcNPV which made the virus an excellent candidate for recombinant protein expression by a baculovirus/insect cell system. It was determined that AcNPV could infect cells of various tissues in a broad range of insects in the Lepidoptera order while most other baculoviruses are much more limited in their selection of hosts (Jarvis, 2009). The DNA of the AcNPV also contained the gene encoding for the polyhedrin protein which could be removed at no detriment to the normal functions of the AcNPV (Smith et al., 1983). This introduced the possibility of replacing the polyhedrin gene with a GOI and not interfering with transcription of the modified viral DNA into messenger ribonucleic acid (mRNA) since removing the polyhedrin gene produced no ill effects.

In order to insert the GOI into the baculovirus DNA, naturally occurring mechanisms such as bacterial transformation and homologous recombination were utilized. A bacterial plasmid, pUC8, was inserted with the gene encoding human interferon β (hIFN-β) into its circular DNA strand along with 5' and 3' flanking regions that are identical to the same regions that flank the polyhedrin gene of the AcNPV. Also encoded on this plasmid is the resistance to ampicillin which will be important when determining which bacteria took up the designer plasmid. Bacteria undergo transformation which is the uptake of DNA, such as plasmids, from the environment. By introducing the designer plasmid to compatible bacterial cells, in this case *E-coli*, transformation is allowed to occur. The cells are then placed on a culture plate which

contains ampicillin in the medium. The plated bacteria experiencing colony growth must contain the gene for ampicillin resistance which was included on the designer plasmid. The surviving colonies are used to produce stocks of the designer plasmid (Baneyx, 1999).

Once an ample supply of the designer plasmid was available and purified, cotransfection with DNA from the AcNPV was performed on cells from Spf. Though the occurrence was minute, the viral and plasmid DNA exchanged genes via homologous recombination before infecting the cell. The cells which contained the chimeric viral DNA would produce hIFN-β instead of the polyhedrin protein. The infected cells were plated on growth medium and the colonies which produced polyhedrin containing occlusion bodies did not recombine with the plasmids while colonies lacking occlusion bodies measured positive for hIFN-β production (Smith et al., 1983). This was the first introduction to manipulating insect cells into producing mammalian proteins. While hIFN-β is comprised of a single chain (187 amino acids) with N-linked glycosylation occurring at amino acid residue 101 (www.uniprot.org/uniprot/P01574), cTSH is a much more complex glycoprotein hormone bicistronically transcribed with multiple post-translational glycosylation sites. Fortunately, advancements in the field of baculovirus technology will provide for the additional demands placed on insect cells to produce complex, multi-chain, mammalian proteins.

Utilization of cell culture bioreactors allows for the mass expression of the GOI inserted by baculovirus infection into host cells in order to produce large quantities of

the designer protein. Culture media, pH, temperature, and dissolved oxygen may be monitored and manipulated via control mechanisms available on the bioreactor. Greater control of the variables results in greater costs of the equipment. Fortunately, various models are available to accommodate for the amount of investment a company or individual is willing to apply towards protein expression.

Upon completion of the cell culture, the protein of interest (POI) must be isolated from byproducts of the expression process such as cellular debris and unwanted proteins also produced by the cultured cells. Purity is especially important for sensitive analysis of the protein (i.e. mass spectrometry) or if the protein will be used as a pharmaceutical agent. One technique made available in the 1940's, which won Moore and Stein the Nobel Prize, and used widely in protein purification is ion exchange chromatography (IEX) (Ward and Swiatek, 2009). Ion exchange resins are available which consists of chemical functional groups that are attached to very small, insoluble beads (i.e. 3-90 μm). These beads are poured and packed tightly into a column. This is known as the stationary phase. Various functional groups are available which will act differently either attracting anions (basic functional groups) or cations (acidic functional groups). These are divided into two further subsets: groups remaining charged regardless of environmental pH (strong ion exchangers) or groups whose charge may be altered depending on environmental pH (weak ion exchangers). Strongly acidic, strongly basic, weakly acidic, and weakly basic functional groups are typically composed of sulfonic acid, quaternary amino, carboxylic acid, and primary, secondary, or tertiary amino groups, respectively. In using ion exchange chromatography, the isoelectric point (pI) of

the protein will dictate the overall charge of the protein dependent on the pH of the environment. If the pH of the environment is less than the pI of the protein, then the protein will be positively charged. Conversely, if the pH of the environment is greater than that of the protein's pI, a negative charge will be carried by the protein (Zhu et al., 2005). The power of ion exchange chromatography utilizes this ability to manipulate the charge of the proteins and enhance separation. Once a strategy has been formed, the media containing the POI is prepared to the proper pH. The media is known as the mobile phase. The media will flow through the ion exchange resin allowing molecules in the mobile phase to interact with the functional groups of the stationary phase. The flexibility of ion exchange allows the user to capture either the POI or the unwanted material to the resin. Any adsorbed molecules may be desorbed from the stationary phase by introducing competing ions (i.e. sodium chloride (NaCl)) that will replace them and effectively block the charges of the functional groups on the stationary phase. The greater the charge of the captured molecules, the greater the concentration of competing ions needed to dislodge the material. For this reason, a gradient elution is used to enhance the degree of separation between molecules of varying ionic strength and thus leading to increasing purity, in theory. Glycoproteins can have various pI's which can be primarily explained by the variations in the oligosaccharide chains due to the presence or absence of sialyl and/or sulfate groups (Chiba et al., 1997). Charge heterogeneity has been attributed to the multiple glycosylation patterns which translate into multiple pI values for bovine, ovine, and human TSH (Green and Baenziger, 1988a,b). Understanding the effect glycosylation has on the pI of glycoproteins will

help to define the parameters for ion exchange chromatography which will be used for the purification of rcTSH.

An end result of ion exchange chromatography is a collection of eluent containing the POI in a solution of salt. The next step in the purification process is to concentrate the POI and remove salt. This may be achieved with tangential flow filtration (TFF) (Ward and Swiatek, 2009). As demonstrated in figure 3, a filter with pores of a specific diameter is utilized to separate molecules of sizes above and below the pore diameter. Driving pressure to push the starting material parallel the TFF membrane is generated by a pump. As material flows tangential to the membrane, part of the driving pressure is translated into force perpendicular to the membrane. The perpendicular force will push material towards the membrane carrying water and any molecules smaller than the pore diameter over into the filtrate. The advantage of applying driving force parallel to the filter is reducing the amount of material adhering to the membrane leaving pores open and maintaining filtration efficacy. Filtration systems that apply force perpendicular to the filter create a buildup of material too large to flow through the pores. This will restrict flow by blocking the pores, increasing back pressure, and greatly reducing filtration efficiency.

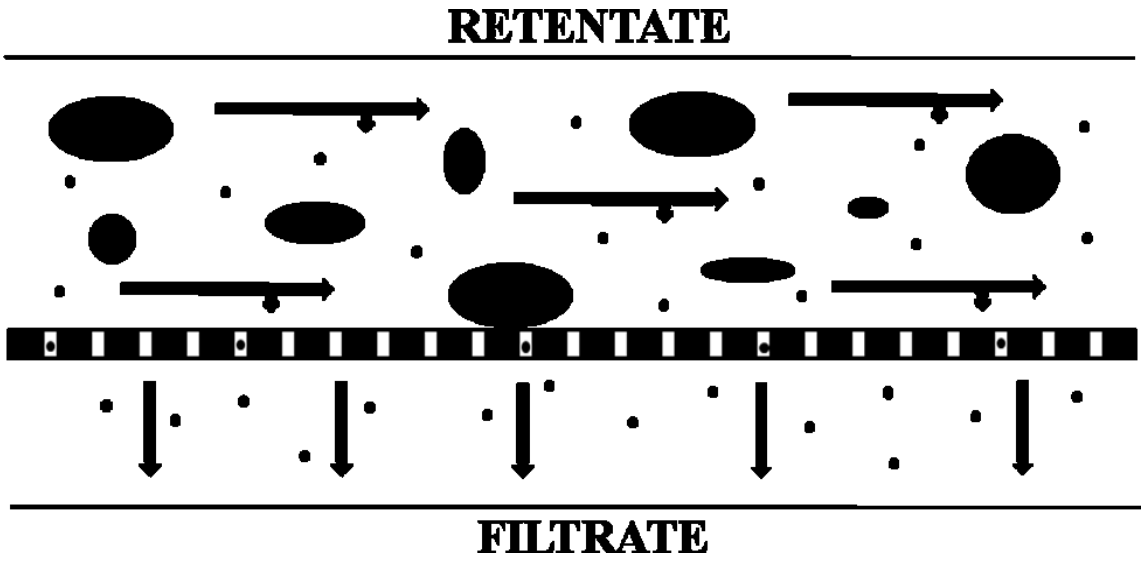

Figure 3. Tangential Flow Filtration. In TFF, pressure is applied to the retentate by a pump. The driving pressure created by the pump is predominately distributed between forces parallel and perpendicular to the TFF membrane.

In order to visualize the effectiveness of a purification process, gel electrophoresis and western blot analysis can demonstrate the purity of a sample as well as show that the detected protein is the POI. The fundamentals of gel electrophoresis date back to 1791 when Faraday introduced his laws of electrolysis. Researchers such as Kohlrauch and Tiselius expanded upon Faraday's laws of particle size and movement through an electric field and developed moving boundary electrophoresis which is a defined front or line of particles migrating through a medium pushed by an electrical current (Vesterberg, 1993). By acting as a sieve, the medium could separate individual components of the sample based on the sizes of the molecules. Shapiro et al. (1967) published their success in estimating the molecular weight of various polypeptides by SDS-PAGE. Sodium dodecyl sulfate is an anionic detergent which will denature proteins by binding to the polypeptide backbone unfolding the protein into a long, strait structure. Sodium dodecyl sulfate will also bind to proteins at a constant rate of 1.4g SDS to 1 g of protein (Daban et al., 1991). This will ensure the amount of anionic charge provided by the SDS will be proportional to the mass of the protein and that the intrinsic charges of the proteins are voided by the SDS and will not affect protein migration towards the anode (Shapiro et al., 1967). When a sample containing unknown amounts of various proteins is treated with SDS and applied to an electrophoresis gel, separation will occur due to the true size of the unfolded proteins.

Various substances have been used as gel electrophoresis mediums such as free solution, paper, gelatin, agar, and polyacrylamide. Raymond and Weintraub (1959) performed zone electrophoresis using acrylamide gels and published their findings

(Vesterberg, 1993). Acrylamide gels are formed by the cross-linking of long, acrylamide chains by a polymer such as bisacrylamide. The pore sizes produced by the acrylamide/bisacrylamide cross-linking may be manipulated allowing for various sieving properties depending on the gel formulation (i.e. greater bisacrylamide content yields smaller pore sizes). Raymond and Weintraub (1959) found that acrylamide gels presented multiple characteristics beneficial to electrophoresis such as:

- Once the gel is formed, it is colorless, clear, elastic, water insoluble, and stable.
- After electrophoresis, the gel can be dried and then stored. The stored gel could later be rehydrated by soaking in water which will return the gel to its approximate original size before shrinking during the drying process.
- Staining of the gel produced sharper bands (less sample diffusion) as well as reduced background staining.

The visualization of the proteins applied to the gel as distinct bands with excellent contrast to reduced background staining was greater than any other medium available during the time of this experiment (Raymond and Weintraub, 1959). Pre-casted polyacrylamide gels are presently available in different configurations (gel size, well size, sieving properties) by various manufacturers but can still be mixed, poured, and casted by hand.

Once SDS treated proteins have undergone electrophoresis on a polyacrylamide gel, stains are applied to the gel. The amount of sample proteins on the gel, the affect the stain will have on the sample proteins if further research is needed (i.e. mass spectrometry), and the specificity of the stain to a POI are factors to be considered.

Coomassie brilliant blue (CBB), silver, and fluorescent staining are all available for protein visualization each having its advantages and disadvantages. Coomassie brilliant blue was originally developed as a textile dye and was later adopted by researchers as a detection tool for proteins. This anionic dye when in acidic solution will reversibly, non-specifically bind to the exposed ammonia (NH_3^+) groups of proteins (Fazekas de St. Groth et al., 1962). The detection limit of CBB R-250 towards microgram amounts of protein ranges from 0.3 - 1 μg/band which is the least sensitive of the three; however, costs are lower and working time is shorter (Sasse and Gallagher, 2009). Alternative protocols are being developed to decrease the needed amount of protein to produce a visual band while maintaining the ability to further analyze the in-gel proteins after exposure to CBB (Pink et al., 2010). Silver staining has much greater sensitivity at the 2 - 5 ng/band amounts but requires a longer and more detailed protocol (Sasse and Gallagher, 2009). Researchers introduced silver staining of polyacrylamide gels to detect proteins and peptides in 1979 and the fundamentals of their silver staining protocol are still in use (Switzer et al., 1979). After gel electrophoresis, the proteins are fixed to the gel and then sensitized to the subsequent addition of silver ions. Image development is performed on the gel to expose the protein bands. Fixation, sensitization, silver attachment, and visualization all occur using various chemicals. One of the chemicals used to increase the sensitivity of the silver stain to small, nanogram amounts of protein is formaldehyde. Unfortunately, post staining analysis such as mass spectrometry of the proteins after exposure to formaldehyde is difficult as modifications to proteins are induced by the chemical. Formaldehyde may induce cross-linking and

the attachment of methylol and imidazolidione adducts to amino/thiol and N-terminal groups, respectively. These changes can alter the sizes of the peptides which construct the total protein as well as hinder cleavage points normally accessible by enzymatic digestion (Metz et al., 2006).

A third type of stain which combines the advantages of Coomassie blue and silver staining is fluorescence. An example of a fluorescent dye, SYPRO Ruby, is an organic compound with ruthenium chelated to the complex (Berggren et al., 2000). Ruthenium, a luminescent metal, is highly excited when exposed to UV light or a laser. When an electrophoresis gel is exposed to SYPRO Ruby, the molecules of the dye will bind electrostatically to the basic amino acid residues (i.e. lysine, arginine, histidine) of the sample proteins in a similar manner as CBB. Detection without the use of visualization equipment is possible by ultraviolet (UV) light; however, fluorescence of the stained proteins are fully realized with the use of a laser scanner or charged-coupled device (CCD) camera (Patton, 2000). Exciting the protein-bound ruthenium complexes with an energy source coupled with precise detection equipment will produce sensitivities equaling that of silver staining while leaving further downstream analysis of the proteins available due to the binding mechanism shared with CBB.

Non-discerning dyes present the total protein content applied to an electrophoresis gel creating an image of the variances in molecular weights and/or isoelectric points of the sample proteins. In order to determine the location of the POI on the gel, a much more precise method must be employed. The heightened specificity of western blot analysis of a polyacrylamide gel utilizes antibodies developed towards

the POI. After electrophoresis, the proteins embedded in the gel are transferred electrophoretically to a protein adsorbent material such as nitrocellulose or polyvinylidene flouride (PVDF) resulting in the sample proteins maintaining the same relative locations on the transfer membrane as on the gel. Since nitrocellulose and PVDF bind proteins indiscriminately, a blocking agent is applied to the membrane to cover any open binding sites that the antibodies added in the following steps might inhabit. The primary antibody specific to the POI is introduced to the membrane. A secondary antibody produced towards the animal species the primary antibody was developed in is tagged with a probe (i.e. radioactive, chemiluminescent, and colorimetric) and exposed to the membrane. Depending on the probe, visualization techniques will develop an image of the locations where the secondary antibody has bound the primary antibody-POI complex on the membrane (Towbin et al., 1979).

If the purified POI is to be used as a pharmaceutical agent, efficacy trials will determine the protein's ability to elicit a biological response. *In vivo* and *in vitro* systems/protocols are numerous providing many options to the researcher. A few factors in determining which system to use include: accessible equipment, finances, type of cell that expresses receptor to POI, reagents for measuring biological endpoint, time constraints, and the technical skill to design and perform the experiment.

In vitro studies can provide an estimate to the *in vivo* activity of the POI. The success of an *in vitro* system depends on the ability to emulate an *in vivo* environment by providing the necessary nutrients to the cultured cells or tissues promoting growth, maintenance, and the ability to respond to stimulatory or inhibitory actions of a ligand.

The number of nutritional delivery variables one wishes to manage during a culture will dictate the complexity of the system.

The supply of nutrients to the tissue or cell culture is either statically or dynamically controlled. In static cultures, media composed of varying substances at specific concentrations is added to the biological sample contained in a vessel (i.e. Petri dish, test tube, flask). The concentration of the substances will decrease in the media as the biological sample utilizes them in accordance to the Michaelis-Menten kinetics of enzyme reaction on a substrate (Sugiura and Kanamori, 2011). While less intricate and costly than dynamic culture systems, availability of sustenance slowly decreases in static cultures which might reduce the effectiveness of the biological sample during treatments. Carrel and Burrows (1911) performed possibly the first documented static cultures on thyroid tissue fragments (Pulvertaft et al., 1959). The primary culture consisted of the thyroid tissue sample placed in the plasma medium of the same animal donor. Secondary and tertiary cultures were also performed where cells from the previous culture were taken and cultivated in new plasma medium. The nutritional concerns for cell maintenance during static culture are observed in their writings:

> "The death of the culture is bound up with the changes induced in the plasma by the growing cells. This is shown by the fact that on secondary transplantation the cells continue to multiply. It may be caused by exhaustion of the nutriment or by accumulation of metabolic products or by both of these factors together." (Carrel and Burrows, 1911).

In contrast to static cultures, a dynamic system maintains a constant concentration of nutrients in the vessel containing the biological sample. This is achieved by pumping media to the culture vessel at the same rate as the biological sample is consuming the media components. Dynamic cultures such as perifusion systems greater emulate *in vivo* performance than static promoting biological sample longevity and reactiveness to a ligand (Hassan and Merkel, 1994). In 1980, perifusion experiments were first conducted utilizing mammalian thyroid tissue to determine the response of rat thyroid cells to TSH delivered in a dynamic system (Attali et al., 1984; Grau et al., 1986). A basic perifusion system consists of culture chambers designed for housing tissue samples with afferent tubing delivering fresh media to the chamber from a reservoir via a peristaltic pump and efferent tubing delivering the media with the products released by the sample tissue to a collection device. Because the tissue sample will be stationary in the vessel, the importance of vessel shape and size is important so as to allow for maximum contact by all cells of the culture to the mobile media. Care must be taken to not overload the chambers with tissue which might restrict media flow as well as reduce the surface area of the biological sample exposed to the media as fluids tend to take the path of least resistance and will follow the same route. Another consideration when using a perifusion system is the fluid flow through the tubing and the chamber, both of which resemble pipes. Changes in pipe radius and length, temperature, and fluid velocity combined with Taylor dispersion will create diffusive effects altering hormone profiles (Shorten and Wall, 2001). While these alterations might be too minute for the average perifusion experiment, using alike culture chambers as well as tubing

material, radius, and length will aid in ensuring diffusive properties are similar across experimental groups (Grau et al., 1986).

Forskolin, a potent activator of adenylate cyclase, initiates a cascade resulting in the production of cAMP which in the thyroid gland ultimately effectuates T3 and T4 release (Laurberg, 1976). The actions of forskolin are a useful tool to demonstrate the viability of cells or tissue samples after the experimental treatments are applied. If cell/tissue death occurs during the experiment, products such as hormones will be released possibly emulating a stimulatory response to a releasing ligand. A viable sample should demonstrate this response to the releasing hormone as well as forskolin if the stimulatory cascade utilizes cAMP as a second messenger.

The measurements of serum hormones allows researchers to closely monitor the amount of the POI used during an experiment as well as determine its effectiveness through the measurement of the hormone whose release is stimulated by the POI. Very early bioassays could only detect the presence of a hormone. For example, serum or urine from a human female would be subcutaneously injected into a male *Bufo* toad. If the toad produced spermatozoa which could be detected in the urine within 3 hours, a positive pregnancy would be diagnosed due to the elevated presence of human chorionic gonadotropin in the human female's serum or urine causing spermatozoa production in the toad (Mainini, 1947). A different methodology was needed to quantify hormone concentrations. In 1960, the first immunoassay was developed to detect human serum insulin concentrations (Goldsmith, 1975). Researchers discovered hormones labeled with a radioactive element (i.e. I^{125}) would compete with the non-labeled hormone for

specific antibody binding. If the concentration of I^{125} bound hormone added to a sample was kept constant, the amount of non-labeled hormone in the sample would dictate how much bound hormone would undergo antibody binding. The amount of radioactive material measured in each sample would be inversely proportional to the amount of non-labeled hormone present. A set of standards with known concentrations of hormone is used to calculate a standard curve to which samples of unknown hormone concentrations may be measured (Yalow and Berson, 1960). Using the same principles as radioimmunoassay, chemiluminescent technology also utilizes competitive binding; however, hormones conjugated with radioactive isotopes are instead labelled with a substance such as alkaline phosphatase which in the presence of the substrate 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt (AMPPD) will produce an unstable intermediate that emits light. The light is measured via a photomultiplier tube (PMT) to produce a result (Reimers et al., 1996).

A non-competitive binding, or sandwich, assay also may be used in place of competitive binding. Instead of two forms of the same hormone competing for antibodies to bind, the sample hormone in a sandwich assay freely binds to stationary antibody sites. Labelled antibody is introduced to the sample tube which binds to the captured hormone which is now effectively "sandwiched" between two antibodies. Sandwich assays are considered to produce greater sensitivities due to the increased specificity of using two antibodies while competitive binding, only utilizing one antibody, will be more prone to non-specific binding by hormone fragments (Prudom et al., 2010). Radioactivity is measured or substrate is added to illuminate the stationary

sample hormone and measured by a PMT, depending on the assay type. Unlike competitive binding assays, the result from a non-competitive assay will be proportionate to the unknown sample concentration.

Statistical analysis of the data will provide the final validation as to whether or not the null hypothesis of the experiment is rejected. Experimental groups are formed where a number of individuals are assigned to a particular group and administered either a treatment or a control dose. As the number of groups increase, so does the complexity of the statistical analysis when comparing more than two populations. Analysis of variance (ANOVA) is a method of comparing a population parameter across multiple groups to test if the null hypothesis, that all groups share the same population value, should be rejected. Analysis of variance provides for this by incorporating the F-test which accounts for variance within the experimental groups as well as between the experimental groups. This differs greatly from a t-test which compares just one population directly to another. Once the F-test value is calculated, the corresponding p-value is determined. If the calculated p-value is less than 0.05, the null hypothesis is rejected (Ott and Longnecker, 2001).

Should the null hypothesis be rejected indicating a significant difference between the group means, the Tukey's W Procedure may be employed to determine which means are different. Tukey's W Procedure is very similar to the t-test but uses the studentized range distribution instead of the t distribution. If the t-test were used to compare two sample means from a pool of multiple means, the probability is high that eventually a type I error will occur even if all the sample means are not significantly different unless

the error rate is set to a very small α. Tukey's W Procedure compares the difference between two of the sample means to the W test statistic calculated using the total number of sample means, degrees of freedom (ANOVA), error rate, number of observations per group, and the mean square within samples (ANOVA) to aid in avoiding the type I error. It is also the preferred comparison of multiple means from sample sizes that are the same or slightly varied (Ott and Longnecker, 2001).

The objective of this research is to determine if rcTSH is comparable to the "Gold Standard", bTSH, which has been discontinued for use in the TSH stimulation test used to determine thyroid status in canines. Necessity for FDA approval as an injectable pharmaceutical requires rigid purification techniques so as not to introduce a possible source of an anaphylactic response by the patient. As the purification techniques are refined, *in vitro* experimentation will be performed to evaluate the stimulatory effects of rcTSH and bTSH on cAMP production in cell culture and T4 release in static and perifusion tissue culture. Upon refinement of the purification technique, bioassays including goldfish, rats, and canines will reveal if rcTSH elicits a similar response by the thyroid gland as bTSH when used according to the TSH stimulation test protocol.

CHAPTER II

IN VITRO TRIALS

OVERVIEW

The efficacy of rcTSH on canine and bovine thyroid tissue was tested utilizing primary cell, static tissue, and perifusion tissue cultures. While purification techniques were examined in order to produce a substance suitable for injection, culture systems provided estimations as to the physiologic responses of *in vivo* testing. *In vitro* testing is performed to determine if the biological actions of rcTSH emulate those of bTSH, the "Gold Standard" for determining the canine thyroidal output in response to an injection of an exogenous thyrotropic substance.

The initial experiment, primary cell culture, was performed on canine thyroid tissue. Thyroid cells were liberated and subsequently cultured in preparations for exposure to either positive (forskolin) control, negative (media) control, bTSH (1600 ng, 8000 ng, and 16000 ng doses), or rcTSH (1600 ng, 8000 ng, and 16000 ng doses). Equivalent immunoreactive TSH concentrations were made for doses of bTSH and rcTSH. Following treatments and incubation, the media was assayed for cAMP, a second messenger in the signaling pathway for the cTSH receptor.

The results demonstrated no logical cAMP expression in accordance with the treatment type and concentration. All treatment means were similar to the negative control ($P>0.05$) (mean=0.10025) except the positive control ($P<0.05$) (mean=0.08100)

and the medium bTSH ($P<0.05$) (mean=0.08750) doses which expressed lower cAMP concentrations. In fact, all treatment means were lower than the negative control except for the low rcTSH treatment.

The next experiment, static tissue culture, allowed for the measurement of a true biological endpoint to TSH actions which is the expression of T4 by thyroid tissue. With the inconclusive results of the cAMP experiment, concentrating on the efficacy of the recombinant hormone was of primary importance. Bovine thyroid tissue was exposed to media (control) and the same concentrations of rcTSH as used in the cAMP experiment while incubating in a warm water bath (37°C). Media was periodically harvested and replenished and assayed for T4 concentrations.

Similar results were obtained in the static culture as in the cAMP experiment where treatments were similar ($P>0.05$) to the control at almost all data points. A gradual decrease in T4 release was seen in all treatment groups with no response to either rcTSH or forskolin. This could be an indication that the tissue samples were not being maintained by the culture media and/or the environmental conditions in the water bath resulted in tissue death.

The final *in vitro* experiment utilized a perifusion culture machine (Endotronics, Inc., Coon Rapids, MN) to provide better environmental controls as well as provide a constant supply of fresh media to the culture chambers housing the thyroid tissue. Due to a limited number of perifusion chambers available, groups were assigned for media (control), bTSH (16,000 ng), and rcTSH (16,000 ng). A fractionator was used to collect media at specific intervals during the course of the experiment and all were measured for

T4 concentrations. While the small number of data points negated the ability to provide a meaningful statistical analysis, graphical representation demonstrated the heightened thyroidal response of rcTSH and bTSH over the control.

INTRODUCTION

For the last 30 years, baculovirus mediated recombinant protein expression has provided an alternative to producing proteins which emulate their endogenous homologues in immuno- and bio-activity (Jarvis, 2009). With the ability to accept codon optimized RNA encoding for the protein of interest (POI) in addition to performing mammalian glycosylation, baculovirus systems are ideal candidates to manufacture a foreign glycoprotein in greater concentrations than their mammalian counterparts and have the enhanced ability for complex glycosylation unlike bacterial culture systems. This technology offers a tantalizing future for the production of proteins for use in both the veterinary and human pharmaceutical industry.

Hypothyroidism, an endocrinopathy in the canine, represents a common yet often misdiagnosed disease to the veterinarian (Graham, 2009). Clinical signs along with a battery of serological tests (i.e. T3, T4, Free T4, TSH, TGAA) provides insight to the functional status of the thyroid gland; however, not all the necessary information to make a firm diagnosis may be provided. To abate this concern, a TSH stimulation test would quantify the patient's thyroidal response to an injection of exogenous TSH. Once considered the standard protocol for diagnosing canine hypothyroidism, the TSH

stimulation test was performed using bTSH. A comparison between serum T4 concentrations before and 4-6 hours after the bTSH injection would reveal if the thyroid gland reacted appropriately to the bolus of bTSH (Panciera, 1999). With bTSH no longer approved as a veterinary pharmaceutical and the elevated expense of recombinant human TSH (rhTSH) extra-label use, veterinarians are forced to base decisions on "snapshots" of thyroid function by submitting serum from random blood draws for thyroid hormone assays.

To produce rcTSH, cells from Spf of the order Lepidoptera are infected by a baculovirus containing recombinant DNA (Jaques and Jarvis, 2010). Wild-type baculovirus infection will force the insect cells to produce the protein, polyhedrin, in large quantities. Substitution of the DNA encoding for polyhedrin with the codon optimized sequence for both α and β chains of cTSH located on a designer plasmid occurs via homologous recombination. Insect cells successfully infected will lack the visibile presence of the polyhedrin protein and are subsequently cultured for rcTSH expression (Jarvis, 2009).

After culture, purification techniques are applied to the media in order to isolate the rcTSH from unwanted molecules. *In vitro* experimentation provided a source of information as to the potency of the recombinant stimulatory hormone as the purification methodology was refined in preparation for *in vivo* injection. The objective to performing *in vitro* experiments was to evaluate the efficacy of rcTSH and compare the stimulatory effects to bTSH. Comparisons will be made by: measuring the ability to

stimulate an increase in cAMP concentrations in cultured thyroid cells and measuring T4 release by thyroid tissue samples in static and perifusion culture systems.

MATERIALS AND METHODS

Expression of rcTSH

United States patents US 7,479,549 B2 and US 7,838,492 B2 protects the methods of production and use of rcTSH as proposed by inventors Dr. John (Scott) T. Jaques and Dr. Donald L. Jarvis. The patents detail the alterations made in the codon sequences of amino acids, used to maximize production of rcTSH α and β chains by lepidopteran cells. Also discussed in the patents are the various cell lines infected by the baculovirus, AcP(+)DIEcTSHA/B, each of which providing individual, post-translational glycosylation modifications (Jaques and Jarvis, 2009; Jaques and Jarvis, 2010).

To summarize, plasmids containing rcTSH DNA undergoes homologous recombination with the baculovirus effectively exchanging the polyhedron encoded region for the α and β chains of rcTSH DNA. The baculovirus infects the lepidopteran cells instructing cellular mechanisms to produce rcTSH, not polyhedron. The infected cells are cultured with fresh medium for 5 days. Centrifugation removes the cells from the culture media containing rcTSH. Expression of rcTSH was provided by Expression Systems, Woodland, CA.

Purification of rcTSH

Frozen (-80°C) media containing rcTSH remained refrigerated at 3° C during thaw. Once thawed, the pH of the media was elevated to 9.5 using sodium hydroxide (NaOH). The media was centrifuged at 3000 revolutions per minute (rpm) for 15 minutes to concentrate particulate matter at the bottom of the container. Media was poured through a glass wool filter to capture sediment formed during centrifugation. Subsequently, the supernate was then applied to a strong anion exchange column (AEX) (Column-Kontes Chromaflex 420830-3020, Resin-GE Healthcare HiTrap Capto Q). The cationic charge of the stationary phase attracts molecules with negative charges in a 9.5 pH environment (Zhu et al., 2005). With an approximate pI of 8.5 (work performed by Dr. Evan Shave of Dr. Gyula Vigh's lab, Chemistry Department, Texas A&M University), most rcTSH does not bind to the column. The column is washed with three column volumes of running buffer to collect unbound rcTSH suspended in the mobile phase on the AEX column.

Once the harvested flow through media is collected, the pH is adjusted to 5.0 with acetic acid. The adjusted media is then applied to a strong cation exchange column (CEX) (Column-Kontes Chromaflex 420830-3020, Resin-GE Healthcare HiTrap Capto S). The rcTSH will retain a positive charge and adsorb onto the negatively charged groups on the cation resin (Zhu et al., 2005). Three column volumes of buffer remove material suspended in the mobile phase of the CEX column. To desorb the rcTSH from the CEX functional groups, a 1 M NaCl solution is applied to the CEX to allow the salt

molecules to replace the rcTSH on the stationary media and the rcTSH is collected in the flow through mobile phase.

The final step in the purification process is to remove the NaCl from the CEX elution. This is achieved using tangential flow membranes. The media flows tangential to a porous membrane containing pores of 10 um in diameter. Water, along with molecules smaller than the membrane pores, cross over to the filtrate of the membrane while larger than 10 µm molecules remain in the retentate (Ward and Swiatek, 2009). The total media volume and NaCl concentration of the retentate will decrease in preparation for lyophilization.

To visualize the effectiveness of the purification protocol, a SDS-PAGE gel was performed using rcTSH. Total volume for each well of the Mini-Protean precast gels (BioRad 456-1044) are 50 µl so to avoid overfilling the wells, loading samples were kept to a 45 µl volume. The electrophoresis buffer concentrate (BioRad 161-0732) required a 1:3 dilution accounting for 10 µl of the preparation. Adding 2 µl of loading dye diluted at a 1:5 ratio with DI water leaves 33 µl for the sample. A total of 2100 ng of rcTSH was present in the 33 µl. The concentrations of rcTSH were measured by chemiluminescence (Siemens Immulite 1000) with canine specific reagents (Siemens LKKT5). Preparations were loaded by pipette into the center wells flanked by molecular weight markers (BioRad 161-0324). Power settings (200V, 120mA, 24W for 30 minutes) applied to the gel were based upon Bio-Rad's recommendations for the

electrophoresis unit.

Upon the completion of electrophoresis, the gel was removed from the unit and rinsed with deionized (DI) water. The gel was subsequently stained with Coomassie Blue (BioRad 161-0786) according to the manufacturer's specifications. The result of the stain is illustrated in figure 4. The technique of electro-elution was performed on the stained gel. Squares of the gel were excised from each lane in which rcTSH was loaded into the well. Gel slices were pooled together according to their vertical location in each lane and placed into an electro-elution chamber provided with the Electro-Eluter (BioRad 165-2976). The six chambers filled with gel slices were placed into the electrophoresis unit and exposed to an electrical current which removed the Coomassie stain as well as residual SDS from the gel and the chamber through a dialysis membrane. The dialysis membrane retained the larger rcTSH in the electro-elution chamber which was no longer embedded in the polyacrylamide gel.

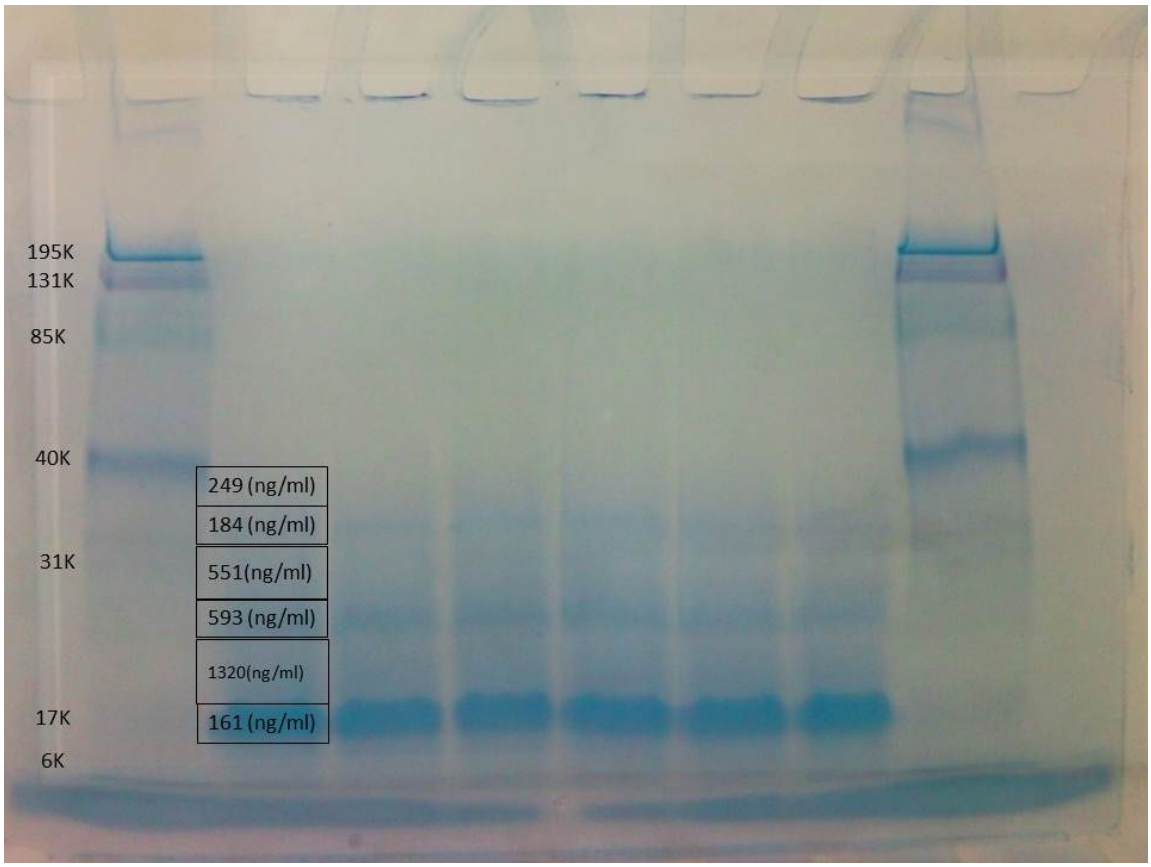

Figure 4. Coomassie Blue Stain of rcTSH on SDS-PAGE Gel. Concentrations of rcTSH present at the different molecular weight ranges. Most of the rcTSH fell between 20K and 35K could be due to varying carbohydrate structures.

The buffer, removed from the electro-elution chambers, was measured for TSH immuno-activity providing the concentrations present at each vertical step. The darkest band at 17K most likely represent the separated α and β chains which are produced individually during the baculovirus expression. Dimerization of the two chains occurs as a post translation event. The bulk of the rcTSH falls between 20K and 31K with the variance in size likely due to the degree of glycosylation present on the individual rcTSH molecules. Another consideration is the position of the rcTSH molecules as migration down the gel occurs. If the lengthwise position of the rcTSH molecules is parallel to the bottom of the gel during migration, size of the rcTSH molecules will be a larger determinant of the final position on the gel. If the length of the rcTSH molecule is perpendicular to the bottom of the gel when power is applied, the molecules will weave through the gel as a needle does through fabric seeming smaller in size.

Primary Cell Culture

For the primary cell culture experiment, thyroid tissue was collected from mongrel beagles at the Texas A&M University Veterinary College and transferred in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 2% penicillin-streptomycin. Tissue was minced with a Stadie-Riggs tissue slicer and rinsed three times in DMEM at 75 ml per 3-4 grams of tissue. After rinsing, tissue was placed in an Erlenmeyer flask containing DMEM and 0.3% collagenase and incubated in a 37° C water bath for 60 minutes while agitated with a magnetic stir bar. Following incubation, the suspension was poured through double

thick sterile gauze and into 50 ml conical tubes for centrifugation for 15 minutes at 1000 x g. The supernatants were removed and the pellets re-suspended in DMEM and combined into one tube. The pooled tube was centrifuged for 15 minutes at 1000 x g, decanted, and re-suspended 3 times to ensure proper washing. The final pellet was re-suspended in 20 mL of DMEM and 10% FCS.

To prepare for dilution, the concentration of cells in the suspension was calculated using a hemocytometer loaded with a solution of 100 µL (0.04%) Trypan Blue and 100 µl of suspension. A final dilution of the cell suspension with DMEM, 10% FCS, and 1% L-glutamine to a concentration of 400,000 cells/ml is achieved according to the following calculations:

(Total cells) / (# cells/ml) = total ml of final diluted cell suspension

where: Total cells = (# of cells on grid)*2*10,000*(# ml suspension)

and: # cells/ml = number of cells to place in each culture well/ml

One ml was placed in each of 64 culture wells and placed in a 37° C incubator at 95% O_2 and 5% CO_2. This was marked as Day 0 of the culture.

On days 2 and 3 of the culture, medium was removed and replaced with 1 ml of DMEM, 10% FCS, and 1% L-glutamine. The media was again removed on day 4 and 1 mL of serum-free DMEM was added in preparation for treatments on day 5. On the day of the treatments, eights groups (n=8) were formed. The groups were formed as follows: negative control (DMEM), positive control (forskolin), bTSH low, bTSH medium,

bTSH high, rcTSH low, rcTSH medium, and rcTSH high doses. All TSH doses were equivalent immunoreactive concentrations as measured by an immunoassay specific for cTSH. The serum-free media from day 4 was removed and replaced with DMEM + treatment equaling a total volume of 1 ml. After 24 hours, media is removed from the culture wells and assayed for cAMP (Cayman Chemicals 581001) concentrations.

Static Tissue Culture

Bovine thyroid tissue was collected at slaughter from the Texas A&M University Rosenthal Meat Science Center and transported to the lab in Krebs-Ringer Bicarbonate (KRB) buffer with 5% bovine serum albumin (BSA). Thyroid tissue was cut into approximately 100 mg slices using the Stadie-Riggs tissue slicer and placed into 13 x 100 glass culture tubes with 8 mL of KRB+BSA and incubated in a 37° C water bath (Precision). Media was collected and replaced every 20 minutes for 1 hour (4 wash collections). Treatment protocols were developed for four groups (n=5): control (media only), 1,600 ng rcTSH, 8,000 ng rcTSH, and 16,000 ng rcTSH. After the fourth collection of media, treatments were administered so the total ng of rcTSH was contained in 8 mL of KRB+BSA. Treatment media was collected every 20 minutes for 1 hour (3 treatment collections). Tissue cultures were again washed with media every 20 minutes for 1 hour (3 wash collections). Forskolin enriched media (8 ml) was applied to the tissue cultures every 20 minutes for 40 minutes (2 forskolin collections). The final wash was performed every 20 minutes for 40 minutes (2 wash collections). Every

incubation period was performed in a 37° C water bath (Precision). All collections were assayed for T4 (Siemens Immulite 1000, Reagent LKCT5) concentrations.

Perifusion

Bovine thyroid tissue was collected at slaughter in the Texas A&M University Rosenthal Meat Science Center and transported to the lab in KRB buffer with 5% BSA. The perifusion system (Endotronics, Inc., Coon Rapids, MN) was immediately turned on to fill tubing with media and to reach a temperature of 37˚ C prior to placing tissue in chambers. After removing non-thyroidal tissue, 50 mg slices were loaded into the perifusion chambers. Once the chambers were loaded onto the perifusion system with afferent and efferent tubing connected, KRB+BSA media was pumped at a rate of 1 ml/min. Tubes (13x100) were used to collect 10 ml fractions. An initial wash step was performed by continuing to run media for 60 minutes with collection of media at 10 minute intervals to allow any T4 released by the thyroid tissue due to handling would settle to a baseline. The treatment phase consisted of introducing the treatment dose via an injection port on the afferent tubing located proximal to the tissue chamber with: media (control) (n=4), 25,000 ng bTSH (n=2), and 25,000 ng rcTSH (n=2). Treatments were slowly injected into the injection hub at the perifusion chambers at the 60 minute interval. Thyroid tissue was allowed to return to non-stimulatory, baseline T4 release. Forskolin induced release of T4 was performed at minute 140 to illustrate that the tissue was viable at the end of the experiment. Each afferent tube was placed into a flask of stock forskolin diluted at 1 to 1000 with media and run for 20 minutes. A final wash

step was performed for 40 minutes to return T4 release to a non-stimulated concentration. All fractions were measured for T4 concentration (Siemens Immulite 1000, Reagent LKCT5)

RESULTS

Primary Cell Culture

On day 5 of the cell culture, before the treatment dosages were given, each well was observed under a microscope to view the progress of the culture. All wells displayed good cell division and growth. Cells could be found dispersed within the well while others would form circular structures as if a cell-thick slice from a thyroid follicle were taken and placed on the slide. No fully intact thyroid follicles were observed.

Statistical analysis of the data was two-fold: utilizing the ANOVA table to analyze the hypothesis that the means of the treatment groups were statistically similar and, if the ANOVA table demonstrated a significant difference in the means, the Tukey's W test would determine which data groups were significantly similar and different. ANOVA analysis determined the group means were not similar ($P<0.05$) thereby rejecting the null hypothesis that all means were equal. Figure 5 demonstrates the means of the treatment groups as a bar graph. The only treatment group with a higher expression concentration of cAMP from the media-only negative control was the lowest rcTSH dose. All other groups, including the positive control, had a mean

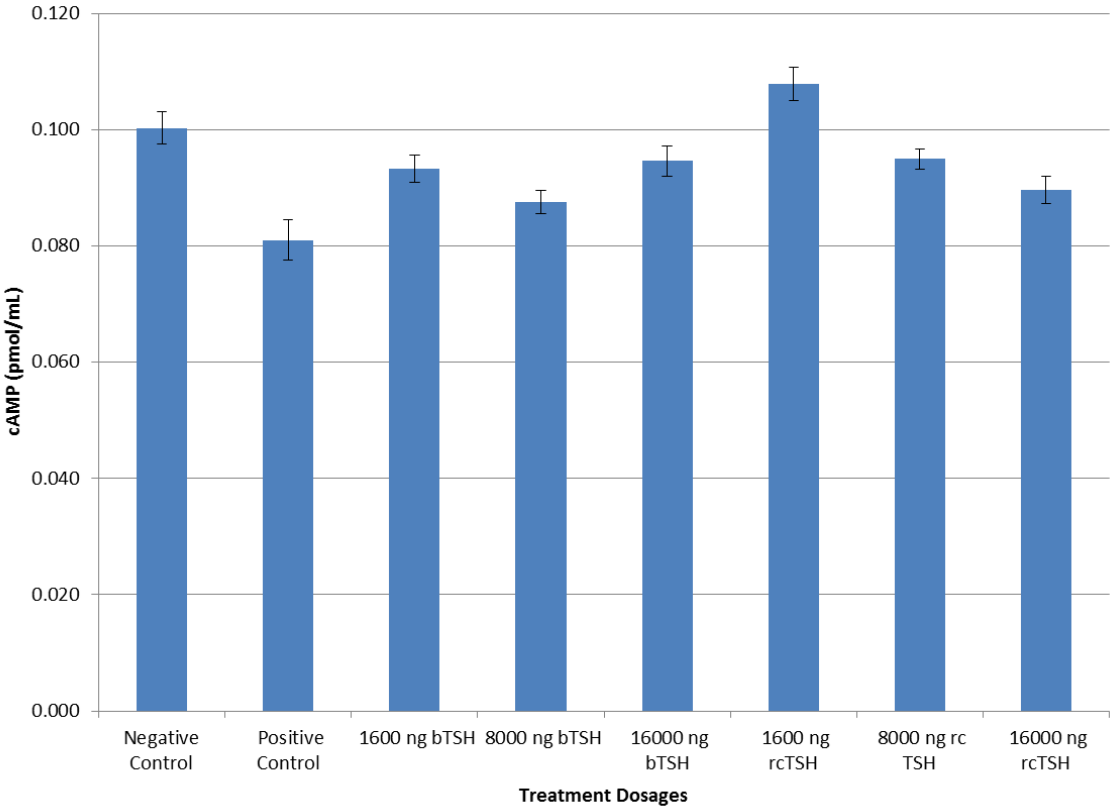

Figure 5. Bar Graph of cAMP Production by Cultured, Canine Thyroid Cells. Presented is a bar graph displaying the average means of cAMP production by each treatment group. Vertical bars represent the standard error of the means.

Table 2. Comparison Between Primary Cell Culture Treatment Groups. Red squares indicate a significant difference ($P<0.05$) between the two treatment groups. The relationship between the significantly different groups is provided in each square.

	Neg. Control	Pos. Control	Low bTSH	Med bTSH	High bTSH	Low rcTSH	Med rcTSH	High rcTSH
Neg. Control		NC>PC		NC>bTSH				
Pos. Control	NC>PC		bTSH>PC		bTSH>PC	rcTSH>PC	rcTSH>PC	
Low bTSH		bTSH>PC				rcTSH>bTSH		
Med bTSH	NC>bTSH					rcTSH>bTSH		
High bTSH		bTSH>PC				rcTSH>bTSH		
Low rcTSH		bTSH>PC	rcTSH>bTSH	rcTSH>bTSH	rcTSH>bTSH		Low>Med	Low>High
Med rcTSH		bTSH>PC				Low>Med		
High rcTSH						Low>High		

response to treatment lower than the negative control. Table 2 displays which treatment groups were different ($P<0.05$) and the relationship between the two different values.

Static Tissue Culture

Upon initiation of the water bath incubation, water temperature measured to 37°C and all tissue slices were set appropriately in the culture tubes with equivalent amounts of media. During the treatment phase of the experiment where TSH was exposed to the tissue, increased temperature of the water bath was observed over a one hour period. Also, a rack of tubes had been added to the water bath during the one hour period increasing the water level. This resulted in elevating the culture tubes out of the rack and into the water bath exposing those tubes to fresh water. Any displaced tubes were secured back into the rack while media was immediately changed in all tubes and transferred into another water bath set to 37° C.

Performing the same statistical procedures as done on the cAMP data, the ANOVA analysis revealed significant differences ($P<0.05$) in the means at the first, third, fourth, sixth, and ninth media-only incubations. The treatments for the entire course of the experiment are represented in figure 6 as line graphs.

The groups which were significantly different ($P<0.05$) are listed in table 3 for the individual incubation stages. On the first, third, and fourth media-only incubations, the low rcTSH dose averaged a significantly greater response than the high dose. The sixth and ninth media-only incubations resulted in the control group being significantly greater than the high and medium TSH dosage, respectively. One caveat to keep in

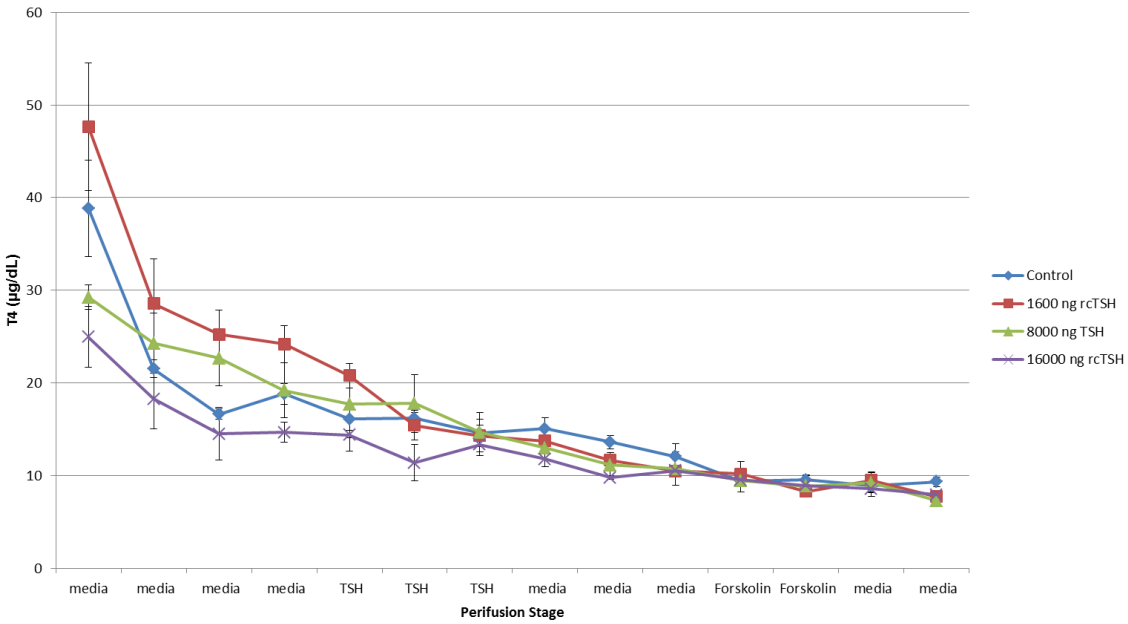

Figure 6. T4 Release by Bovine Thyroid Tissue in Static Culture. The average means of the treatment groups at each point where media was changed. Vertical bars represent the standard error of the means.

Table 3. Comparison Between Static Culture Treatment Groups. Red squares indicate a significant difference ($P<0.05$) between the two treatment groups. The relationship between the significantly different groups is provided in each square.

	First Media	Third Media	Fourth Media	Sixth Media	Ninth Media
Control				Control>High	Control>Med
Low rcTSH	Low > High	Low > High	Low > High		
Med rcTSH					Control>Med
High rcTSH	Low > High	Low > High	Low > High	Control>High	

mind, some cultures were contaminated and data may be skewed and should be interpreted with caution as to significance.

Perifusion

This experiment was designed to compare control, bTSH, and rcTSH with each group consisting of data from 4 perifusion chambers. Unfortunately, at the time of the experiment, one fractionator was inoperable allowing for only two replicates of bTSH and rcTSH. With such small sample sizes for the TSH treatments, the weight behind a statistical analysis would not produce valid statistical inference. It was decided to use the data to manufacture a graphical representation of the perifusion experiment to determine the relationship between the stimulatory actions of bTSH and rcTSH. Figure 7 depicts the results from the perifusion experiment. The baseline T4 expressions by the three groups before applying the treatments are not equal. To allow for a more subjective comparison, figure 8 displays same lines from figure 7 shifted so the baseline T4 before treatments are all equal. This provides a basal starting point for the experimental groups. Figure 8 shows a small elevation in T4 expression by the control group followed by a greater response by rcTSH with the greatest increase observed by the bTSH treatment. Forskolin injection resulted in the expression of T4 by all groups providing evidence of viable tissue throughout the experiment.

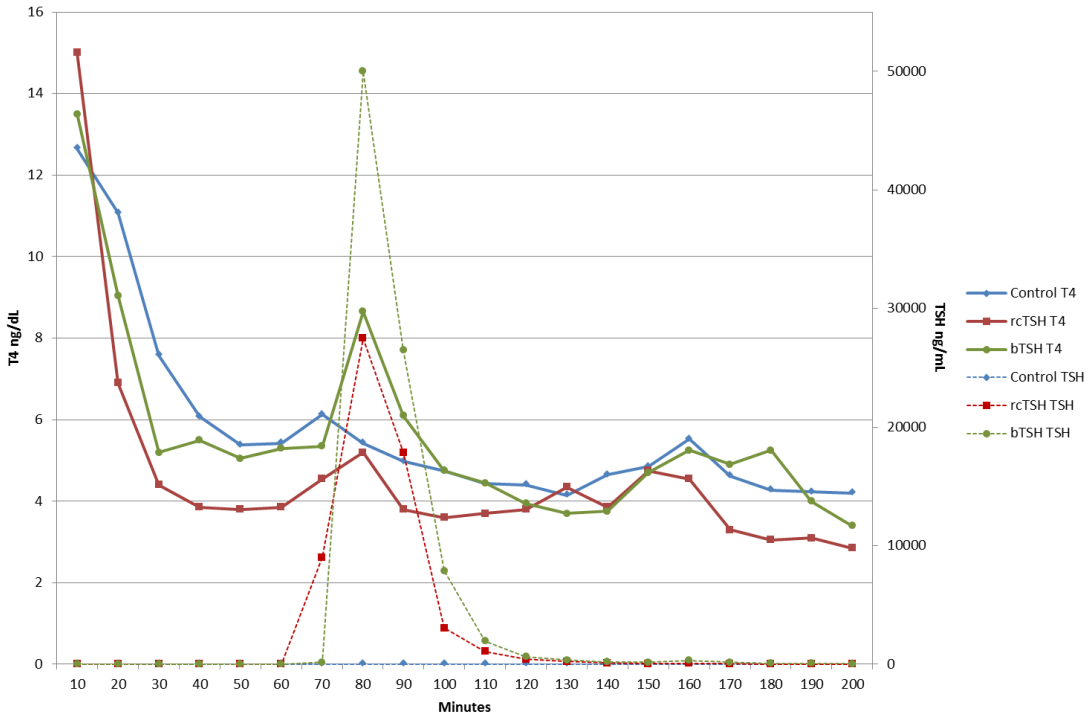

Figure 7. T4 Release by Bovine Thyroid Tissue in Perifusion Culture. Solid lines represent the T4 release by the thyroid tissue while broken lines represent the TSH concentration in the media at the time of release. Even though control concentrations are greater than rcTSH at most all time points, it is due to the greater baseline T4 release before stimulation. The slight rise in T4 concentration experienced by the control at the time of the treatment dose could be attributed to the added pressure applied to the tissue by the force of the treatment injection into the injection port located on the afferent tubing.

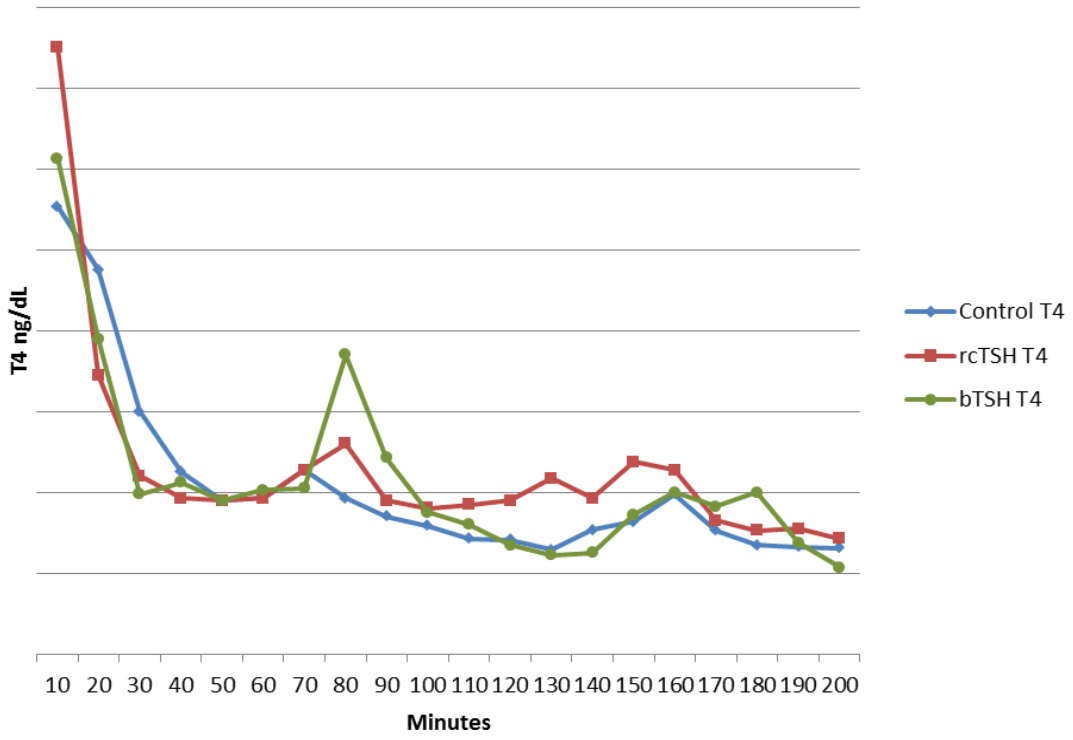

Figure 8. Line Graphs from Figure 7 with Normalized Baseline T4 Release. By shifting all line graphs so the baseline T4 before stimulation are equal, better visual representations of the treatments when compared to the control are given.

DISCUSSION

In performing *in vitro* investigations on a possible pharmaceutical agent, the time and expenses to house live subjects are removed and replaced by uncertainties of replicating a true *in vivo* experience. In this day and age, it is always beneficial from an animal standpoint to do as much as possible before using live animals in research. Results should be analyzed with caution when using *in vitro* models as negative results in an experiment do not necessarily translate into a faulty product but rather the consequence of improper procedure or technique.

The cAMP response to TSH stimulation resulted in inverted data where the media-only negative control was greater in cAMP concentration in all but one (low dose rcTSH) treatment. Past research using dispersed thyroid cell cultures yielded thyroid cells with cAMP activity unaffected by TSH stimulation as well (Rapoport, 1976). A gentler approach was eventually utilized to maintain fully intact or, at a minimum, very large fragments of thyroid follicles to place onto cell culture plates to develop into full monolayer cell cultures. With tight junctions remaining intact and an extended length of time to culture before initiating treatments, monolayer thyroid follicular cells retained the sensitivity to TSH binding by responding appropriately with cAMP production (Roger et al., 1997). Dispersed thyroid cells cultured for 5 days before treatment produced unexpected results not consistent with the varying doses of TSH administration.

Other possible variables attributing to the inconsistencies of the cell culture experiment include: the extra-follicular cells that were also plated in the culture as

cellular dispersion with collagenase did not discern between cell types and some of the experimental subjects were positive for heartworms. The non-follicular thyroid cells requisitioned valuable resources reserved for the follicular cells while producing data that did not meet expectations. The movements of the heartworm microfilaria present in the culture plates would mechanically inhibit the attachment and growth of the cell culture. Ultimately, the measurable endpoint of rcTSH administration will be T4 release by the thyroid. For this reason, the cell culture experiment was not repeated and T4 release by intact thyroid tissue sample under different culture conditions was examined.

The static culture experiment was the first investigation as to the T4 output by thyroid tissue when exposed to rcTSH. While not all tubes were ejected from the rack, all were exposed to elevated water temperature for at least 30 minutes. Elevated temperatures, exposure to fresh water, or improper media could explain the lack of response by the thyroid tissue to TSH and forskolin exposure as the maintenance of viable tissue was not executed. As cell death occurs due to high temperatures, hypotonic solutions, or lack of nutrients, any stores of molecules (i.e. proteins, steroids, etc.) could be released explaining the presence of T4 in the incubation media even though no response to stimulating agents appeared to occur.

Perifusion experiments were performed in parallel with the static culture. The added control of the different variables during perifusion over static culture was justified in not repeating the static culture experiment. Not obtaining statistical analysis was unfortunate; however, the graphical representation provided motivation for future *in vivo* experimentation. While response was not as great as bTSH, rcTSH did stimulate the

bovine thyroid tissue housed in the culture chambers to release T4. Since culture media was used to inject a treatment sample into the control chambers, the slight rise and decline in T4 observed by the control might be attributed to the mechanics of the injection. As fluid was injected into the chambers, pressure was added which could cause a mechanical release of T4 from the thyroid tissue housed in the chamber. This is quite different from a stimulatory reaction by the thyroid tissue to the presence of a thyrotropic hormone. To distinguish between the amount of T4 release caused by injection force and TSH stimulation, the difference between the control T4 release curve and that of the treatments represent TSH stimulatory effects.

CHAPTER III

IN VIVO TRIALS

OVERVIEW

The efficacy of rcTSH was tested *in vivo* utilizing goldfish and rat bioassays as a prelude to performing the endpoint *in vivo* experiments in the canine. Bioactivity of rcTSH was measured against bTSH which was considered the "Gold Standard" for performing a TSH stimulation test. Goldfish, rats, and canine, bloodhound crossbreeds were given injections of exogenous TSH in separate experiments to determine if rcTSH and bTSH were similar in eliciting T4 responses compared to controls (saline injected).

The goldfish assay performed by Dr. Thomas Miller (Zoology Department, Texas A&M University) resulted in similar T4 response profiles between bTSH and rcTSH. The glaring difference was the increased amount of rcTSH needed to equal the same T4 release caused by bTSH. Approximately five times the dose was needed to generate the equivalent T4 response using rcTSH compared to bTSH (Miller, 2011).

For the rat bioassay, equal doses of bTSH and rcTSH were given to rats of approximately equal sizes (50g) with post injection blood collections at five hours. Activity at the two doses (5,000 ng and 10,000 ng) were compared resulting in similar ($P>0.05$) responses by the groups given exogenous TSH (5000 ng rcTSH mean=4.45, 5000 ng bTSH mean=5.19, 10000 ng rcTSH mean=5.76, 10000 ng bTSH mean=6.63) with significantly different ($P<0.05$) responses between treatment groups and the control

group (mean=2.96). Even though exogenous treatment (TSH) groups were different from control ($P<0.05$), rcTSH treatments had consistently lower T4 response as compared to the bTSH.

The canine experiment will dictate the true future of rcTSH as a possible FDA approved pharmaceutical to be used in the diagnosis of canine hypothyroidism. As in the rat bioassay, canines were given equal immunoreactive doses of rcTSH and bTSH to be compared to each other and against a control group at post injection blood collection times of 2, 4, 6 ,and 8 hours. Statistical analysis proved T4 release responses to rcTSH (2 hour mean=2.775, 4 hour mean=4.138, 6 hour mean=3.728) and bTSH (2 hour mean=3.435, 4 hour mean=5.398, 6 hour mean=5.598) were significantly similar ($P>0.05$) while significantly different ($P<0.05$) to the control group (2 hour mean=1.525, 4 hour mean=1.465, 6 hour mean=1.558). At the eight hour time frame, there was not a significant difference ($P>0.05$) observed between the control group (mean=1.70) and the rcTSH group (mean=2.93). This time frame falls outside the normal TSH stimulation protocol of drawing a post injection sample at 4-6 hours.

The rat and the canine experiments reveal that rcTSH is similar ($P>0.05$) to the "Gold Standard" bTSH in T4 response. Further steps will be pursued for FDA approval as an approved, veterinary diagnostic tool for determining hypothyroidism in the canine.

INTRODUCTION

For the last 30 years, baculovirus mediated recombinant protein expression has provided an alternative to producing pharmaceutical agents which emulate their endogenous homologues in immuno- and bio- activity (Jarvis, 2009). With the ability to accept codon optimized RNA encoding for the POI in addition to performing mammalian glycosylation, insect cells are programmed by a baculovirus to manufacture a foreign glycoprotein in large quantities which will stimulate a physiologic reaction from the target cells. This technology offers a solution for the veterinarian to increase the accuracy of diagnosing true canine hypothyroidism.

Hypothyroidism, an endocrinopathy in the canine, represents a common yet often misdiagnosed disease to the veterinarian (Graham, 2009). Clinical signs along with a battery of serological tests (i.e. T3, T4, Free T4, TSH) provides insight to the functional status of the thyroid gland; however, not all the necessary information to make a firm diagnosis will be received at times. To abate this concern, TSH stimulation test would quantify the patient's thyroidal output after injection with exogenous TSH. Once considered the standard protocol for diagnosing canine hypothyroidism, the TSH stimulation test was performed using bTSH. A comparison between serum T4 concentrations before and 4-6 hours after the bTSH injection would reveal if the thyroid gland reacted appropriately to the bolus of bTSH (Panciera, 1999). With bTSH no longer approved for veterinary use and the elevated expense of rhTSH, veterinarians are

forced to base decisions on "snapshots" of thyroid function by submitting serum from random blood draws for thyroid hormone assays.

Baculovirus expression of rcTSH could provide valuable information to aid in the diagnosis of canine hypothyroidism. Cells from *Spodoptera frugiperda* of the order Lepidoptera are infected by a baculovirus containing recombinant DNA. Wild-type baculovirus infection will force the insect cells to produce the protein, polyhedrin, in large quantities. Substitution of the DNA encoding for polyhedrin with the codon optimized sequence for both α and β chains of cTSH located on a designer plasmid occurs via homologous recombination. Insect cells successfully infected will lack the visibile presence of the polyhedrin protein and are subsequently cultured for rcTSH expression.

The media harvested after culture will contain not only rcTSH but other by-products of cellular protein translation. The unwanted material must be removed in order to isolate the rcTSH before injection else any foreign proteins might cause an anaphylactic response. One objective for this experiment was establishing a purification protocol to provide a pharmaceutical agent safe for injection.

Upon the purification of rcTSH from the cell culture media, the major objective to determine and compare the ability of rcTSH and bTSH to elicit T4 release from the thyroid gland using the TSH stimulation test protocol was pursued. Three bioassays were developed for this comparative study: goldfish, rats, and canine species. The goldfish bioassay would be used for an alternate publication so the TSH stimulation test

was not performed. Instead, dose response curves were generated for rcTSH and bTSH for comparison.

MATERIALS AND METHODS

Expression of rcTSH

Filed by Jaques and Jarvis (2009, 2010) , United States patents US 7,479,549 B2 and US 7,838,492 B2 protects the methods of production and use of rcTSH as proposed by inventors Dr. John (Scott) T. Jaques and Dr. Donald L. Jarvis. The patents detail the codon optimizations, alterations made in the codon sequences of amino acids, used to maximize production of rcTSH α and β chains by lepidopteran cells. Also discussed in the patents are the various cell lines infected by the baculovirus, AcP(+)DIEcTSHA/B, each of which providing individual post-translational glycosylation modifications.

To summarize, plasmids containing rcTSH DNA undergo homologous recombination with the baculovirus effectively exchanging the polyhedron encoded region for the α and β chains of rcTSH DNA. The baculovirus infects the lepidopteran cells instructing cellular mechanisms to produce rcTSH, not polyhedron. The infected cells are cultured with fresh medium for 5 days. Centrifugation removes the cells from the culture media containing rcTSH. Expression of rcTSH is provided by Expression Systems, Woodland, CA.

Purification of rcTSH

Frozen (80˚C) media containing rcTSH remained refrigerated at 3°C during thaw. The pH of the media was elevated to 9.5 using NaOH. The media was then centrifuged at 3000 rpm for 15 minutes. After pouring through a glass wool filter to capture sediment formed during centrifugation, the supernate is applied to an AEX (Column-Kontes Chromaflex 420830-3020, Resin-GE Healthcare HiTrap Capto Q) column. The cationic charge of the stationary phase attracts molecules with negative charges in a 9.5 pH environment (Zhu et al., 2005). With an approximate pI of 8.5 (work perform by Dr. Evan Shave of Dr. Vigh's lab, Chemistry Department, Texas A&M University), most rcTSH will not bind to the column. The column was washed with three column volumes of running buffer to collect rcTSH suspended in the mobile phase on the AEX column.

The harvested flow through media receives acetic acid to lower the pH to 5.0. The adjusted media is then applied to a CEX (Column-Kontes Chromaflex 420830-3020, Resin-GE Healthcare HiTrap Capto S) column. The rcTSH will retain a positive charge and adsorb onto the negatively charged groups on the cation resin (Zhu et al., 2005). Three column volumes of buffer remove material suspended in the mobile phase of the CEX. To desorb the rcTSH from the CEX functional groups, a NaCl solution is applied to the CEX in an increasing molar gradient.

The final step in the purification process is to remove the NaCl. The media flows tangential to a porous membrane containing pores of 10 μm in diameter. Water, along with molecules smaller than the membrane pores, cross over to the filtrate of the

membrane while larger than 10 um molecules remain in the retentate (Ward and Swiatek, 2009). The total media volume and sodium chloride concentration of the retentate will decrease in preparation for lyophilization.

To visualize the effectiveness of the purification protocol, SDS-PAGE and western blot gel protocols are performed in accordance with the Mini-PROTEAN 3 Cell (Bio-Rad 165-3301) system. Multiple sample types were analyzed including: separated α and β chains of bTSH (Sigma), bTSH (Sigma), de-glycosylated bTSH (Sigma), bTSH (National Institute of Health (NIH)), and rcTSH (SWT-4). Separation and de-glycosylation of bTSH was performed by Dr. Thomas Miller of Dr. Duncan McKenzie's lab for an alternate publication. Subsequent to loading the samples into the wells of the Mini-Protean precast gel (Bio-Rad 456-1044), all were measured for TSH immunoactivity by chemiluminescence (Siemens Immulite 2000) with veterinary canine reagents (Siemens LKKT5). Once the TSH concentrations were determined, calculations were performed to compute the ratio of sample, buffer (including SDS), loading dye, and water so all preparations contained approximately the identical, total milligram amount of immunoreactive TSH. Total volume for each well of the Mini-Protean precast gels (Bio-Rad 456-1044) are 50 µl so to avoid overfilling the wells, loading samples were kept to a 40 µl volume. Electrophoresis buffer concentrate (Bio-Rad 161-0732) required a 1:3 dilution accounting for 10 µl of the preparation. Adding 2 µl of loading dye diluted at a 1:5 ratio with DI water leaves 28 µl for the sample. Depending on sample concentration, the total volume needed to load a uniform amount of mg TSH onto the gel

is supplemented with DI water. Preparations were loaded by pipette into duplicate wells of two gels sharing the same electrophoresis chamber. Power settings (200V, 120mA, 24W for 30 minutes) applied to the gel were based upon Bio-Rad's recommendations. Due to an outage of an upright refrigeration unit, electrophoresis was performed at room temperature instead of a refrigerated environment.

Silver staining (Bio-Rad 161-0447) one of gels will present a picture of the proteins present in each sample according to their size dependent migration. The silver staining protocol was followed as instructed by the Bio-Rad handbook. Figure 9 demonstrates how effective the ion exchange columns and the tangential flow membrane were in isolating rcTSH from the culture media. The rcTSH sample, loaded on the far right lane, contains three distinct bands: two lower molecular weight bands possibly representing the separate α and β rcTSH subunits and a band of higher molecular weight material possibly representing intact, glycosylated rcTSH molecules.

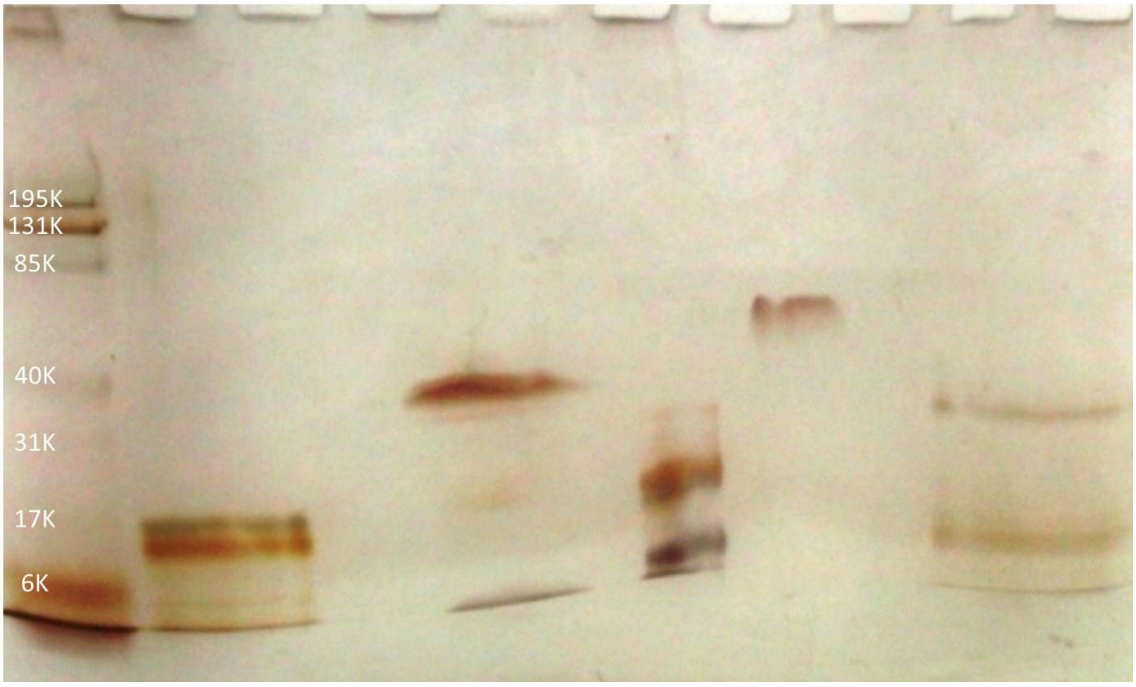

Figure 9. Silver Stain of rcTSH on SDS-PAGE Gel. Silver stain of SDS-PAGE gel loaded with samples (from left to right): molecular weight marker, bTSH α and β subunits(Sigma), bTSH (Sigma), de-glycosylated bTSH (Sigma), bTSH (NIH), and rcTSH (SWT-4).

To determine if the higher molecular weight band is rcTSH, a western blot is performed on the duplicate gel. The proteins from the unstained gel were transferred to the western blot membrane using an electrophoretic transfer cell (Bio-Rad 170-3930) for the Mini-Protean 2 Cell electrophoresis unit. Antibodies to cTSH acquired from Siemens were diluted with phosphate buffered saline (PBS) at a 1:1000 dilution for use with the western blot reagents (Vector Labs Vectastain ABC-AmP). Figure 10 shows a distinct band which developed in the same, approximate location as the suspected rcTSH band in the silver stain gel. Due to the separate expression of rcTSH α and β subunits during culture, the expectation to visualize bands at molecular weights on the silver stain for the intact rcTSH and subunits while development of a band on the western blot for only the intact rcTSH molecule were met. Also note that cTSH antibodies did react with bTSH as demonstrated by the presence of bands on the western blot in the Sigma, de-glycosylated Sigma, and NIH bTSH lanes.

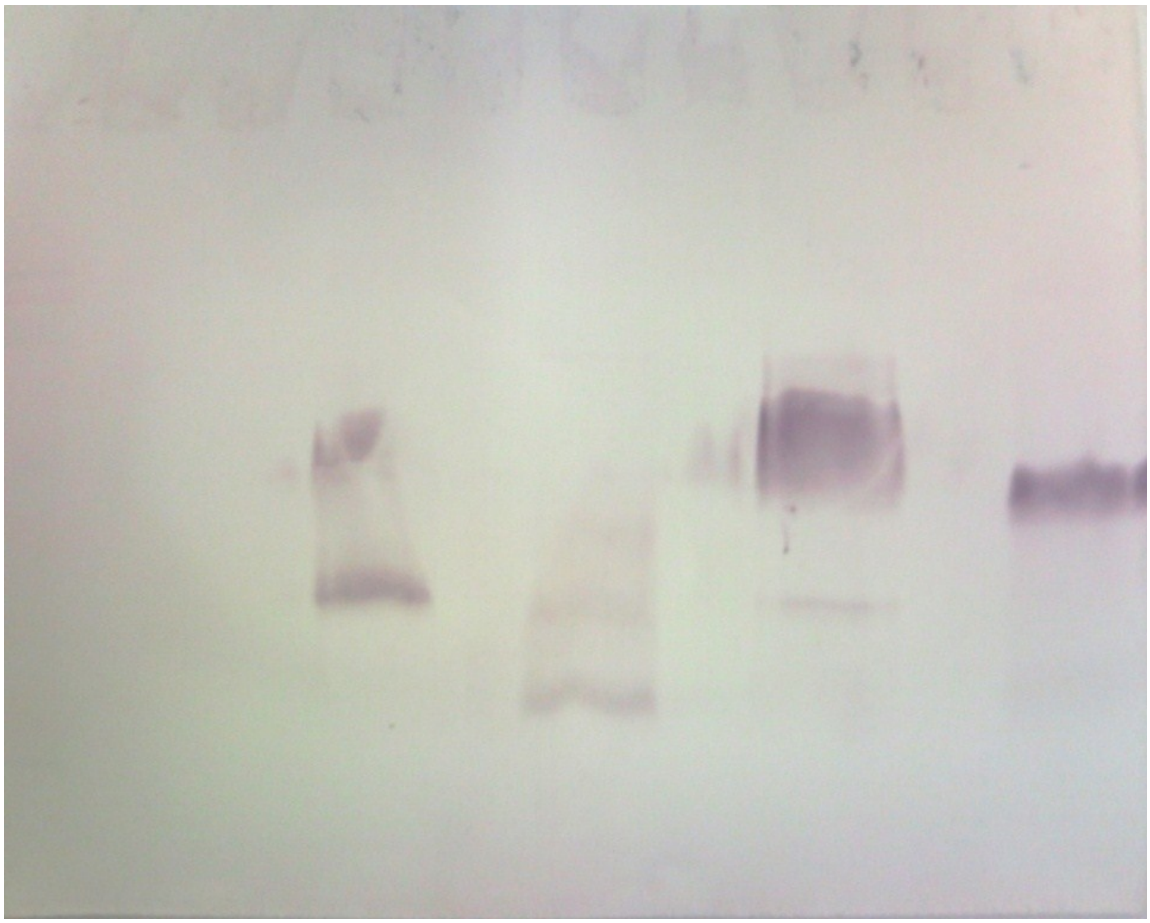

Figure 10. Western Blot of Duplicate SDS-PAGE Gel from Figure 9.

Goldfish

Thomas Miller, a former graduate student (PhD) of Dr. MacKenzie's Lab in the Zoology Department at Texas A&M University and I assisted one another while we performed our experiments for our research. While evaluating the biological activity of different TSH preparations (bTSH (Sigma), bTSH(NIH), rcTSH (SWT-4), Thyrogen (hTSH)) in red drum and goldfish, the now Dr. Miller helped with my project by utilizing rcTSH as a source of exogenous TSH with a glycosylation pattern that differed from that of bTSH. A comparison between the bioactivity of rcTSH and bTSH was performed by injecting intraperitoneally (IP) multiple groups of goldfish (n=7) with varying doses of bTSH (0.1-21.5 ir-ng/µl) or rcTSH (5-100 ir-ng/µl) and harvesting blood at 5 hours post injection for plasma T4 concentration determination (Miller, 2011).

Rodent

Black and white hooded rats were donated by Dr. George Stoica's Lab (Veterinary Physiology and Pharmacology, Texas A&M University) were randomly assigned into groups (n=10) to receive either media for control (saline), 5,000 ng of bTSH, 10,000 ng of bTSH, 5,000 ng of rcTSH, or 10,000 ng of rcTSH. Treatment injections were given IP. Blood was harvested 5 hours post IP injection into sterile red top tubes (Monoject). Serum T4 (Siemens Immulite 2000, Reagent L2KCT6) concentrations were determined after centrifugation of the blood collection tubes for 15 minutes at 2500 rpm. All rats weighed approximately 50g.

Canine

Twelve hound crossbreeds were procured from the Texas Department of Justice and housed at the Texas A&M University Laboratory Animal Resources and Research (LARR) facility. Canines were fed twice every 24 hours and given water ad lib. After two weeks of acclimation, an IV catheter was placed cephalically on each dog on the morning of the experiment. Once all catheters were place, blood samples were drawn for general health chemistries and hypothyroid screens. Immediately following the baseline blood draw, treatments were injected through the catheter port of saline (n=4), 3,000 ng/kg bTSH (n=4), or 3,000 ng/kg rcTSH (n=4). Blood samples were taken at 0, 2, 4, 6, and 8 hours post treatment injections into red top tubes (Monoject). The tubes were centrifuged for 15 minutes at 2500 rpm to separate serum from the red blood cells. T4 concentrations were subsequently measured using canine specific regents (Siemens Immulite 2000, Reagents L2KCT6).

RESULTS

Goldfish

The response to bTSH and rcTSH in the production of T4 five hours after injection paralleled one another but at a different dosage frame in goldfish. At every point, the rcTSH required a larger dose to evoke the same T4 response as the smaller bTSH dose. For example, bTSH at a dose of approximately 21.5 ir-ng/µl elicited a slightly larger T4 response than rcTSH at 100 ir-ng/µL as shown in figure 11 (Miller,

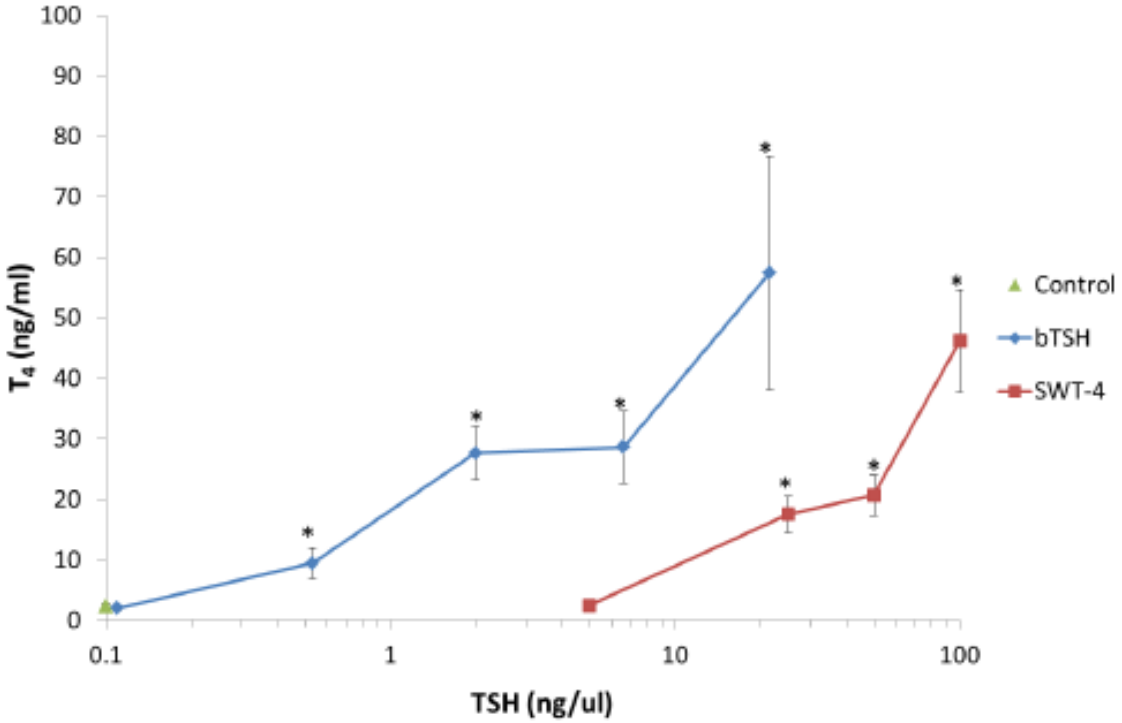

Figure 11. Response of Goldfish Thyroid Tissue to bTSH and rcTSH. Dr. Miller's data compares the response of goldfish thyroid tissue to bTSH and rcTSH (SWT-4). While the line graphs representing the T4 release to TSH stimulation parallel one another, the rcTSH required much larger doses than bTSH to elicit the same response (Miller, 2011).

2011). While the potency of rcTSH was not equal to that of bTSH, a response to rcTSH by goldfish thyroid tissue was evident.

Rodent

Like the goldfish, data gathered from an exogenous TSH stimulation performed on rats produced encouraging findings. Using ANOVA analysis rejected the null hypothesis that all means were similar ($P<0.05$). The Tukey's W Procedure was performed to determine which groups were similar. In comparison of the 5,000 ng dose, both rcTSH and bTSH were significantly different ($P<0.05$) from the control group while being significantly similar ($P>0.05$) to each other as shown in figure 12. Analysis of the 10,000 ng dose was not different from the 5,000 ng dose treatment: significant differences ($P<0.05$) were observed between groups receiving TSH and the control (saline) group with similar stimulatory effects ($P>0.05$) between groups receiving exogenous TSH which is shown in figure 13. In both cases, the mean T4 response from bTSH was greater than rcTSH but not significantly different ($P>0.05$).

Figures 14 and 15 represent the serum TSH concentrations for the 5,000 ng and the 10,000 ng dosed groups, respectively. No similarities ($P<0.05$) existed between the treatment groups of the 5,000 ng dose group. In the 10,000 ng dose individuals, the groups receiving TSH were similar ($P>0.05$) in serum TSH concentration with both being greater ($P<0.05$) than the control. Determining TSH concentration after injection is not a top priority in this research. When glycosylation studies are initiated will longevity in circulation be a more relevant consideration.

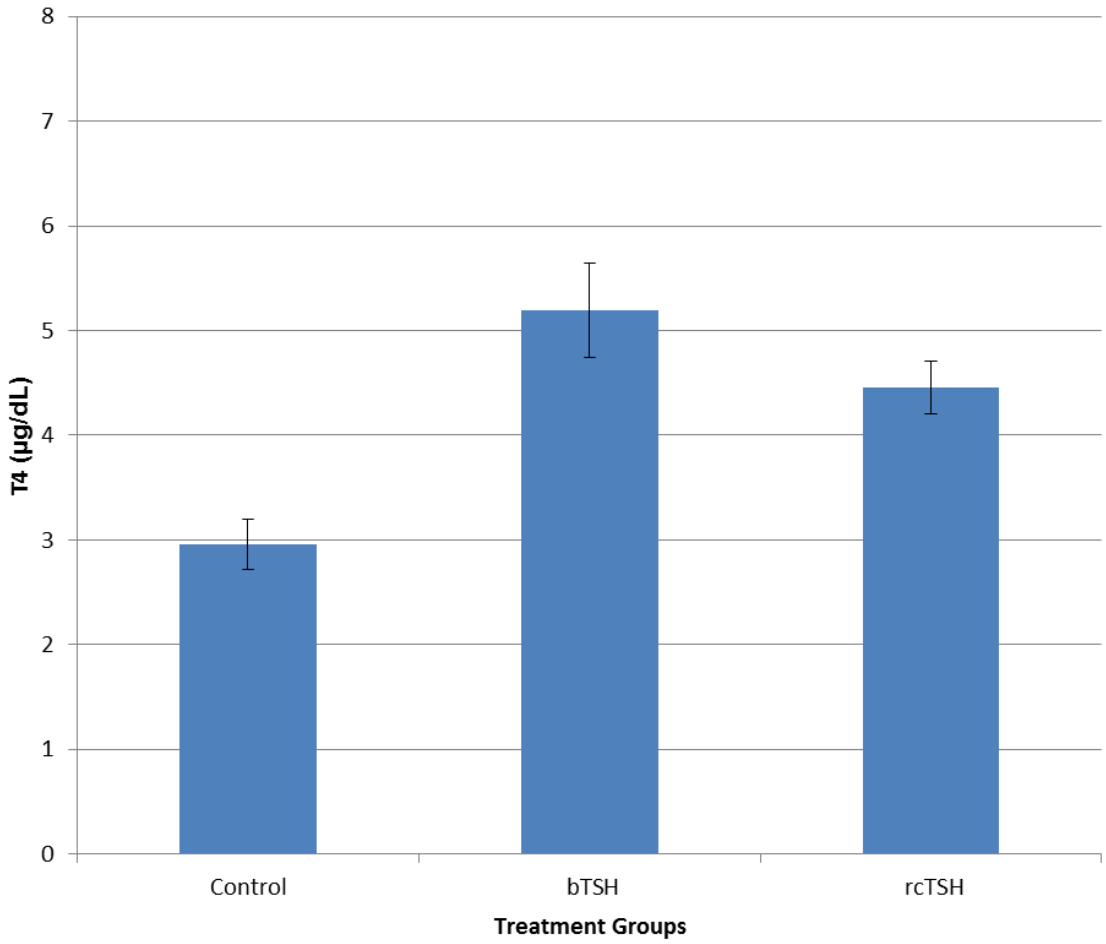

Figure 12. Response of Rat Thyroid Tissue to 5,000 ng of bTSH and rcTSH. Both TSH analogs were significantly greater ($P<0.05$) than control while significantly similar to each other ($P>0.05$).

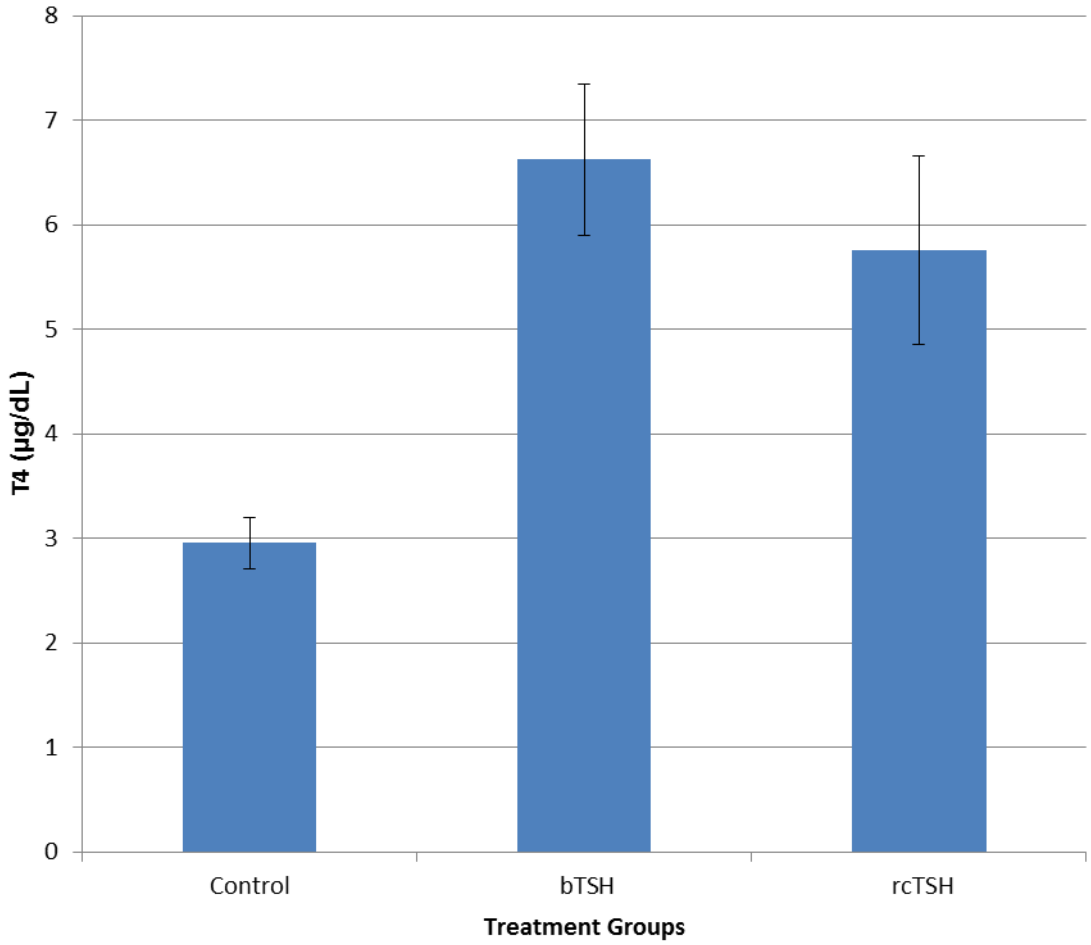

Figure 13. Response of Rat Thyroid Tissue to 10,000 ng of bTSH and rcTSH. The means of T4 response in rats exposed to 10,000 ng of exogenous TSH performed in parallel to the groups depicted in figure 9. Similarly, TSH groups were significantly similar to one another ($P>0.05$) but significantly different from control ($P<0.05$).

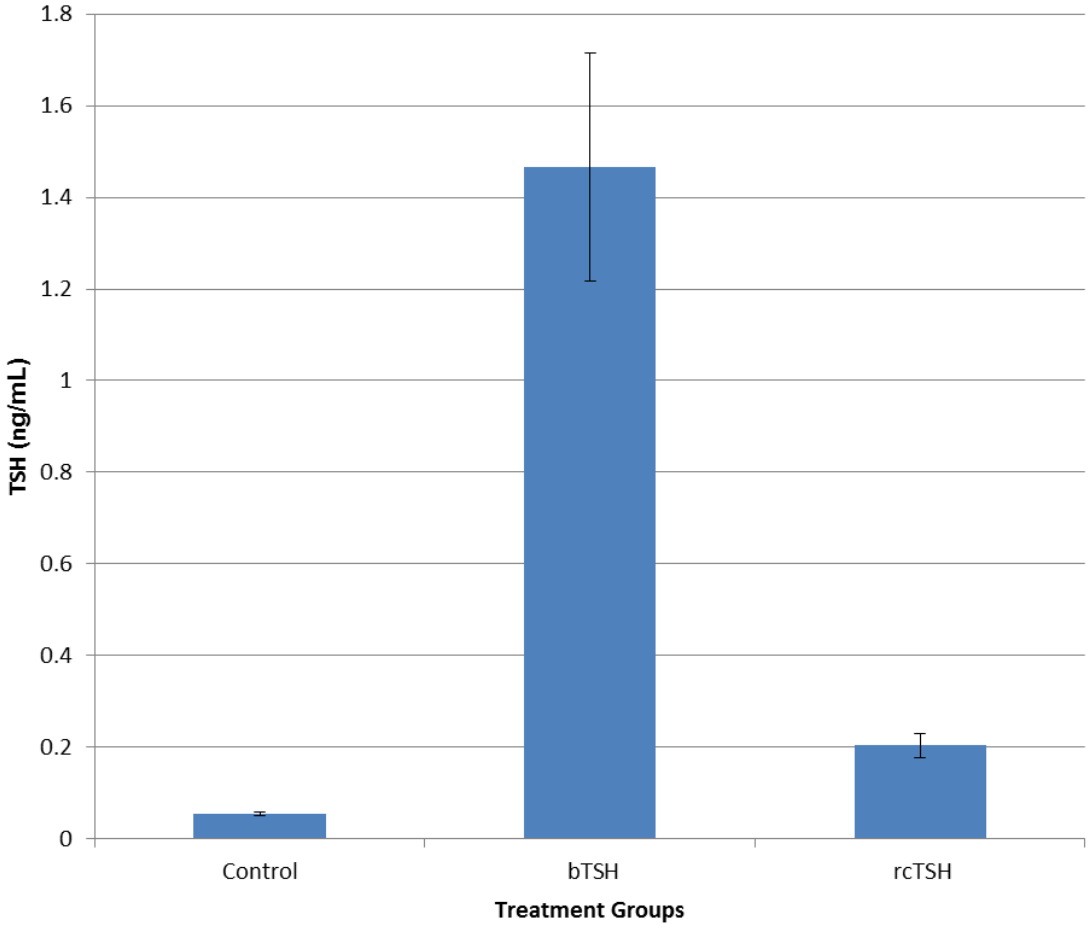

Figure 14. Serum TSH Concentrations of Rats from Figure 12. Differences were observed between all treatment groups ($P<0.05$).

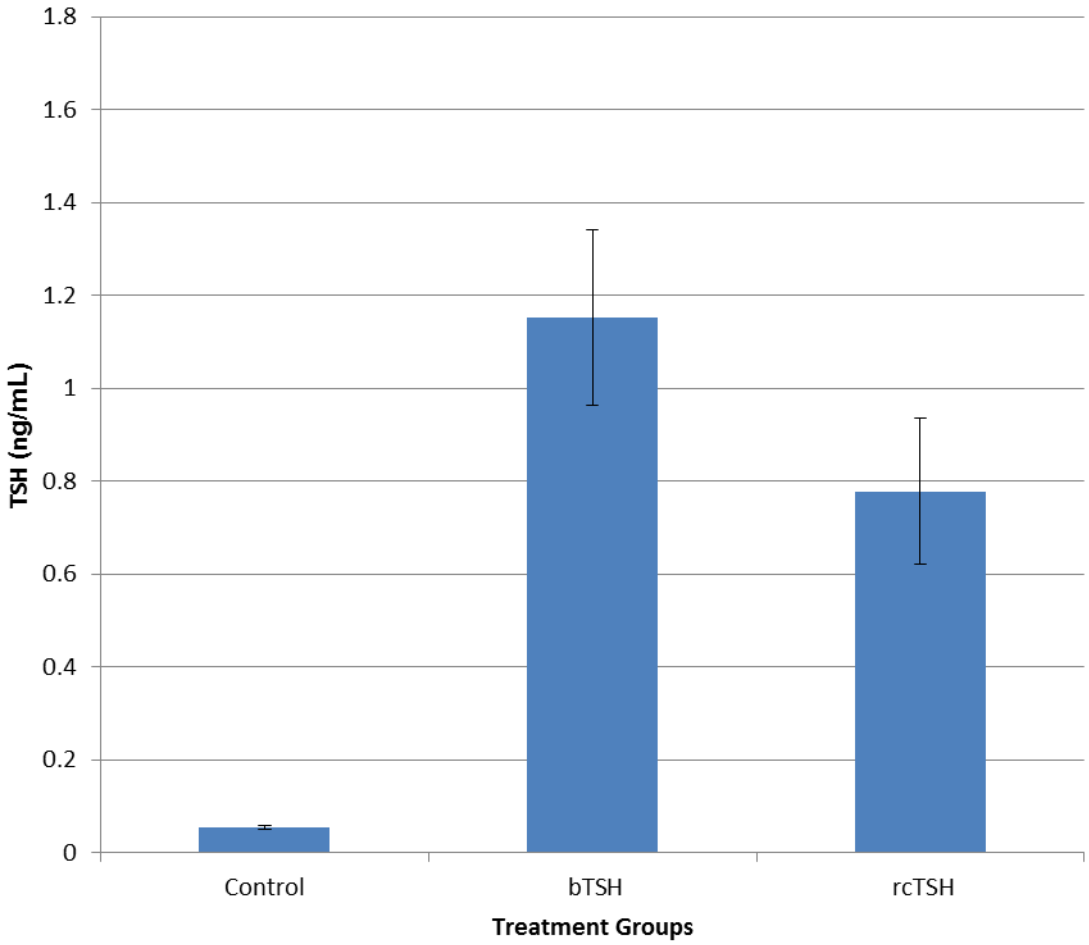

Figure 15. Serum TSH Concentrations of Rats from Figure 13. Both treatment groups were greater ($P<0.05$) than the control and similar ($P>0.05$) to each other.

Canine

Performing TSH Stimulation tests on the species which the recombinant hormone was specifically design for brought excitement. Our husbandry for these test animals was arduous as they were not accustomed to walking on a leash and running without supervision in a field which was a new and clumsy experience for all individuals. For two weeks, our goal was to acclimate the canines to their new surroundings and develop a sense of trust with their new handlers through feedings and shared activities to reduce the stress during the experiment.

Catheters were placed on all individuals successfully; however, some individuals removed their catheters during the course of the experiment. Any individuals removing their catheters were subjected to a blood draw via cephalic venipuncture with a 22 gauge x 1 inch needle and 5 ml syringe at collection times. Attempting to place another catheter resulted in a quicker removal of the catheter by the subject. E-collars were also employed to aid in preventing catheter removal.

Analysis using ANOVA rejected the null hypothesis that all means were significantly similar ($P<0.05$) leading to the Tukey's W Procedure. At the baseline sample, the Tukey's comparison resulted in the non-significant difference ($P>0.05$) between all groups indicating a normalized baseline T4 for all individuals. The serum

T4 concentrations at the 2, 4, and 6 hour post injection blood collections did not significantly differ ($P>0.05$) between the treatment groups. Both groups receiving TSH treatments were greater ($P<0.05$) in T4 response to the injection from control (saline) group at all 3 time points. The only time point where T4 response to a preparation of TSH was not significantly different ($P>0.05$) between control (saline) and a treatment group (rcTSH) was at the 8 hour draw.

Figure 16 displays the means for serum T4 concentrations for all treatment groups at their respective blood draws. Serum levels of T4 were greater in response to bTSH than rcTSH at all time points. Serum TSH concentrations visualized in figure 17 are consistently greater for bTSH until hour eight. The presence of sialic acid on the rcTSH molecules could be reason for its elevation over bTSH in serum concentration at that blood collection time.

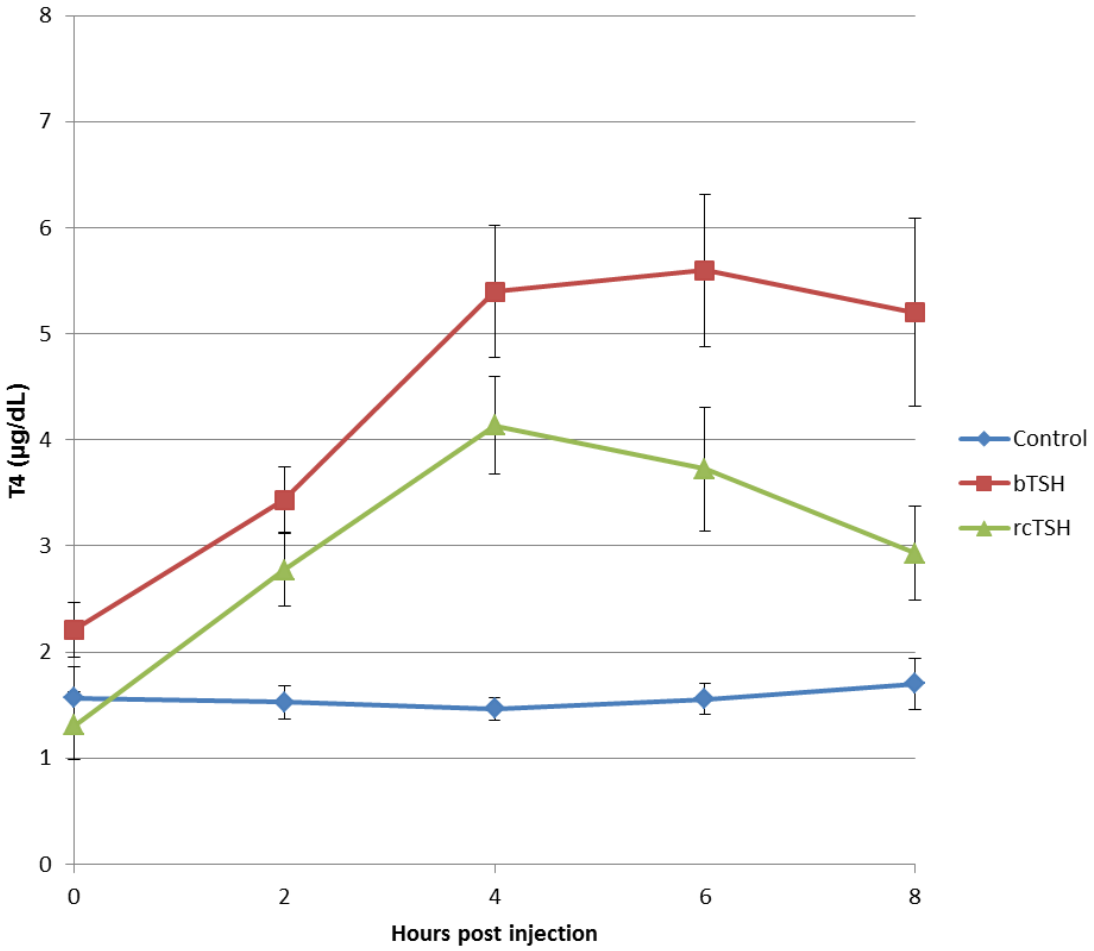

Figure 16. Response of Canine Thyroid Tissue to bTSH and rcTSH. The mean T4 response by canines at various hours after receiving exogenous TSH or control. At all data points, bTSH and rcTSH were significantly similar ($P>0.05$). Both exogenous TSH treatments were significantly different ($P<0.05$) from control except for rcTSH at the 8 hour post sampling.

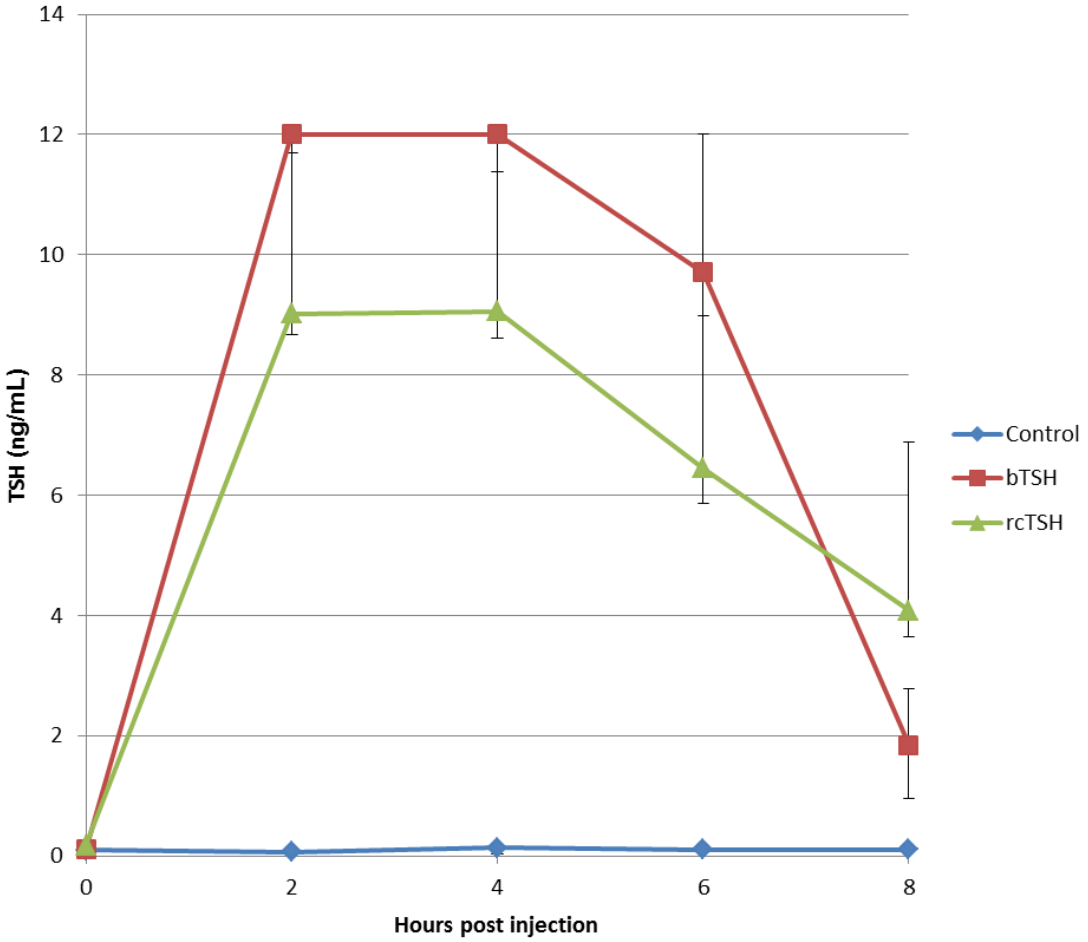

Figure 17. Serum TSH Concentrations of Canines from Figure 16

DISCUSSION

All *in vivo* experiments demonstrated similar conclusions: rcTSH does elicit a response by thyroid tissue in the species used in the preceding experiments; a greater amount of rcTSH is needed to evoke a T4 response equal to that of bTSH; and rcTSH was significantly similar to bTSH in causing T4 release. It was shown in goldfish that canine pituitary extract had a statistically equivalent response in T4 stimulation as bTSH (Miller, 2011). Recombinant cTSH was designed by Dr. Jaques to emulate endogenous cTSH (at least 98% homology for both α and β chains) with the exception of optimizing the nucleic acid sequence to promote a greater expression of rcTSH in lepidopteran cells (Jaques and Jarvis, 2010). Any changes to nucleic acid sequences away from the true cTSH sequence in order to increase rcTSH expression could be tested for efficacy by utilizing the *in vivo* methods described in this chapter.

The amino acid sequence of rcTSH was designed to mimic endogenous cTSH which stimulated T4 production and release to the same efficacy as bTSH in goldfish. It is therefore interesting to observe rcTSH not promoting an increase in serum T4 concentrations as bTSH. An obstacle when using insect cells to produce a mammalian protein, other than different codon sequences encoding for amino acids, is the glycosylation machinery in insect cells do not natively produce the complex carbohydrate moieties as mammalian cells. The enzymes to remove sugars are available to the insect cell while the enzymes to build the sugar chains like mammalian cells are not (Jarvis, 2003). Fortunately, methods have been discovered to manipulate insect cells to act more mammalian. Insect cell lines have progressed from Sf9 to SfSWT-

1/SfSWT-3 and SfSWT-4 each increasing in its ability add complex mammalian carbohydrate structures (Shi and Jarvis, 2007). The rcTSH produced by the SfSWT-4 cell line was used for all the experiments described in this dissertation due to its ability to build complex, biantennary oligosaccharides with terminal sialylation. Sialic acid attached to the termini of an oligosaccharide provides protection to the glycoprotein from hepatic removal from circulation as well as a slight negative charge to the molecule allowing for an increase in water solubility (Morell et al., 1971). Endogenous cTSH and bTSH, however, contain sulfated termini to which hepatic endothelial and Kupffer cells have receptors. This allows the liver to quickly clear molecules containing a specific oligosaccharide chain terminating with sulfonation. These properties are similar to those found LH. Rapid clearance of these molecules activates a negative feedback at the pituitary to release more of LH or TSH which will be quickly cleared again by the liver. This equates to pulsatile hormone concentrations in the bloodstream increasing the biological effect by the hormones (Strott, 2002).

While rcTSH may numerically not incite a T4 response equivalent to bTSH, statistically both are stimulatory. As seen in figure 17, rcTSH remained higher in circulation at the 8 hour post injection demonstrating the effectiveness of terminal end sialylation of the carbohydrate moieties present on rcTSH. Further investigation is needed to determine the biological activity of rcTSH with sialic acid compared to sulfonation of the oligosaccharide termini; however, rcTSH has been shown to have the same efficacy as bTSH, but in most cases, not as elevated in T4 response to the exogenous challenge.

CHAPTER IV

CONCLUSIONS

The purpose of these experiments was to determine, using *in vitro* and *in vivo* techniques, if a recombinant form of cTSH produced by baculovirus achieved the same statistical efficacy (release of T4) as the "Gold Standard" bTSH when used as an exogenous source for the TSH stimulation test. Developing techniques to produce recombinant sources of protein hormones is essential for disease diagnoses and treatment in the health care field. A protein produced by a baculovirus was approved by the FDA as the first recombinant vaccine for acquired immune deficiency syndrome (AIDS) to undergo trials (Luckow and Summers, 1988). No longer can extracts from other species be used for fear of disease transmission. While human medicine spares no expense in developing and bringing pharmaceutical drugs to market, the veterinary field is not quite as receptive to the high cost of products destined for the use in veterinary health. Fortunately, the popularity of pet insurance is on the rise which could provide more opportunities for research in customizing pharmaceuticals for the pet industry.

To belay cost considerations, a baculovirus capable of infecting cells of the Lepidoptera order was used to create a recombinant, glycosylated canine thyroid stimulating protein hormone. This would provide a more direct alternative to aid in determining the functional status of the thyroid gland ass compared to performing the ancillary tests like T3, T4, and Free T4, to name a few. Stimulating thyroidal production of T4 by rcTSH was compared against an older "Gold Standard" of bTSH which had

been used in the past for a TSH stimulation test in the canine which is no longer sanctioned by the FDA as a pharmaceutical agent. The *in vitro* and *in vivo* experiments designed for the comparative efficacy study demonstrated the similar ($P>0.05$) potency of rcTSH to bTSH in stimulating activity in thyroid follicular cells with the exception of the cAMP cell culture and the static tissue culture. The issues preventing appropriate data collection from these two experiments have been addressed; however, the alternative testing protocols (perifusion, bioassays) presented offer either greater control of variables during the experiment (perifusion system control over media temperature, %CO2, %O2, dynamic nutritional delivery) or a viable bioassay providing physiologic responses to an exogenous thyroid stimulatory glycoprotein.

The choice to investigate rcTSH was decided by the need for a better diagnostic tool to identify hypothyroidism in the canine as compared to the current "backdoor test" to identify true hypothyroid animals. Providing a consistent source of TSH with FDA approval and the availability of a more improved hormone assay for the measurement of canine specific TSH may provide for better and more accurate ways to asses hypothyroidism in the canine. An alternative goal to this research is to lay the groundwork for the production of two glycoproteins of similar structure to TSH, bovine LH and more importantly bovine FSH. The use of LH and primarily FSH in the cattle industry is quite prevalent in superovulation protocols and as possible treatments for conditions inhibiting reproduction such as cystic ovaries.

While short term goals have been identified, the scope of this technology can benefit in the production of many other hormones (specifically the glycoproteins) in

other species whether it is for agriculture or preservation. Further recombinant diagnostic tools could be considered for veterinary as well as human medicine. The ability to design specific recombinant proteins could also provide the basis for creating antibodies to develop better hormone assays.

From a business perspective, improvements in glycosylation will occur possibly producing a more potent ligand resulting in requiring a smaller dose for a physiologic response. Other production consideration addresses the concerns that the metabolic strain on the insect cells to produce a mammalian glycoprotein might inhibit the insect cells from proper growth and stability. A new cell line (SfSWT-5) has been developed to help address those metabolic concerns (Aumiller et al., 2011). As baculovirus technology advances towards the goal of producing fully glycosylated, recombinant mammalian protein hormones at a high yield rate, the impact of this system will continue to affect many species in many fields of physiology.

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APPENDIX A

IN VITRO DATA

A1. Primary Cell Culture Data

Data Pt.	cAMP		Data Pt.	cAMP		Groups (i,j)	|Yi - Yj|	W-|Yi-Yj|
1a	0.10400		5a	0.09400		1,2	0.01925	-0.00796
1b	0.11000		5b	0.10700		1,3	0.00700	0.00429
1c	0.09300		5c	0.10400		1,4	0.01275	-0.00146
1d	0.10400		5d	0.09000		1,5	0.00563	0.00567
1e	0.09400		5e	0.09500		1,6	0.00763	0.00367
1f	0.08700		5f	0.08500		1,7	0.00525	0.00604
1g	0.10600		5g	0.09100		1,8	0.01063	0.00067
1h	0.10400		5h	0.09100				
						2,3	0.01225	-0.00096
2a	0.06900		6a	0.09500		2,4	0.00650	0.00479
2b	0.07800		6b	0.10700		2,5	0.01363	-0.00233
2c	0.10200		6c	0.11200		2,6	0.02688	-0.01558
2d	0.08000		6d	0.10100		2,7	0.01400	-0.00271
2e	0.08600		6e	0.11700		2,8	0.00862	0.00267
2f	0.07400		6f	0.11700				
2g	0.08100		6g	0.11200		3,4	0.00575	0.00554
2h	0.07800		6h	0.10200		3,5	0.00138	0.00992
						3,6	0.01463	-0.00333
3a	0.09500		7a	0.09900		3,7	0.00175	0.00954
3b	0.09100		7b	0.10000		3,8	0.00362	0.00767
3c	0.08200		7c	0.09500				
3d	0.10500		7d	0.09100		4,5	0.00713	0.00417
3e	0.09600		7e	0.08800		4,6	0.02038	-0.00908
3f	0.09000		7f	0.10100		4,7	0.00750	0.00379
3g	0.09600		7g	0.09600		4,8	0.00213	0.00917
3h	0.09100		7h	0.09000				
						5,6	0.01325	-0.00196
4a	0.08800		8a	0.09100		5,7	0.00038	0.01092
4b	0.08600		8b	0.10400		5,8	0.00500	0.00629
4c	0.09800		8c	0.08800				
4d	0.09000		8d	0.09200		6,7	0.01288	-0.00158
4e	0.09000		8e	0.08200		6,8	0.01825	-0.00696
4f	0.08600		8f	0.08700				
4g	0.08300		8g	0.08900		7,8	0.00537	0.00592
4h	0.07900		8h	0.08400				

A2. Static Tissue Cultre Data

Data Pt.	wash	wash	wash	wash	tsh	tsh	tsh	wash	wash	wash	forsk	forsk	wash	wash
1a	35.9	19	16	15.5	11.3	14.7	10.7	12.8	13.1	8.7	8.5	7.8	8.3	9.8
1b	31.5	22.1	16.6	17.6	17.7	17.1	15.9	15.4	14.7	13.6	10.5	10.4	11.7	9.6
1c	33.9	22.2	16.8	22.1	16.2	17.6	16.9	18.9	15.3	15.8	9.2	9.2	8.8	10.8
1d	33.5	24.4	18.6	18.7	16.9	16.7	17.9	15.7	13.9	13.2	9.6	10.6	8.1	8.3
1e	59.4	19.9	15.2	20.2	18.5	14.8	11.6	12.6	11.1	9	9.2	9.7	7.7	8.1
2a	71.2	31.7	21.7	25.9	23.9	20.5	13.3	18.4	11.5	9.8	10.7	8.5	11.8	8.2
2b	46.8	46.1	29.4	21.3	21.7	14.8	14.7	11.1	11.6	9.8	10.4	8.5	9.3	8.3
2c	40.3	22.3	28.4	21.9	22.8	16.7	16.8	12.1	10.3	12.7	14.2	9.2	10.3	8
2d	29.5	22.8	30	31.2	18.7	14.5	16.1	12.9	11.4	10	9.8	9	9.8	7.9
2de	50.5	19.9	16.7	20.6	16.8	10.7	10.7	14.3	13.6	10.2	5.8	6.3	6.3	6.3
3a	30.1	12.8	13.1	11.1	8.6	9.8	8.4	7.4	7.4	6.5	6.3	6	6.4	6.3
3b	31	22.4	23.7	13.6	12.7	16.4	12.8	11.1	10.5	9.6	8.5	7.7	7.5	7.1
3c	29.4	31.5	30.9	24.3	28.4	25.1	21.3	15.8	15.4	16.2	11.7	12.5	12.7	9.3
3d	24.1	28.6	25.5	26.4	23.4	24.9	16.5	19.1	12.4	13.6	12.7	10.2	9.5	7.6
3e	31.6	26.1	20.2	20.6	15.4	12.8	14.4	11.5	10.1	7.8	8	7.9	10	6.2
4a	24.3	17.2	13.7	13	13.3	6.7	11.2	10.6	9.6	10.2	8.6	8.8	8.9	7.6
4b	23.5	15.8	11.4	15.2	13.7	12	13.1	11.7	10.4	11.2	8.8	7.4	6.1	7.6
4c	14.2	11.3	9	13.1	11.3	9.5	13	10	9.2	9.3	10	9	7.5	8.1
4d	34.1	30.6	25.3	18.7	21.1	18.5	17.9	14.8	9.7	11.6	11.3	10.3	9.6	8
4e	28.7	16.4	13.2	13.3	12.5	10.3	11.5	11.9	10	10.4	9.1	9.1	10.8	8.3
p-values:	0.015	0.21	0.022	0.027	0.245	0.189	0.919	0.427	0.03	0.764	0.93	0.68	0.897	0.022
Groups (i,j)	|Yi-Yj|													
1,2	8.82	7.04	8.6	5.36	4.66	0.74	0.28	1.32	1.94	1.56	0.78	1.24	0.58	1.58
1,3	9.6	2.76	6.04	0.38	1.58	1.62	0.08	2.1	2.46	1.32	0.04	0.68	0.3	2.02
1,4	13.88	3.26	2.12	4.16	1.74	4.78	1.26	3.28	3.84	1.52	0.16	0.62	0.34	1.4
2,3	18.42	4.28	2.56	4.98	3.08	2.36	0.36	0.78	0.52	0.24	0.74	0.56	0.28	0.44
2,4	22.7	10.3	10.72	9.52	6.4	4.04	0.98	1.96	1.9	0.04	0.62	0.62	0.92	0.18
3,4	4.28	6.02	8.16	4.54	3.32	6.4	1.34	1.18	1.38	0.2	0.12	0.06	0.64	0.62
Groups (i,j)	W-|Yi-Yj|													
1,2	10.05039	6.572158	1.26946	2.537085	4.244522	7.492067	5.8756	4.313505	1.338249	3.266472	3.046982	1.629933	3.033476	0.141131
1,3	9.270393	10.85216	3.82946	7.517085	7.324522	6.612067	6.0756	3.533505	0.818249	3.506472	3.786982	2.189933	3.313476	-0.29887
1,4	4.990393	10.35216	7.74946	3.737085	7.164522	3.452067	4.8956	2.353505	-0.56175	3.306472	3.666982	2.249933	3.273476	0.321131
2,3	0.450393	9.332158	7.30946	2.917085	5.824522	5.872067	5.7956	4.853505	2.758249	4.586472	3.086982	2.309933	3.333476	1.281131
2,4	-3.82961	3.312158	-0.85054	-1.62292	2.504522	4.192067	5.1756	3.673505	1.378249	4.786472	3.206982	2.249933	2.693476	1.541131
3,4	14.59039	7.592158	1.70946	3.357085	5.584522	1.832067	4.8156	4.453505	1.898249	4.626472	3.706982	2.809933	2.973476	1.101131

A3. Perifusion Data

Minutes:	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180
1a	9.2	10.9	7.1	5.9	5.2	4.9	6.1	5.8	5.5	4.7	4.2	4.2	3.8	4	5.1	9.2	6.9	5.4
1b	12	9.8	7.4	5	4.1	4.1	4.8	4.3	3.7	3.3	3.2	3.3	3	2.9	3.7	3.7	2.7	2.9
1c	15	14.8	8.9	7	6.1	6.6	7.1	5.3	5.3	5.5	5.4	5.3	5.4	6.6	5.2	4.5	4.7	4.7
1d	14.4	8.8	6.9	6.4	6.1	6.1	6.5	6.3	5.4	5.5	4.9	4.8	4.4	5.1	5.4	4.7	4.2	4.1
2a	15	9.9	6.3	5.3	5.2	5.2	6	7.2	4.8	4.8	4.7	4.9	5.7	4.8	5.8	5.5	3.8	3.5
2b	15	3.9	2.5	2.4	2.4	2.5	3.1	3.2	2.8	2.4	2.7	2.7	3	2.9	3.7	3.6	2.8	2.6
3a	15	5.6	5.9	6.4	5.9	6.4	6.6	10	6.2	4.8	4.6	4.3	4.3	4.2	5.2	6	4.9	4.4
3b	12	12.5	4.5	4.6	4.2	4.2	4.1	7.3	6	4.7	4.3	3.6	3.1	3.3	4.2	4.5	4.9	6.1

APPENDIX B

IN VIVO DATA

B1. Rodent Data @ 5000 ng TSH

Data Pt.	T4		Groups (i,j)	|Yi - Yj|	W-|Yi-Yj|		TSH		Groups (i,j)	|Yi - Yj|	W-|Yi-Yj|
1a	4.17000		1,2	2.23500	-1.07817		0.075		1,2	1.413	0.018101
1b	3.81000		1,3	1.49300	-0.33617		0.046		1,3	0.1497	1.281401
1c	2.33000						0.051				
1d	2.38000		2,3	0.74200	0.41483		0.04		2,3	1.2633	0.167801
1e	2.17000						0.072				
1f	2.25000						0.049				
1g	3.17000						0.043				
1h	3.09000						0.043				
1i	2.60000						0.071				
1j	3.61000						0.05				
2a	6.61000						0.067				
2b	3.90000						0.123				
2c	5.87000						1.97				
2d	5.00000						2.8				
2e	3.23000						1.83				
2f	6.60000						1.42				
2g	6.60000						1.44				
2h	3.69000						2.32				
2i	7.69000						1.3				
2j	2.74000						1.4				
3a	6.09000						0.328				
3b	4.89000						0.225				
3c	3.71000						0.27				
3d	4.17000						0.126				
3e	4.71000						0.165				
3f	4.22000						0.153				
3g	5.04000						0.156				
3h	5.70000						0.341				
3i	1.59000						0.047				
3j	4.39000						0.226				

B2. Rodent Data @ 10,000 ng TSH

Data Pt.	T4		Groups (i,j)	|Yi - Yj|	W-|Yi-Yj|		TSH		Groups (i,j)	|Yi - Yj|	W-|Yi-Yj|
1a	4.17000		1,2	3.66900	-1.26978		0.075		1,2	1.0978	-0.60154
1b	3.81000		1,3	2.79800	-0.39878		0.046		1,3	0.7238	-0.22754
1c	2.33000						0.051				
1d	2.38000		2,3	0.87100	1.52822		0.04		2,3	0.374	0.122258
1e	2.17000						0.072				
1f	2.25000						0.049				
1g	3.17000						0.043				
1h	3.09000						0.043				
1i	2.60000						0.071				
1j	3.61000						0.05				
2a	3.04000						1.3				
2b	3.96000						1.1				
2c	7.97000						1.68				
2d	7.64000						1.19				
2e	7.44000						1.69				
2f	8.10000						0.031				
2g	8.16000						0.177				
2h	9.77000						2.16				
2i	3.59000						1.09				
2j	6.60000						1.1				
3a	2.03000						0.149				
3b	2.36000						0.097				
3c	2.12000						0.176				
3d	5.24000						0.774				
3e	9.42000						1.46				
3f	6.58000						1.51				
3g	7.97000						0.893				
3h	6.76000						0.528				
3i	8.21000						1.37				
3j	6.87000						0.821				

B3. Canine Data

Data Pt.	2 hr post	4 hr post	6 hr post	8 hr post
1a	1.79	1.57	1.28	1.25
1b	1.55	1.47	1.45	1.34
1c	1.07	1.17	1.52	1.98
1d	1.69	1.65	1.98	2.23
2a	3.97	6.62	6.88	6.75
2b	2.86	4.47	5	4.59
2c	3.97	6.3	6.65	6.5
2d	2.94	4.2	3.86	2.97
3a	1.84	3.92	4.03	2.65
3b	2.81	3.46	2.9	2.76
3c	2.95	3.69	2.73	2.12
3d	3.5	5.48	5.25	4.19
Groups (i,j)	|Yi-Yj|			
1,2	1.91	3.9325	4.04	3.5025
1,3	1.25	2.6725	2.17	1.23
2,3	0.66	1.26	1.87	2.2725
Groups (i,j)	W-|Yi-Yj|			
1,2	-0.79182	-2.16056	-1.90816	-1.17681
1,3	-0.13182	-0.90056	-0.03816	1.095685
2,3	0.458179	0.511938	0.261844	0.053185

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