Tissue Specific Regulation of the Extent and Timing of Thyroid Hormone Responses during Amphibian Development.

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ABSTRACT

TISSUE SPECIFIC REGULATION OF THE EXTENT AND TIMING OF THYROID HORMONE RESPONSES DURING AMPHIBIAN DEVELOPMENT

by

MICHELLE WOLFE

Chairperson: Professor David Jennings

There are two main patterns of development within animals: direct and indirect. Direct developers are animals such as humans, whose offspring are basically smaller versions of the adult. Indirect developers have a larval stage that can be dramatically different than the adult, and consequently go through a transformation known as metamorphosis. Frogs are a well-known example of vertebrate indirect development, developing first into an aquatic, herbivorous tadpole that later transforms into a terrestrial, carnivorous frog. This transformation is largely regulated by a single hormone - thyroid hormone (TH). Changes in TH play a vital role in tissue transformations such as, differentiation and growth of the limbs, remodeling of the gut tube and liver, as well as resorption of larval features such as the tail. In addition, the timing of metamorphic changes can differ substantially among species. For instance, the average rate of metamorphosis for a majority of frogs/toads (anurans) is between 3-5 weeks, yet there are a multitude of frogs and toads that take several months or even years to go through this process (Duellman and Trueb, 1994; Gilbert, 2010; Petranka, 2007; Provenzano and Boone, 2009). At

the other extreme are frogs that have reduced or even eliminated the free-living larval period such as the Eastern Spadefoot toad, *Scaphiopus holbrookii*, which has one of the shortest larval periods found in metamorphosing frogs or direct developing frogs, like *Eleutherodactylus coqui* which have no free-living larval stage. This diversity of developmental patterns sparks many questions about the precise molecular and developmental roles TH has on metamorphosis in frogs. How can a single regulator produce such a wide range of responses, not only among species that differ in metamorphic timing, but also among tissues within a single individual? The vast majority of recent studies that examine tissue specific responses to TH have focused on the genes that code for TH binding proteins or for TH receptors (Buchholz et al., 2011; Hollar et al., 2011). However, a very important aspect is being overlooked in these studies, which is the actual level of the TR proteins themselves.

There are three distinct mechanisms that regulate tissue responses to TH: deiodinase enzymes, cytosolic thyroid hormone binding proteins (CTHBPs), and thyroid hormone receptors (TRs) (Buchholz *et al.*, 2006; Morvan-Dubois *et al.*, 2008). The objective of the current study is to develop a technique that will allow protein level analysis of two of the three components implicated in the regulation of tissue specific responses to TH during tadpole metamorphosis: CTHBPs and TRs. I hypothesize that changes in the affinity and/or capacity of thyroid hormone receptors and cytosolic thyroid hormone binding proteins to bind TH throughout metamorphosis underlie the timing and extent of tissue remodeling. In fresh tissue, saturation binding assays suggest a difference in binding capacity among tissues.

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CHAPTER I

INTRODUCTION

Throughout an organism's life, development never ceases. The entire life cycle of an organism involves all stages of growth and differentiation between fertilization and death. During development organisms are constantly generating new cells such as epithelium, blood cells, muscle cells, neurons and lymphocytes (Gilbert, 2010). Despite this life-long process, most developmental studies focus on early development and the initial formation of adult anatomy. In animals there are two main categories of development: direct and indirect. Direct developers are animals, including humans, where the juvenile is a smaller version of the adult. In contrast, indirect developing species go through a dramatic transformation, known as metamorphosis, from a larval stage into an adult (Gilbert, 2010; Laudet 2011). Among vertebrates, the most well-known indirect developers are amphibians, particularly frogs (anurans). During metamorphosis the aquatic, herbivorous tadpole becomes a terrestrial, carnivorous frog.

Metamorphosis of a larval tadpole into a juvenile frog is mainly regulated by a single hormone - thyroid hormone (reviewed in Buchholz *et al.*, 2006; Buchholz *et al.*, 2011; Denver, 2013; Tata, 2006). Changes in thyroid hormone (TH) play a vital role in tissue transformation and sensitivity, regulating *de novo* growth and differentiation of limbs, remodeling of the gut tube and liver as well as larval tissue death and degeneration of the tail (Laudet, 2011). During this metamorphic transition these tissue specific responses occur at different times. For instance,

relatively early in metamorphosis is limb formation, followed by remodeling of internal organs such as the liver, lastly the tail will degenerate.

Throughout metamorphosis each organ has a unique set of genes that are induced by TH, that are specifically associated with histological changes occurring in the tissues. In addition, TH is even responsible for changes in gene expression of tissues that do not appear to go through morphological changes, such as, the liver and kidneys (Buchholz *et al.*, 2011; Kulkarni *et al.*, 2010; Kulkarni and Buchholz, 2013). Even more intriguing, the timing of metamorphic changes can differ substantially among species. For example, the average rate of metamorphosis for the majority of anurans is between 3-5 weeks, yet there are a multitude of frogs and toads that take several months or even years to go through metamorphosis (Duellman and Trueb, 1994; Gilbert, 2010; Petranka, 2007; Provenzano and Boone, 2009). At the other extreme are frogs that have reduced or even eliminated the free-living larval period such as the Eastern Spadefoot toad, *Scaphiopus holbrookii*, which has one of the shortest larval periods found in metamorphosing frogs (Hollar *et al.*, 2011) or direct developing frogs, (*e.g.*, *Eleutherodactylus coqui*) which have no free-living larval stage and the embryo directly forms adult features (Callery and Elinson, 2000; Elinson, 2013; Jennings and Hanken, 1998; Kulkarni *et al.*, 2010). The diversity of developmental timing and life history patterns sparks questions about the precise molecular and developmental roles TH has on metamorphosis in frogs (Buchholz *et al.*, 2011; Elinson, 2013; Gomez-Mestre *et al.*, 2012; Kulkarni et *al.*, 2010). How can a single regulator produce such a wide range of responses, not only between species that differ in metamorphic timing, but also among tissues

within a single individual? The variation seen among extant anurans provide natural experiments that can be used to examine evolutionary changes in tissue responses to TH, and how these changes contribute to metamorphic diversity.

There are three distinct mechanisms that regulate tissue responses to TH: deiodinase enzymes, cytosolic thyroid hormone binding proteins (CTHBPs), and thyroid hormone receptors (TRs; Buchholz *et al*., 2006; Morvan-Dubois *et al*., 2008). Deiodinases are intracellular enzymes involved in the activation or deactivation of thyroid hormones. While deiodinase enzymes are important, they are beyond the scope of this study and require different analytical techniques than CTHBPs and TRs (Brown, 2005). CTHBP's have been proposed to regulate TH actions by altering the availability of TH within cells. The role of CTHBPs in metamorphosis of frogs is still largely speculative as metamorphic changes in CTHBP levels and actions have not been reported (Buchholz *et al.*, 2011; Yamauchi and Tata, 1997). In contrast, thyroid hormone receptors have been demonstrated to play a crucial role in tissue responses to TH because they bind to DNA and regulate specific genes. In vertebrates there are two distinct types of TR: TR α and TR β. Each form of TR is expressed at different times and in different tissues during metamorphosis suggesting they are important for tissue specific responses to TH (Buchholz *et al*., 2006; Chan and Privalsky, 2009; Cheng *et al*., 2010).

The vast majority of recent studies that examine tissue specific responses to TH have focused on the genes that code for TH binding proteins or for TH receptors (Buchholz *et al.*, 2011; Buchholz and Hayes, 2005; Hollar *et al.*, 2011). However, a very important aspect is being overlooked in these studies, which is the actual level

of the TR proteins themselves. Previous studies rely on the assumption that changes in messenger RNA (mRNA) levels are directly related to changes in protein levels. Yet this assumption may not be valid because there are multiple regulatory steps, such as localized translation of mRNAs, cytoplasmic polyadenylation and transcript clearance that can alter the relationship between mRNA and protein levels (Jung *et al.*, 2011; Walser and Lipshitz, 2011; Villalba *et al.*, 2011).

The overall objective of the current study is to develop a technique that will allow protein level analysis of two of the three components implicated in the regulation of tissue specific responses to TH during tadpole metamorphosis: CTHBPs and TRs. This will help determine whether TH binding characteristics of CTHBPs and TRs vary among tissues and developmental stages. Thus, I am looking at the nuclear and cytoplasmic components of this complex but precise relationship. I hypothesize that changes in the affinity and/or capacity of thyroid hormone receptors and cytosolic thyroid hormone binding proteins to bind TH throughout metamorphosis underlie the timing and extent of tissue remodeling.

CHAPTER II

REVIEW OF LITERATURE

General Concepts

In vertebrates the endocrine system maintains homeostasis and ensures that all bodily functions are carried out efficiently and effectively. The endocrine system secretes hormones directly into circulation rather than using ducts. A hormone is a mediator molecule released in one part of the body that regulates activity of cells in other parts (Hadley and Levine, 2007). Hormones help control specific target tissues, not all cells in the body, by interacting with receptors. Hormone receptors are protein molecules located either on the surface of a cell or in the interior of the cell. The ultimate function of these protein receptors is to identify, bind and induce the biological actions of a specific hormone in a particular target cell and/or tissue (Hadley and Levine, 2007).

Thyroid hormone receptors are internal, which can be found in the cytoplasm or nucleus. In addition, TH may bind other proteins in the cytoplasm known as cytosolic thyroid hormone binding proteins which will eventually transport TH to the nucleus. Once transported, the activated nucleoplasmic receptor must translocate to the chromatin. The binding of the thyroid hormone receptor complex to chromatin results in de-repression of specific DNA sequences that are transcribed into mRNA. Finally the mRNA is translated in the cytoplasm and codes for a functional protein specific to that cell type resulting in an altered gene expression (Figure 2).

Figure 1. Central Dogma. First DNA sequences (genes) are transcribed into an intermediate (mRNA) which is then translated to form a functional protein.

Conservation of TH pathway in vertebrates

Thyroid hormone plays a significant role in the development, regulation and metabolism of vertebrates, and abnormal thyroid activity underlies a number of human pathologies (Das *et al*., 2010). An observation in 1912 helped demonstrate the importance TH has in regulating post-embryonic growth and development in all vertebrates. In this experiment a mammalian thyroid gland extraction triggered precocious metamorphosis in tadpoles (reviewed in Tata, 2012). Thyroid hormones are conserved across a wide range of vertebrates from the most primitive chordates to humans. In addition, all vertebrates have two forms of thyroid receptors, TRα and TRβ which are structurally and functionally highly conserved from frog to man (Furlow and Neff, 2006; Hollar *et al.*, 2011). Another similarity found in both human and anuran development are the high levels of TH throughout the postembryonic stage and all vertebrates including frogs have a peak in TH at some point throughout their lifetime (Buchholz *et al.*, 2011; Das *et al.*, 2010). These significant findings make frogs a great model for researchers, allowing them to be: 1) to examine the developmental actions of TH within vertebrates; 2) address issues pertaining to

tissue specific responses to TH; and 3) better understand the hypothalamic-pituitary-thyroid axis (Das *et al.*, 2010; Tata 2006).

Hypothalamus and pituitary gland

There are multiple mechanisms involved within thyroid hormone physiology; both central and peripheral systems are influenced by neuroendocrine control (Figure 3). In vertebrates the hypothalamus and the pituitary gland make up the neuroendocrine system which controls activity of the rest of the thyroid axis during metamorphosis (Buchholz *et al.,* 2011). In amphibians, the initial systemic response is controlled by environmental signals regulating secretions of corticotropin-releasing hormone (CRH) in larval stages; whereas, thyrotrophin-releasing hormone (TRH) is the primary neuroendocrine regulator of adult frogs. In larvae, CRH communicates with the anterior pituitary stimulating it to secrete both thyroid stimulating hormone (TSH) and adrenocorticotropic hormone (ACTH; Laudet, 2011). The pituitary develops during premetamorphic stages, after hatching, but prior to formation of the larval thyroid gland (Denver, 2013). The pituitary gland is a critical regulator of metamorphosis as removal of the pituitary stops thyroid function completely because there is no TSH production (Brown, 2005; Huang *et al.*, 2001).

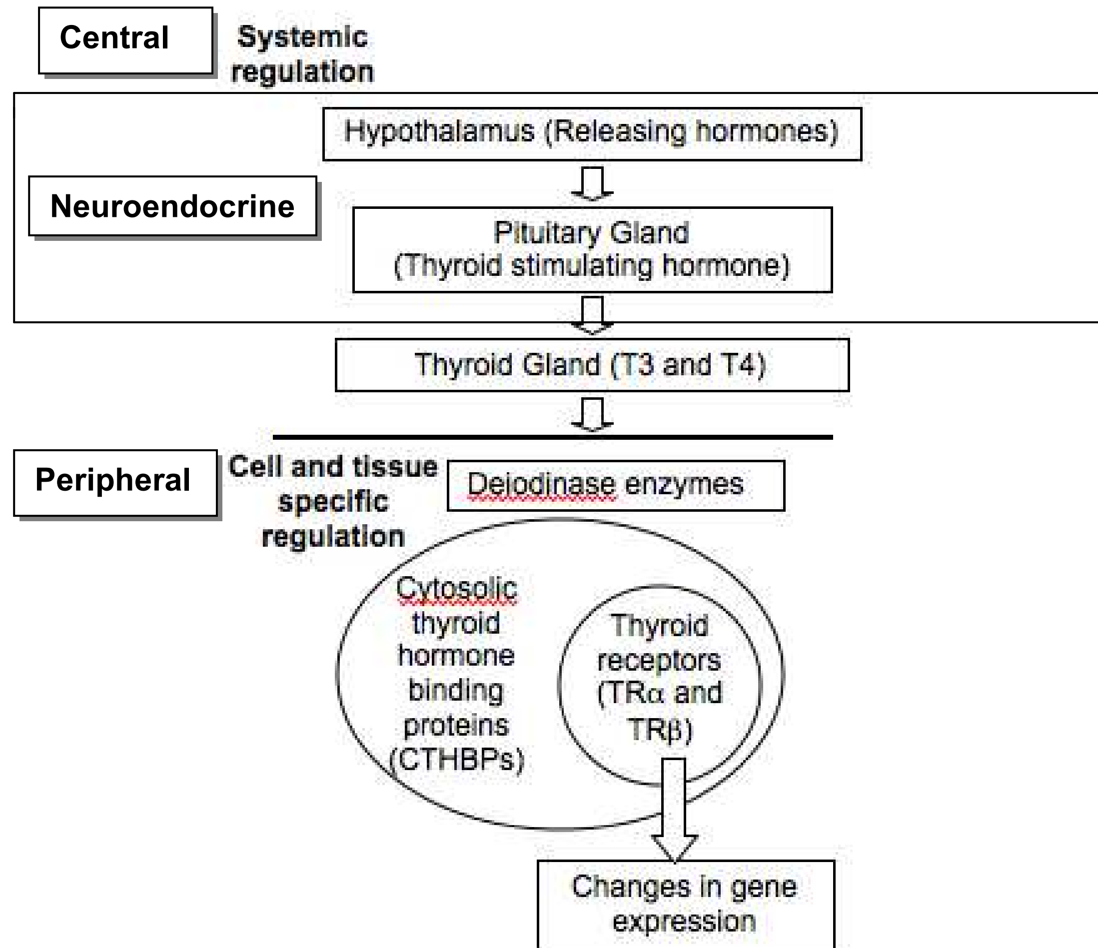

Figure 2. Diagram of systemic regulation/cell and tissue regulation. Systemic regulation (top) and cell and tissue regulation (bottom) of the thyroid axis in vertebrates

Once released by the pituitary both TSH and ACTH act on peripheral glands that produce hormones that regulate the onset and rate of metamorphosis. TSH is a key regulator that assists in the growth, synthesis and release of hormones in the thyroid. ACTH acts on the interrenal glands within tadpoles to synthesize and secrete a stress hormone known as corticosterone (CORT). Interrenal glands are homologous to the adrenal glands in mammals (Denver, 2013). Both CORT and TH

help the brain and pituitary mature while simultaneously exerting feedback on the hypothalamus and pituitary (Buchholz *et al.*, 2011; Denver, 2013).

Thyroid gland

The thyroid gland begins to develop in the amphibian embryo and, by the time of hatching is functionally mature (Denver, 2013). The thyroid produces two distinct forms of TH, thyroxine (T4) and 3, 5, 3'-triiodothyronine (T3), that are released into circulation (Figure 4). Both forms of TH are lipid soluble hormones that readily enter target tissues and bind to internal receptors. T4 is the main product of the thyroid gland found in the bloodstream and is converted into T3, in peripheral tissues (Buchholz *et al.,* 2011; Cai and Brown, 2004; Huang *et al.*, 2001; Tata, 2013). T3 is considered the active hormone in vertebrates as it has 10-15 times higher affinity for TH receptors than does T4 (Buchholz *et al.,* 2011; Denver, 2013). Thyroid hormones have a crucial function in establishing tissue specific responses and timing during metamorphosis. Inhibition of endogenous TH synthesis or binding to receptors effectively blocks metamorphic changes; whereas, accumulation of exogenous TH will stimulate metamorphosis sooner than normal (Cossette and Drysdale, 2004). Both T3 and T4 can change the dynamics of metamorphosis. For example, adding low levels of exogenous T4 to premetamorphic tadpoles induces normal morphological changes, while adding low amounts of exogenous T3 in early and late events occurring simultaneously (Cai and Brown, 2004).

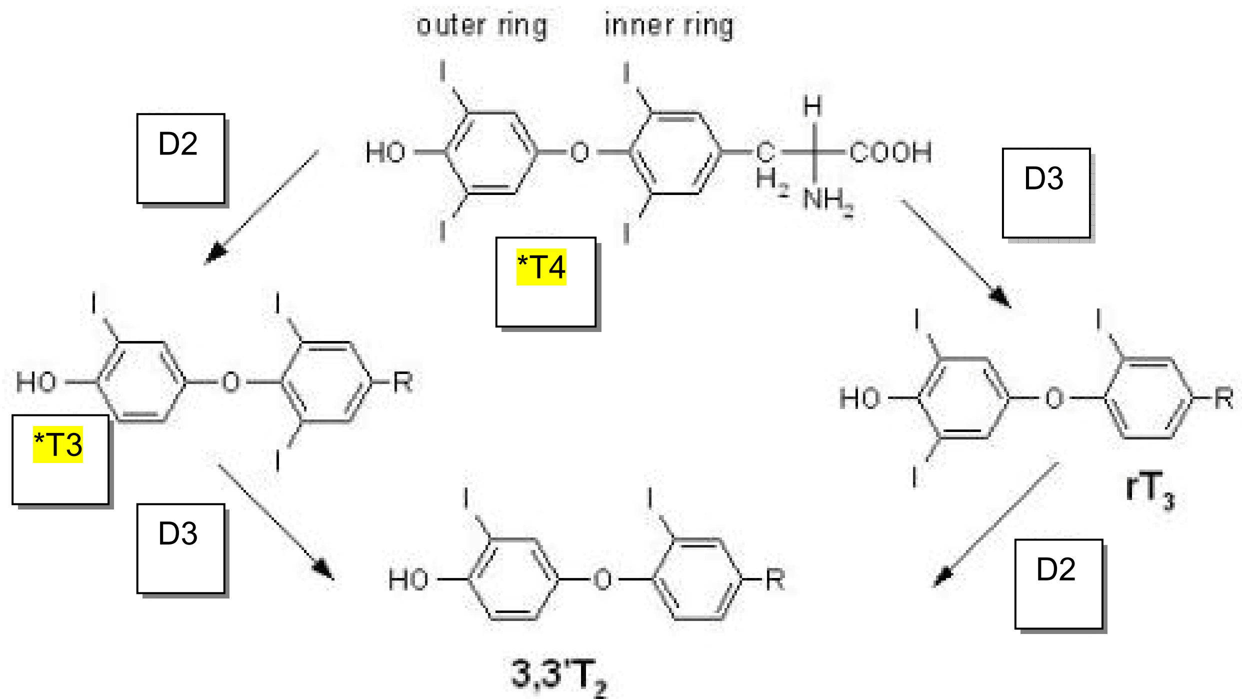

Figure 3. Overview of the deiodination of thyroid hormones. Structural formulas of thyroxine (T4), tri-iodothyronine (T3), di-iodothronine (T2) and reverse T3 (rT3). D2 activates thyroid hormone by removing an iodine molecule from the outer ring to convert T4 into the more active T3, whereas D3 inactivates thyroid hormone by removing an iodine molecule from the inner ring converting T3 into T2. T4 can be converted into rT3 when D3 eliminates an iodine molecule from the inner ring. Finally, rT3 can be converted into T2 when D2 eliminates an iodine molecule from outer ring.

*Two main hormones involved in amphibian metamorphosis.

Modified from http://upload.wikimedia.org/wikipedia/commons/a/a5/Iodothyronine_deiodinase.png

Cell and tissue specific regulation

During amphibian metamorphosis there are three distinct mechanisms that potentially regulate tissue responses to TH: deiodinase enzymes, cytosolic thyroid hormone binding proteins (CTHBPs) and thyroid receptors (TRs) (Buchholz *et al.,* 2006; Morvan-Dubois *et al.,* 2008). Deiodinases are intracellular enzymes involved in the activation or deactivation of thyroid hormones. Once in the cytoplasm, TH can

then bind to CTHBPs or move into the nucleus and bind to TRs. Which protein TH binds to dramatically influences the process of metamorphosis. Binding to CTHBPs alters the availability of TH, while binding to TRs can directly alter the rate and specificity of gene transcription (Figure 5).

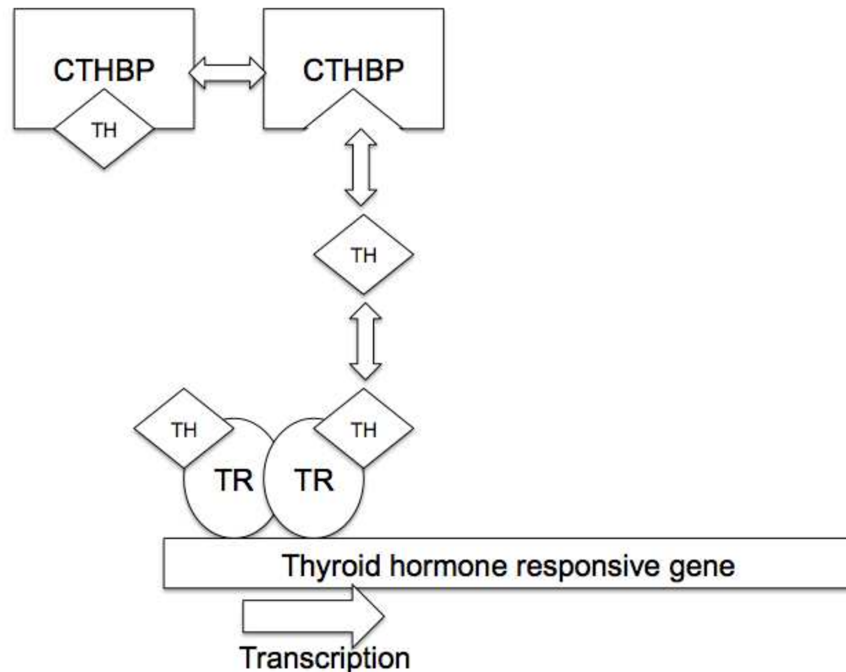

Figure 4. Thyroid hormone interactions with CTHBPs and TRs.

Deiodinase and its role with TH

In vertebrates, there are three types of deiodinases (types I, II, and III). The three types differ in how they interact with TH and their tissue distribution during development (Brown, 2005; Buchholz *et. al.*, 2011; Denver, 2013). Both type II and type III have been detected in tadpole tissues; however type I enzyme activity has not. Adult frogs have a type I gene however little is known about its role and expression (Denver, 2013).

The two main deiodinases found in tadpole tissues, type II iodothyronine deiodinase (D2) and type III iodothyronine deiodinase (D3) have tissue and developmental stage specific expression patterns. D2 and D3 help regulate intracellular T3 concentrations and their expression directly correlate with the asynchronous morphological changes seen in tissues throughout metamorphosis (Denver, 2013). D2 activates thyroid hormone by removing an iodine molecule from the outer ring to convert T4 into the more active T3, whereas D3 eliminates an iodine molecule from the inner ring of the hormone causing inactivation of the hormone through formation of reverse T3 (Figure 4;Cai and Brown, 2004).

D2 and D3 expression is correlated with the onset of metamorphic tissue changes which are divided into early and late events depending on how they respond to concentrations of TH. Early induction by low plasma concentrations of TH causes development of the limbs, brain, and the spinal cord; whereas, late events including remodeling of the intestines, gill and tail resorption as well as the progress of the negative feedback loop in the pituitary are induced by high plasma TH (Brown, 2005; Denver, 2013). D2 expression is seen in the earliest tissues that will grow and differentiate, such as the limbs. D2 controls a negative feedback loop between the thyroid and pituitary gland (Brown, 2005; Cai and Brown, 2004; Huang *et al.*, 2001; Opitz and Kloas, 2010). In addition, D2 is expressed in late-responding tissues that remodel or die such as the gut tube and tail (Cai and Brown, 2004). For instance, D2 activity emerges in the tail at the late climax stage in *X. laevis* right before tail resorption. At the climax of metamorphosis, D2 activation is exclusive to the TSH-producing cells within the anterior pituitary gland. Increased D2 activation

produces a high enough concentration of T3 that suppresses TSH synthesis. This establishment of a negative feedback loop helps orchestrate the end of metamorphosis (Brown 2005; Huang *et al.*, 2001).

D3 protects cells from responding to thyroid hormone by converting T4 to rT3 or T3 to T2. The clearest demonstration of this protective role is seen in the process of tail resorption. D3 reaches a peak before the climax stage then falls dramatically to a much lower level when TH concentration is highest. This suggests that D3 protects the tail from the rise in TH during both premetamorphic and prometamorphic stages. Once the D3 activity drops at the very end of metamorphosis, the tail degenerates, as TH is not inactivated (Brown, 2005; Denver, 2013).

D2 and D3 have a balanced and interactive role which coordinates the precise timing of metamorphic changes (Brown, 2005; Huang *et al.*, 2001). There is a shared relationship between D2 and D3 in the role of tail resorption such that D2 is up-regulated and D3 is down-regulated during the end of metamorphosis. This same process is seen in the development of the liver as well (Brown, 2005; Cai and Brown, 2004). Deiodinases are significant regulators in amphibian metamorphosis because they play a part in establishing tissue competence by controlling the availability of T3 at the tissue level. However, TH availability is not the entire story and there are two other regulatory levels that strongly impact tissue responses; cytosolic thyroid hormone binding proteins and nuclear thyroid hormone receptor proteins. Ultimately, TH acts rapidly through their receptor (TRs) which leads to tissue specific changes in gene expression.

CTHBPs

Cytosolic thyroid hormone binding proteins are thought to play a role in the process of TH-regulated gene transcription. CTHBPs are multifunctional proteins that have a 10- to 100-fold weaker binding affinity for TH than TRs. CTHBPs have been reported in both mammals and amphibians (Buchholz *et al.*, 2011; Shi *et al.*, 1996). There are three types of CTHBPs that have been identified in the frog *X. laevis*: aldehyde dehydrogenase 1, pyruvate kinase subtype M2 and protein disulfide isomerase (Yamauchi and Tata, 1997). One role of CTHBPs is to regulate the bioavailability of TH and/or transport of TH to the nucleus. Once inside a cell TH can bind either to cytoplasmic CTHBPs or to nuclear TRs, leading to differing effects. Binding to CTHBP reduces the amount of TH available in the cell and potentially slows the rate of tissue response. In contrast, TH binding to TRs enhances tissue responses since the TH-TR complex binds to DNA and turns on specific TH responsive genes resulting in alteration of gene expression (Hollar *et al.*, 2011). Overall, the function and timing of cytosolic thyroid hormone binding proteins remains unclear (Buchholz *et al.*, 2011; Shi *et al.*, 1996; Yamauchi and Tata, 1997). If CTHBPs are important for tissue specific responses, their levels should differ among tissues and should be correlated with the timing of metamorphic changes. For instance, an increase in CTHBPs affinity or capacity would be predicted in tissues not changing, whereas a decrease in affinity or capacity would be predicted in tissues that are responding.

Thyroid receptors: TR α and TR β

Thyroid receptors are ligand-dependent transcription factors that belong to the nuclear hormone receptor superfamily of transacting zinc-finger proteins (Bilesimo *et al.*, 2011; Cossette and Drysdale, 2004; Das *et al.,* 2010; Furlow and Neff, 2006; Hadley and Levine, 2007). Proteins of this superfamily have three common components; a DNA binding domain positioned in the amino terminal half, a hormone binding domain positioned in the carboxyl terminal half and interaction domain located in between (Shi *et al.*, 1996). TRs regulate target genes by binding to TH response elements (TREs) which are *cis*-acting DNA sites. TRs can bind to TREs as monomers, homodimers and heterodimers. However, TRs bind best to TREs as a heterodimer of another nuclear receptor family, retinoic X receptor (RXR) which is stimulated by 9-*cis* retinoic acid (Cossette and Drysdale, 2004; reviewed in Furlow and Neff, 2006; Shi *et al.*, 1996). This heterodimer complex changes gene expression ultimately leading to associated morphological tissue alterations during metamorphosis (Hollar *et al.*, 2011).

The timing and rate of metamorphic changes based on TR expression has been formalized as the “dual function” model (Buchholz *et al.*, 2006; Hollar *et al.*, 2011). The general principle behind the dual function model is that in the absence of TH, TRs function to repress TH dependent gene expression, while in the presence of TH, TRs function to activate these same genes. The ability of TRs to perform these two different functions is mediated by different types of cofactors interacting with the TR (reviewed in Hollar *et al.*, 2011). TRs interact with coregulatory proteins that facilitate the transcriptional activation or repression of chromatin (Furlow and

Neff, 2006). Corepressors identified in *X. laevis* are nuclear receptor corepressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) both of which inhibit the transcription of genes containing TREs. Conversely, the steroid receptor coactivator (SRC) family identified in *X. laevis* inhibits the binding of corepressors. Expression of SRC allows for acetylation and methylation of histones and consequently stimulates transcription of TH-responsive genes. Tissue differences in coactivator recruitment potentially underlie larval tissue resorption and the formation of adult tissues (Furlow and Neff, 2006).

In vertebrates there are two forms of thyroid receptor, TR α and TR β. Interestingly both α and β isoforms in *X. laevis* are very similar to human TR proteins (over 85% amino acid identity; Furlow and Neff, 2006). While the overall molecules are highly conserved the greatest similarity is found in the two main functional domains, the ligand binding and DNA binding regions (Furlow and Neff, 2006). Each form of TR is expressed at different times and in different tissues during embryonic development and metamorphosis (Figure 6), suggesting they are important for tissue specific responses to TH (Buchholz *et al.*, 2006; Chan and Privalsky, 2009; Cheng *et al.*, 2010). TR α mRNA levels rise early in the larval period, right after hatching and continue to increase until the completion of metamorphosis. Highest levels of TRα mRNA are found in cells that proliferate, such as the limb buds, skin and head cartilages. Conversely, TR β mRNA levels are low during early larval stages, increase as TH levels rise during metamorphosis, peak at mid-metamorphosis, and then decrease at the end of metamorphosis. TRβ mRNA levels are associated closely with circulating TH levels and the highest expression is seen in tail tissue

(reviewed in Buchholz *et al.*, 2006; Cossette and Drysdale, 2004; Furlow and Neff, 2006; Tata, 2006). Changes in TR expression support the dual function model because TRα is present in early tissues/stages when expression of adult genes are repressed whereas, TRβ is expressed during later tissues/stages to activate the adult pattern gene expression.

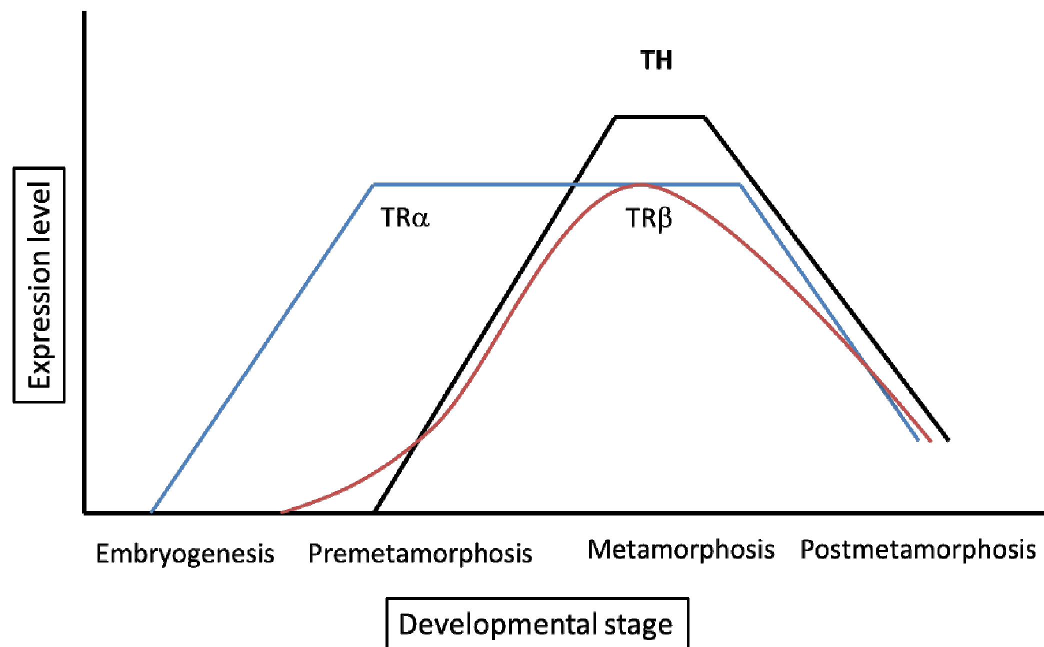

Figure 5. Expression patterns of TH and TRs through development. There are two main isoforms of TRs are TRα and TRβ. TR α mRNA levels rise early in the larval period, right after hatching and peak several days prior to the rise in TH, level off until the completion of metamorphosis and then decrease. TR β mRNA levels are low during early larval stages, increase as TH levels rise during metamorphosis, peak at mid-metamorphosis, and then decrease at the end of metamorphosis.

The importance of TR activation throughout metamorphosis has been emphasized through many experiments. However, two recent examples of this well documented pathway include a transgenic overexpression of a dominant negative

form of TR α (DNTR, Furlow and Neff, 2006). DNTR inhibits ligand-binding and prevents the recruitment of coactivators. The corepressor NCoR is maintained on the promoters of DNTR, even if TH is present, consequently keeping the target genes in a repressed state. This negatively affects many morphological changes, such as the proliferation of the jaw and brain, resorption of the gills and tail, and remodeling of the gut tube. The second approach used a synthetic TR antagonist, NH-3 which inhibits TH-induced transcription by altering receptor conformation and inhibiting binding to DNA. NH-3 treatment altered tadpole development just as effectively as DNTRs and prevented spontaneous metamorphosis (Furlow and Neff, 2006).

Ultimately, changes in the expression of thyroid receptors play a vital role in the timing and rate of amphibian metamorphosis. While there is a general relationship between whole-body levels of TR mRNAs and metamorphosis the tight relationship between tissue TR mRNA level and the onset of metamorphosis disappears when individual tissues are examined (Buchholz and Hayes, 2005; Hollar *et al.*, 2011). The lack of correspondence between tissue specific changes in CTHBP and TR mRNA levels and metamorphic changes suggest that other approaches are needed for a full analysis of how tissue responses to TH are achieved (Hollar *et al.*, 2011; Opitz *et al.*, 2006). Since mRNA levels do not necessarily correlate with protein levels, analysis of protein may provide more accurate information on tissue competence to TH (Walser *et al.*, 2011; Villalba *et al.*, 2011). Protein levels of TRs still remain largely unknown and are in need of further research.

Outstanding issues

The duration of, and size at, metamorphosis are two very important life history traits for amphibians. Variation in each of these traits has costs and benefits. For example, if the larval habitat is too dangerous due to predators or pond drying, the tadpole will undergo metamorphosis precociously in order to survive. The cost is smaller body size which might increase predation risk or harm prey capture ability in the terrestrial environment. There is a wide diversity of metamorphic life history strategies within amphibians (Duellman and Trueb, 1994). For example, there is variation in the length of larval periods. While the average rate of metamorphosis for the majority of anurans is relatively short between 3-5 weeks, there are a multitude of frogs and toads that take several months or even years to go through metamorphosis. Even within a single species or family, larval periods can vary dramatically. The larval period of the American Bullfrog, *Rana catesbeiana*, ranges from a few months in warmer areas to three years in colder climates. In spadefoot toads, the New World genera (*Scaphiopus* and *Spea*) have much shorter larval periods than a closely related Old World genus (*Pelobates*; Buchholz and Hayes, 2005; Duellman and Trueb, 1994; Gilbert, 2010; Petranka, 2007; Provenzano and Boone, 2009). The next category involves a complete loss of metamorphosis seen in salamanders such as the Mexican Axolotl, *Ambystoma mexicanum*, and the neotenic salamander, *Necturus maculosus*. These species reach sexual maturity without undergoing metamorphosis, and they remain aquatic and gilled throughout their lives (Buchholz *et al.*, 2011; Duellman and Trueb, 1994). Finally, at the other extreme are frogs that have reduced or even eliminated the free-living larval period

examples include the Eastern spadefoot toad, *Sc. holbrookii*, which has one of the shortest larval periods found in metamorphosing frogs (Hollar *et al.*, 2011) or direct developing frogs, (*e.g.*, *E. coqui*) which have no free-living larval stage and the embryo directly forms adult features (Callery and Elinson, 2000; Elinson, 2013; Jennings and Hanken, 1998; Kulkarni *et al.*, 2010).

Despite the diversity seen in amphibian life histories, relatively little work has been done on modifications to TH regulation in derived taxa. Spadefoot toads show extreme variation within their larval periods ranging from some of the shortest found in *Scaphiopus* and *Spea* species among frogs to the longest recorded in *Pelobates* species (Buchholz and Hayes, 2005). Both *Scapiopus* and *Spea* live in harsh, dry environments while *Pelobates* are found in mediterranean climates or temperate forests (Hollar *et al.*, 2011). Although there are differences in their natural environments, laboratory studies where tadpoles are reared under the same conditions demonstrate that *Sc. couchii* still have the shortest larval period compared with *Sp. multiplicata* and *Pb. syriacus* (12d vs. 16 d vs. 31d respectively; Buchholz and Hayes, 2005; Hollar *et al.*, 2011). Among these three species TH content of tail tissues was higher in species that have shorter metamorphic periods (Buchholz and Hayes, 2005). While suggestive it is possible that hormone differences within tissues do not reflect differences in blood concentration. For example, tissue-specific difference in deiodinase enzymes could result in tissue TH differences despite similar blood TH levels. Therefore, the variation seen in amphibian larval periods and its relationship to central control of TH is still unknown (Buchholz *et al.*, 2011). In contrast, there is evidence for peripheral control as a

regulator of species differences in developmental rate. Consistent with the differences in their larval periods, tail tips of three species of spadefoot toads in culture shrank at different rates after exposure to T3 (Buchholz and Hayes, 2005). Thus, peripheral control seems to act as a mechanism for achieving shorter larval periods and plays a potential role in larval period diversity (Buchholz *et al.*, 2011; Hollar *et al.*, 2011).

More detailed information is available for amphibians where either the adult stage or the larval stage has been largely eliminated (paedomorphosis vs. direct development). Axolotls are salamanders that do not naturally undergo the full set of metamorphic changes seen in most salamanders. However, axolotls can be induced to finish metamorphosis by treatment with TH (Buchholz *et al.*, 2011; Galton, 1992; Page *et al.,* 2009), whereas *Necturus maculosus* cannot undergo metamorphosis even when treated with TH (Buchholz *et al.*, 2011). Early in life Axolotls have a peak in T4 yet fail to go through metamorphosis because they lack the proper deiodinase conversion of T4 to T3 and have low TR levels. This experimental behavior seen within Axolotls is mostly likely due to central control of TH (Buchholz *et al.*, 2011). In contrast, *Necturus* have a loss of TH-induction genes seen at the peripheral level, suggesting tissue specific responses are no longer functioning at the receptor level (Buchholz *et al.*, 2011; Safi *et al.*, 2006). Direct development is a derived life history strategy that evolved at least a dozen times independently and is found in both frogs and salamanders (Elinson, 2013; Kulkarni *et al.*, 2010). The most well-studied direct developer is the species *E. coqui*, when treated with methimazole, a TH inhibitor development of the limbs, skin, intestine,

muscle and tail are incomplete (Buchholz *et al.*, 2011; Callery and Elinson, 2000). In addition, when treated with high doses of T3 *E. coqui* exhibit a low sensitivity to TH as opposed to other biphasic tadpoles such as *Xenopus* and *Rana*. For instance, doses of 2 and 20nM T3 caused premature metamorphosis in *Xenopus* and *Rana* however *E. coqui* were not affected (Kulkarni *et al.*, 2010). Endogenous TH levels in *E. coqui* are unknown, so the significance of high levels of exogenous TH in causing morphological changes should be interrupted with caution. Direct developers tissues are sensitive to TH and are contingent on TH for proper metamorphic development, whether it is central or peripheral control remains unclear (Kulkarni *et al.*, 2010).

Summary

There are still many questions to be answered about the role TH plays within anuran species. First, the diversity of developmental patterns and life history traits sparks questions about the precise molecular and developmental roles TH has on metamorphosis in frogs (Buchholz *et al.,* 2011; Elinson, 2013; Gomez-Mestre *et al.*, 2012; Kulkarni *et al.,* 2010). Even though the patterns are very different among amphibians, the control mechanisms seem relatively conserved among vertebrates (Callery and Elinson, 2000; Kulkarni *et al.*, 2010). Therefore frogs are an excellent model to examine how a conserved set of mechanisms can be altered in such a way as to produce a diversity of developmental patterns. This is a central question to all studies of development and evolution. Second, TR mRNA levels are not equal to tissue changes. The mRNA levels do not give clear results and still remain an unresolved issue (Hollar *et al.*, 2011). Since mRNA levels do not necessarily

correlate with protein levels, analysis of protein may provide more accurate information on tissue competence to TH. Lastly, the functional roles of CTHBPs are not well understood. The current study may provide tools necessary to address these outstanding questions and lead to a better understanding of the relationship between TH levels and tissue specific responses during metamorphosis in frogs with a wide range of life history strategies.

The specific aims of the current work are to determine (1) if the affinity and capacity of CTHBP and TR to bind to TH are tissue specific throughout metamorphosis, (2) if the ability of CTHBP and TR to bind TH within a tissue changes during development, (3) if changes in the ability of CTHBP and TR to bind TH underlie evolutionary diversity in metamorphic life history strategies. Radioligand binding assays are the primary method used to characterize binding parameters of a wide range of hormone binding proteins and receptors (Jennings *et al.*, 2000; Lattin *et al.*, 2012; Orchinik *et al.*, 2000).

CHAPTER III

MATERIALS AND METHODOLOGY

Animal Collection and Maintenance

Species were chosen based on local availability for field collection, and due to their standard larval period duration. Tadpoles of *Anaxyrus fowleri* were field collected in Madison County, IL on and around the Southern Illinois University Edwardsville campus (August 2011 and May-June 2012). Tadpoles were placed in tubs with 10% Holtfreters solution (10.5g NaCl, 0.15gKCL, 0.3g $CaCl_2$ and 0.6g $NaHCO_3$ in 30L of distilled water). Tubs were aerated, and tadpoles were reared at 25°C. Tadpoles were fed spinach and lettuce until they reached appropriate developmental stages.

Tadpoles of *Bombina orientalis* were obtained by induced breeding of adults maintained as a breeding colony at SIUE. To induce breeding one male and one female were removed from the colony and injected with human chorionic gonadotropin (hCG, Biotang USA). Both male and female frogs were injected into their dorsal lymph sac. Female frogs were injected with 0.12mL of hCG while males received 0.1mL. After injection frogs were held in the dark until oviposition occurred (usually within 24hrs). Once the eggs were deposited they were placed in glass bowls with 10% Holtfreters solution and aerated. Once tadpoles hatched they were feed spinach or lettuce.

The final species collected were a mix of *Hyla chrysoscelis* and *Hyla versicolor*. Tadpoles were collected in Madison County in an inactive swimming pool

(May-July 2013) and placed in tubs with 10% Holtfreters solution at 25°C. Tubs were aerated and tadpoles were fed spinach and beet greens.

Staging

All species of tadpoles were staged according to Gosner (1960). Although there are slight differences among species in developmental timing, this staging table is general for all metamorphic frogs. Each set of stages within anuran development have distinct features; stages 1-18 represent embryonic development and hatching occurs around stage 19. During stages 20-25 development of gill and tail circulation along with pigmentation. Stages 26-39 describe limb bud formation as well as toe differentiation and development. The final stages, 40-46, include forelimb emergence, mouth development, and tail resorption.

Sampling: tissue collection

Our initial assays on *Anaxyrus* and *Bombina* used frozen tissue. The *Anaxyrus* and *Bombina* tadpoles were chosen at stages where limb development is occurring through later stages where the tail is degenerating (stages 26-45). Tadpoles were anesthetized in MS222, placed under a microscope and staged. After staging, tissues responsive to thyroid hormone during metamorphosis (hindlimbs, tail, gut tube and liver) were dissected. Tissues were placed in Eppendorf tubes, labeled by tissue type and stage, and then frozen at -70°C until further processing.

Our second set of assays used fresh samples from the two *Hyla* species (*chrysoscelis* and *versicolor*). Tadpoles were anesthetized using MS222 and staged according to Gosner (1960). Specific tissues (hindlimbs, tail, gut tube, and liver)

were dissected from each tadpole, placed on ice, and then processed. For each tissue type, samples from 12-28 tadpoles were pooled In order to obtain sufficient tissue mass for analysis.

Tissue preparation: frozen samples

On the day of assay, frozen tissue samples were thawed and weighed to the nearest 0.01g. Once weighed, tissue was chopped thoroughly with a clean razor blade, and suspended in 4X volume/tissue mass of assay buffer. Several different formulations of assay buffer were tested (Table 2). Tissue was then homogenized with dounce pestle approximately 10 times and in later assays homogenized 4 times with pestle B and 2 times with pestle A. The sample was centrifuged at 100g for 2 min at 4°C. After centrifugation, the pellet containing unbroken cells was discarded and the supernatant containing nuclear and cytoplasmic components was removed to a new tube. Supernatant was centrifuged at 600g or 800g for 10 min at 4°C to pellet out cell nuclei. The nuclear fraction was then frozen at -70°C until assayed. The supernatant, containing primarily cytoplasmic components, was subjected to a second round of centrifugation 100,000g for 60 min at 4°C. The resulting supernatant, containing cytoplasmic proteins (including CTHBPs), was frozen at -70°C until assayed.

Fresh samples

On the day of the assay the pooled tissue from the *Hyla* species was weighed to the nearest 0.01g. Once weighed the tissue was chopped thoroughly with a clean razor blade, and suspended in 4X volume/tissue mass of preparation buffer (Table

1). Tissue was homogenized with a glass dounce pestle, four strokes with pestle B and two strokes with pestle A. The sample was centrifuged at 500g for 10 minutes at 4°C to remove whole cells. After centrifugation, supernatant was removed and the pellet was resuspended in the original volume of preparation buffer containing 0.5% Triton. The sample was then vortexed and the homogenate filtered through two layers of nylon mesh (215 μm and 64μm, Component Supply Co). After filtration the sample was centrifuged again at 500g for 10 min at 4°C. The resulting supernatant containing nuclear proteins (TRs) was resuspended in buffer and used for the filter binding assay.

Protein assay

Protein content of incubated tissues was measured using Bradford reagent (Sigma). Protein standards were prepared using bovine serum albumin. Protein content of each sample was used to standardize for the amount of tissue added to each incubation tube.

Chemicals

Radiolabeled thyroid hormone [I^{125}] was purchased from Perkin- Elmer (Mass.) and stored at 4°C. Unlabeled T3 was purchased from Acros Organics (New Jersey) and stored at 4°C. Unlabeled T4 was purchased from Sigma (St. Louis, MO) and kept at 25°C. The dextran-coated charcoal suspension (DCC) used to remove endogenous thyroid hormones contained 1g Charcoal (Norite neutral), 0.1g dextran per 100mL, 1.5mM $MgCl_2$. The dextran and Charcoal were purchased from Fisher

(Pittsburg, PA). The Sephadex G-25 columns and Whatman-Optitran BA-S (0.45μm) filters were purchased from GE Healthcare Life Science (Pittsburg,PA).

Assay methodology

There were several methods used to optimize specific binding (Table 2 and 3). Our initial set up was based on Breuner and Orchinik (2009) and Orchinik *et al.*, (2000). In addition, binding affinity and capacity of both cytosolic thyroid hormone binding proteins (CTHBPs) and thyroid receptors (TRs) was assayed using techniques previously used to characterize plasma and cytosolic binding of steroid hormones (Jennings *et al.*, 2000).

Binding assays followed the same general protocol, but had slightly different techniques such as buffer type, or incubation parameters (Table 2 and 3). Standard set up for all tubes included label (50ml), buffer (50μl) or buffer containing unlabeled TH (50μl), to which sample tissue (50μl) was added at the appropriate time. Buffer containing unlabeled TH was used to determine non-specific binding. Once done filling tubes for assay setup, tubes were vortexed and incubated (Table 3). All assays were carried out in triplicate. After incubation bound vs. free TH was separated by rapid vacuum filtration over GF/B and GF/C filters presoaked for 60 minutes in TEM rinse buffer with 0.3% polyethylenimine (Orchinik et al., 2000, Table 1). Following filtration, filters were washed with 6ml of ice-cold rinse buffer (Trisma base 25mM, EDTA 1mM and Molybdic acid 10mM, pH 7.4). After rinsing filter paper samples were poked out and placed into gamma counter vials then capped. Radioactivity bound to filters was measured by gamma counter (Perkin-Elmer Wizard 2). Specific binding was calculated by subtracting total binding from non-

specific binding determined from tubes containing an excess of unlabeled TH (1μM). For an assay to be considered effective non-specific binding should be 10% or less of total binding. In figures, binding is presented as mean ± 1 SEM of triplicates assayed. Since all samples represented a pool of individuals and each pool was only run once statistical comparisons are not possible among assays.

Table 1. Buffer systems used for assay incubation and washes

Buffer/ pH	Ingredients	Citation
1.TEGM with Triton pH 7.4	10mM Trisma base, 1mM EDTA, 20mM Molybdic acid and 10% glycerol	**Breuner and Orchinik, 2009; Orchinik *et al.,* 2000**
TEM Rinse pH 7.4	5mM Trisma base, 1mM EDTA and 10mM Molybdic acid	
2. BUFFER A pH 7.6	250mM sucrose, 2mM MgCl2, 20mM Tris-HCL	**Inoue *et al.,* 1983**
BUFFER B pH 7.6	50mM NaCl, 10% glycerol, 2mM EDTA, 5mM 2-mercaptoethanol and 20mM Tris HCL	
3. Wash Buffer pH 7.4	90mM Tris-HCL, 10mM MgCl2, 1mM EDTA	**Leifert *et al.,* 2009**
Isolation Buffer pH 7.4	250Mm Sucrose, 50mM HEPES and 1mM EDTA	
4. Wash Buffer pH 7.5	177mM Sucrose, 20mM Tris-HCL, 1mM MgCl2 and 0.1mM ZnCl2	**Galton and Schaafsma, 1983**
4. Wash Buffer pH 7.5	150mM Sucrose, 20mM Tris-HCL, 10mM CaCl2 and 0.1mM ZnCl2	

Table 2. Assay summary detailing species, tissues, stages, and conditions

Experiment (Buffer system see Table 1)	**Species**	**Tissue, Sample size**	**Stage (Gosner)**	**Incubation (time, temp)**	**Filter type, PEI**	**Rinse volume**
Experiment #1 TRs vs. CTHBPs and Filter type (Buffer 1)	*Anaxyrus fowleri*	Frozen tail tissue, n=5.	Late stage metamorphosis (stages 39-41)	1 hour, 25C	GF/B and GF/C	6mL
Experiment #2 Protein level (Buffer 1)	*Anaxyrus fowleri*	Frozen tail tissue, n=8.	Late stage metamorphosis (stages 36-41)	1 hour 15 mins, 25C	GF/B only(thick)	6mL
Experiment #3 Incubation Parameters (Buffer 1)	*Anaxyrus fowleri*	Frozen tail tissue, n=8	Late stage metamorphosis (stages 37-45)	4 vs. 18 hours, 25C	GF/B, 0.3% vs. 0.6% PEI, Dextran Coated Charcoal	6mL
Experiment #4 Different tissues: Tail vs. Gut (Buffer 3)	*Anaxyrus fowleri*	Frozen tail vs. gut, n=10	Late stage metamorphosis (stages 37-45)	1 hour, 25C	GF/C	6mL
Experiment #5 Filters vs. Columns (Buffer 4)	*Bombina orientalis*	Fresh tail, hindlimb and liver. n=12	Late stage metamorphosis (stages 37-45)	1 hour, 25C	GF/C vs. G-25 Columns	6mL
Experiment #6 B-max initial estimates (Buffer 4)	*Bombina orientalis*	Fresh tail, hindlimb and liver. n=18	Late stage metamorphosis (stages 37-45)	1 hour, 25C	GF/C	6mL
Experiment #7 Saturation assay (Buffer 4)	*Hyla versicolor and Hyla chrysoscelis*	Fresh tail, hindlimb and liver. n=25	Late stage metamorphosis (stages 42-45)	1 hour, 25C	GF/C	6mL
Experiment #8 Saturation assay (Buffer 4)	*Hyla versicolor and Hyla chrysoscelis*	Fresh tail, hindlimb and liver. n=26	Late stage metamorphosis (stages 42-45)	1 hour, 25C	GF/C	6mL
Experiment #9 Saturation assay (Buffer 4)	*Hyla versicolor and Hyla chrysoscelis*	Fresh tail, hindlimb and liver. n= 28	Late stage metamorphosis (Stages 42-45) and Post-metamorphic frogs (liver only)	1 hour, 25C	GF/C	6mL

CHAPTER IV

RESULTS

Specific Binding: TR vs. CTHBPs and Filter Type

To determine whether TH binding characteristics of CTHBPs and TRs vary among tissues and developmental stages we began with an assay to assess specific binding. Both non-specific and total binding were assessed from cytosolic fractions containing cytosolic thyroid hormone binding proteins (CTHBPs) and nuclear fractions containing thyroid receptor proteins (TRs). Tissue used in this assay was frozen tail tissue from late stage *A. fowleri* tadpoles (n=5, Gosner stages 39-41). In these stages, tadpoles are mature and exhibit subarticular tubercle development, breakdown of the larval mouthpiece, robust hindlimbs that are largely formed, and degeneration of the tail. Our preliminary assay compared two filter types, GF/C and GF/B filters; GF/C is a thinner filter type while GF/B is thicker (Figure 6). The primary goal for this experiment was to determine which filter type resulted in the highest levels of specific binding; defined as the difference between total binding (TB) and non-specific binding (NSB). The only difference between TB and NSB samples is that NSB samples were incubated with an excess of unlabeled TH.

There was a slight difference in results between the filter types used during this radioligand assay. For TRs, total binding that was slightly greater than non-specific binding for GF/B filters, while CTHBP total binding was equal to non-specific binding. For both TRs and CTHBPs, total binding was less than or relatively equal to NSB for GF/C filters. Comparisons among the two fractions assayed indicated that

CTHBPs gave a weaker signal than TRs, particularly in the assay using GF/B filters. However, there was no difference between total binding vs. non-specific binding with any tissue or filter type and specific binding was minimal or non-existent in all trials.

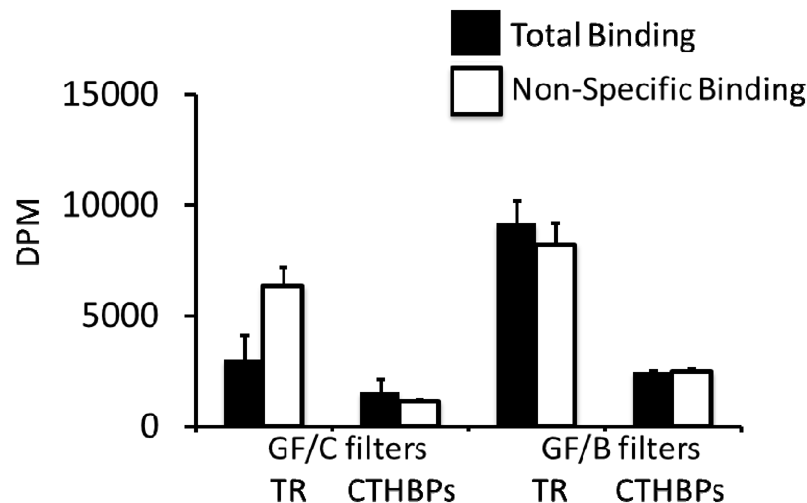

Figure 6. Specific Binding: TR vs. CTHBPs and filter type
Radiolabeled [I^{125}] thyroid hormone binding in frozen tail tissues of late stage metamorphic (Gosner stages 39-41) *Anaxyrus fowleri* tadpoles. Binding of two filter types was tested (GF/C-thin, GF/B-thick). Binding parameters were tested for both TR binding (nuclear fraction) and CTHBP binding (cytosolic fraction). Black bars=total binding, white bars=non-specific binding.

Protein levels

To generate a stronger specific signal, assays using samples with range of protein levels were conducted (Figure 7). Frozen tail tissue from late stage *A. fowleri* tadpoles (n=8) were pooled for this assay (stages 36-41). The high protein sample was undiluted after preparation and was equal to 7μg/μl. An aliquot of this high protein sample was diluted 1 to 1 in assay buffer to yield a low protein concentration of 3.5 μg/μl. CTHBPS were treated similarly; the high protein sample was 5.7μg/μl compared to the low protein of 2.85 μg/μl. Only GF/B filters were used in this experiment as they gave a stronger signal in our preliminary assay. Total binding

was higher than non-specific binding for both TRs and CTHBPs across all trials, and protein level did not alter total or non-specific binding levels.

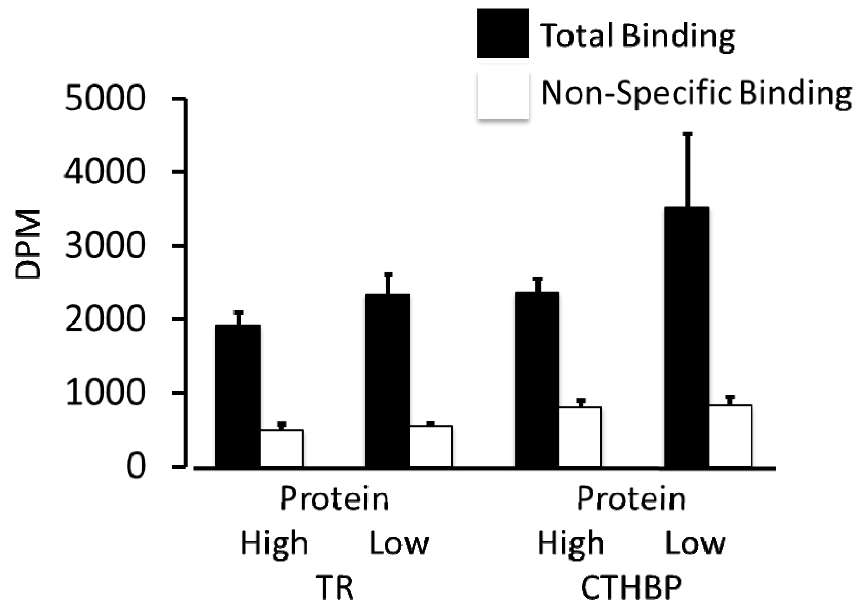

Figure 7. Protein levels
Radiolabeled [I^{125}] thyroid hormone binding in frozen tail tissues of late stage metamorphic (stages 36-41) *Anaxyrus fowleri* tadpoles. Binding of one filter type was tested GF/B. Assays were conducted using samples diluted across a range of protein levels: high 7μg/μl vs low 3.5 μg/μl for TR (nuclear fraction) and high 5.7ug/ul vs low 2.85 ug/ul for CTHBP (cytosolic fraction). Black bars=total binding, white bars=non-specific binding.

Incubation parameters: incubation time, charcoal and PEI %

To maximize specific binding while minimizing tissue breakdown, long (20h) vs. short (3h) incubation times were tested. Frozen tail tissue from late stage *A. fowleri* (n=9, stages 37-45) were assayed, using GF/B filters. In addition, short-term CTHBP samples were treated with dextran-coated charcoal suspension (DCC) to remove endogenous thyroid hormones that might interfere with radioligand binding. Lastly, filters were treated with two concentrations of polyethylenimine (PEI) to determine if specific binding could be enhanced (0.3 % vs. 0.6%; Figure 8a and b).

Sample preparations for TR binding gave an overall higher signal in both short and long incubation trials. However there was no difference between TB and NSB with any of the assay conditions and specific binding was minimal or non-existent in all trials.

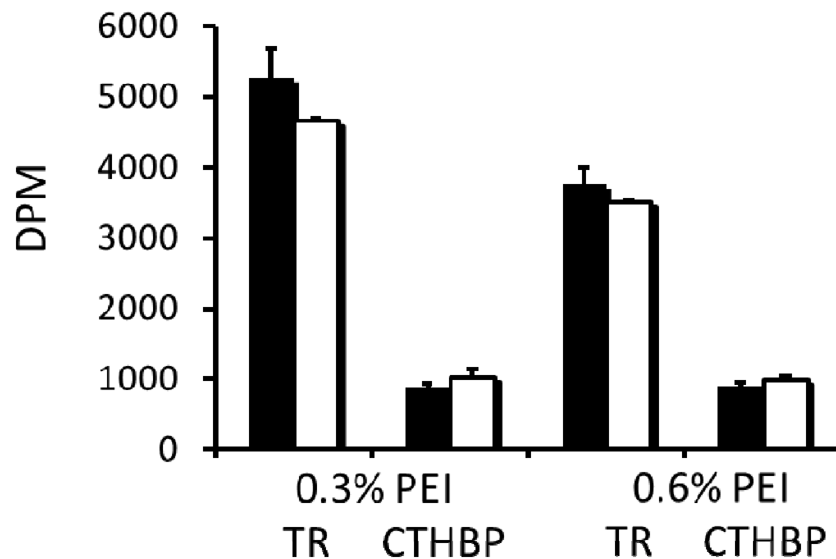

Fig. 8a. Incubation parameters-long incubation 20h- [PEI]

Radiolabeled [I1^{25}] thyroid hormone binding in frozen tail tissues of late stage metamorphic (Gosner stages 39-41) *Anaxyrus fowleri* tadpoles. Binding of one filter type was tested GF/B. Binding parameters were tested for both TR binding (nuclear fraction) and CTHBP binding (cytosolic fraction) under long incubation (20h). Black bars=total binding, white bars=non-specific binding.

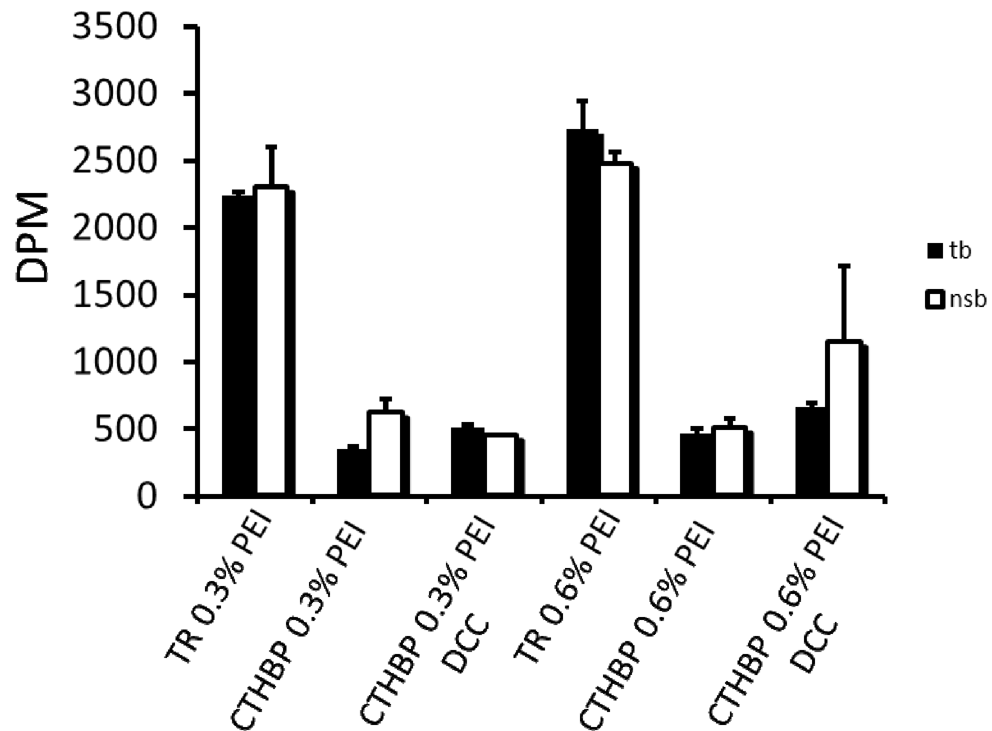

Fig. 8b. Incubation parameters-short incubation 3h-charcoal/ [PEI]

Radiolabeled [I^{125}] thyroid hormone binding in frozen tail tissues of late stage metamorphic (Gosner stages 37-45) *Anaxyrus fowleri* tadpoles. Binding of one filter type was tested GF/B. Binding parameters were tested for both TR binding (nuclear fraction) and CTHBP binding (cytosolic fraction) under short (3h) incubation. Black bars=total binding, white bars=non-specific binding.

Different tissues: tail vs. gut

Both frozen tail and gut tube tissue from late stage *A. fowleri* tadpoles (n=10, stages 37-45) were compared to determine if binding parameters vary among tissue types (Figure 9). In addition, to reduce NSB a new buffer system was implemented and GF/C (thin) filters were used (Leifert *et al.*, 2009). Overall, there was no difference between total binding and non-specific binding with either tissue sampled, and specific binding was minimal or non-existent in all trials.

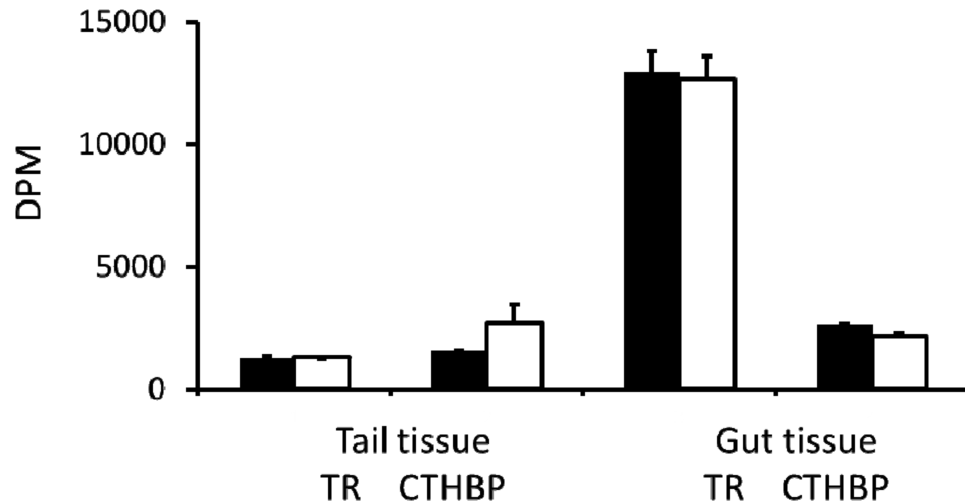

Figure 9. Tail vs. gut tissue
Radiolabeled [I^{125}] thyroid hormone binding in two different tissue types: frozen tail and gut of late stage metamorphic (Gosner stages 37-45) *Anaxyrus fowleri* tadpoles. Binding of one filter type was tested GF/C. Binding parameters were tested for both TR binding (nuclear fraction) and CTHBP binding (cytosolic fraction). Black bars=total binding, white bars=non-specific binding.

Filters vs. columns

Since frozen tissue samples were not yielding clean results, subsequent assays employed only fresh tissue samples. In addition, sample preparations were modified to follow methods that used dialysis to examine adult bullfrog thyroid hormone receptors (Galton and Schaafsma, 1983). Specific modifications included a new buffer system and nylon mesh (215 μm and 64μm) for preparation of nuclei. Lastly Sephadex G-25 columns were tested as an alternative technique for bound vs. free separation. Fresh tail, hindlimb and liver tissue from late stage metamorphic *B. orientalis* tadpoles (n= 12, stages 37-45) were assayed. Sephadex G-25 columns were not effective in separating bound vs. free radiolabel and gave a weak signal for both TRs and CTHBPs in all tissue samples (Figure 10). In contrast, GF/C filters had higher binding for both TRs and CTHBPs in all samples (Figure 10).

Radioligand binding in nuclear fractions containing TRs (Figure 10a) was generally higher than CTHBP binding (Figure 10b) in all tissues examined.

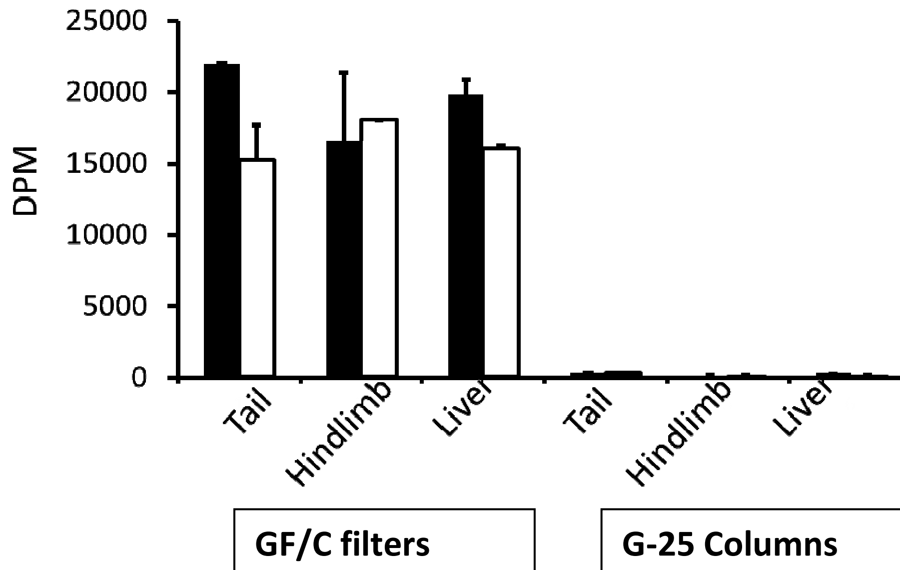

Fig. 10a. TR binding to filters or columns

Radiolabeled [I^{125}] thyroid hormone binding in fresh tail, hindlimb and liver tissue of late stage metamorphic (stages 37-45) *Bombina orientalis* tadpoles. Binding was compared between GF/C filters and G-25 Sephadex columns. Black bars=total binding, white bars=non-specific binding.

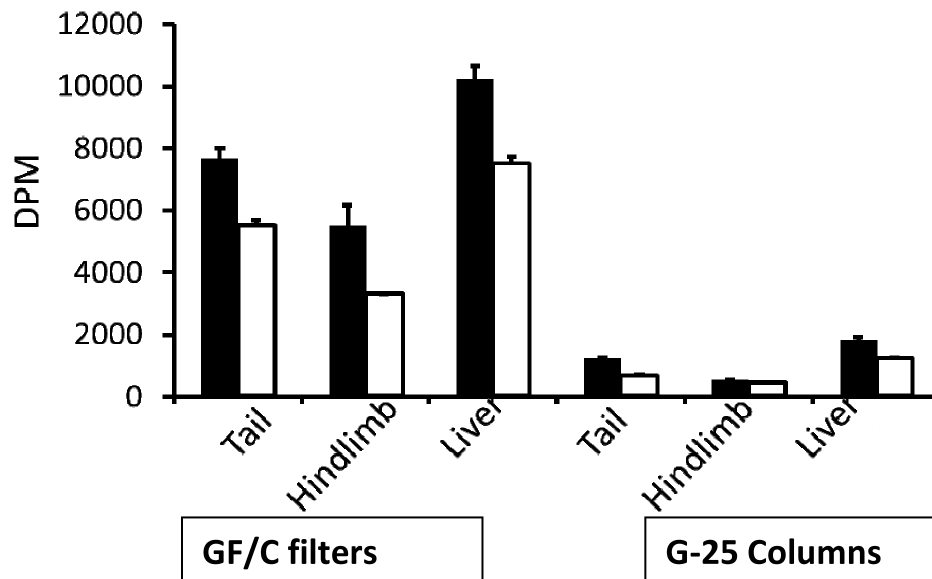

Fig. 10b. CTHBPs binding to filters and columns

Radiolabeled [I^{125}] thyroid hormone binding in fresh tail, hindlimb and liver tissue of late stage metamorphic (stages 37-45) *Bombina orientalis* tadpoles. Binding was compared between GF/C filters and G-25 Sephadex columns. Black bars=total binding, white bars=non-specific binding.

B-max initial estimates: thyroid receptors

Nuclear fractions containing thyroid hormone receptors continually gave a higher signal and became the main focus of our research. Fresh tail, hindlimb and liver tissue was pooled from late stage *B. orientalis* tadpoles (n=18, stages 37-45) assayed at 0.85 nM to test for tissue difference in capacity. At this concentration B-max values differed slightly among the tissue types. The lowest B-max value was associated with tail tissue, intermediate values with hindlimb tissue and the highest value with liver tissue. The overall pattern across all samples suggests a tissue difference in total binding capacity.

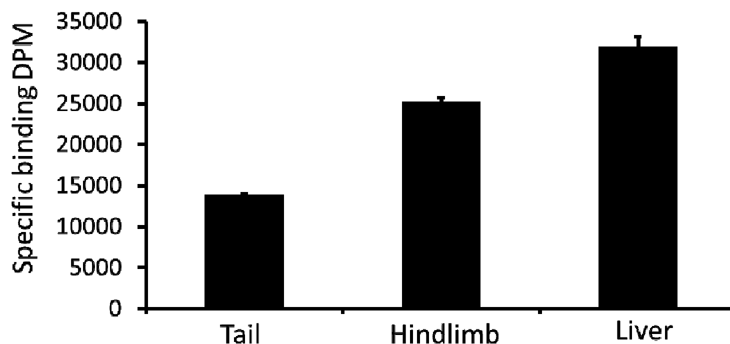

Figure 11. B-max initial estimates: TRs

Specific binding in fresh tail, hindlimb and liver tissue samples from late stage *Bombina orientalis* tadpoles (stages 37-45, n=18) were assayed at a concentration label=0.85 nM. Binding of one filter type was tested GF/C. Binding parameters were tested for TR binding (nuclear fraction).

Saturation assays

Now that we have seen a difference in binding capacity of TRs across different tissues the next step was to do a full saturation curve to examine both binding characteristics: affinity and capacity. Fresh tissue samples of tail, hindlimb and liver from late stage *H. versicolor* and *H. chrysoscelis* (n=25) were assayed

(stages 42-45). These specific stages are characterized by protrusion of forelimbs, formation of an adult mouth and the tail is reduced to a stub. There were five concentrations of label ranging from 0-0.37nM used in this assay. The slopes were relatively equal; however the binding capacity seemed to differ between tissues (Figure 12). Both tail and hindlimb tissue were relatively equal showing a DPM (decay per minute) of approximately 5000, whereas liver tissue was higher with a DPM of 12,000 (Figure 12).

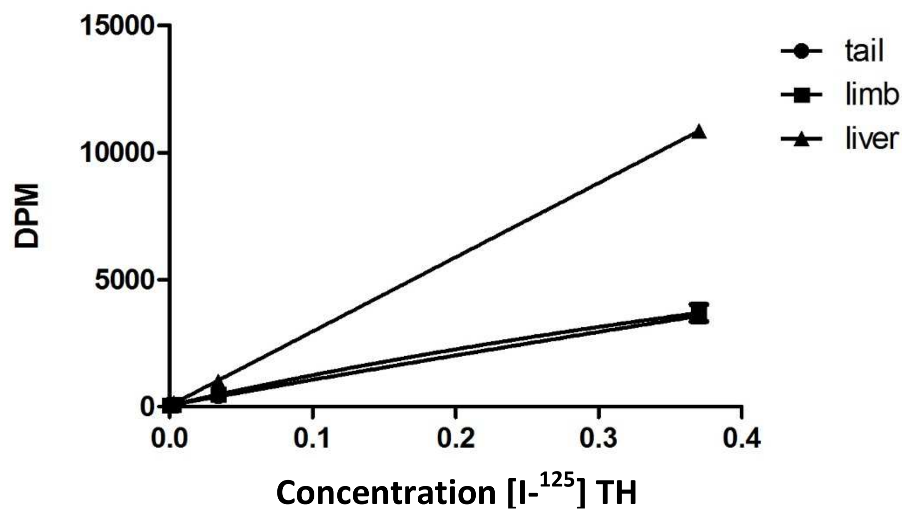

Figure 12. Saturation assay

Saturation binding assay in fresh tissue samples of tail, hindlimb and liver from late stage *H. versicolor* and *H. chrysoscelis* (stages 42-45, n=25) were assayed. One filter type was used GF/C filters and concentration of radiolabeled [I^{125}] thyroid hormone ranged from 0-0.4nM.

As saturation was not achieved at 0.4nM radioligand, a second saturation assay was conducted with radioligand concentrations ranging from 0-5nM. Fresh tail, hindlimb, and liver tissue samples from late stage *Hyla* tadpoles (n=26) were assayed (stages 42-45). The slopes were relatively equal however the binding capacity seems to differ once again. The capacity values were more significant and

consistent. The limb had the lowest signal at 100,000 DPM next was the tail at 140,000 DPM (Figure 13). Liver tissue was the highest at 225,000 DPM and began to level off showing saturation (Figure 13). This data continues to suggest a difference among tissue competence.

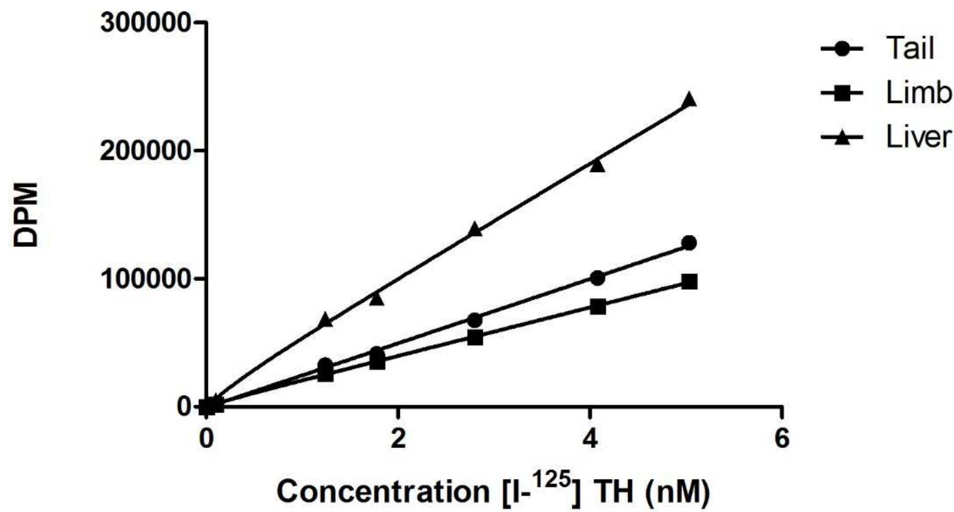

Figure 13. Saturation assay

Saturation binding assay in fresh tissue samples of tail, hindlimb and liver from late stage *H. versicolor and H. chrysoscelis* (stages 42-45, n=26) were assayed. One filter type was used GF/C filters and concentration of radiolabeled [I^{125}] thyroid hormone ranged from 0-5nM.

As saturation was not achieved at 5.0nM radioligand, a third saturation assay was conducted with radioligand concentrations ranging from 0-10.5nM. Fresh tail, hindlimb, and liver tissue samples from late metamorphic stage *H. versicolor* and *H. chrysoscelis* tadpoles (n=28) were assayed (stages 42-45). In addition, we compared liver tissue between late stage metamorphic *Hyla* tadpoles and recently metamorphosed *Hyla* frogs. There were eight concentrations of label from 0-10.5nM used in this assay. The slopes were relatively close; however the binding capacity of each tissue seemed to differ and the signal had increased (Figure 14). The tail

tissue was the lowest at 150,000 DPM. Next the liver in metamorphosed frogs (240,000 DPM) and hindlimb tissue (241,000 DPM) were relatively equal. The liver from the late stage tadpoles was the highest at 280,000 DPM. These results suggest a difference in binding capacity between tissues as well as between stages of the same species. In addition, there is a possibility of two binding sites showing a difference between TRs: α and β.

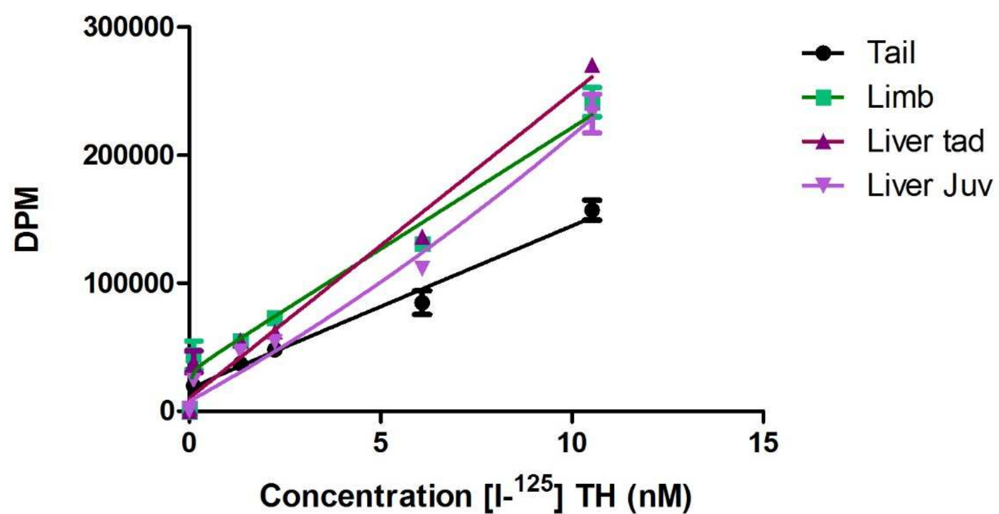

Figure 14. Saturation assay

Saturation binding assay in fresh tissue samples of tail, hindlimb and liver from late stage *H. versicolor and H. chrysoscelis* (stages 42-45, n=28) were assayed. In addition, we compared liver tissue between late stage metamorphic Hyla tadpoles and recently metamorphed *Hyla* frogs. One filter type was used GF/C filters and concentration of radiolabeled [I^{125}] thyroid hormone ranged from 0-10.5nM.

CHAPTER V

DISCUSSION

The overall objective of the current study is to develop a technique that allows protein level analysis of two components implicated in the regulation of tissue specific responses to TH during tadpole metamorphosis: CTHBPs and TRs. I hypothesized that tissue specific changes in the affinity and/or capacity of TRs and CTHBPs to bind TH throughout metamorphosis underlie the timing and extent of tissue remodeling. To understand the specific tissue responses to TH during metamorphosis in amphibians, we compared different tissues (*e.g.* hindlimbs, tail and liver) in late stage metamorphic tadpoles (Gosner stages 36-45) through radioligand binding assays. To examine tissue specific changes in TH receptor proteins during amphibian development and metamorphosis the current work examined the binding affinity and binding capacity of CTHBPs and TRs using vacuum filtration. This technique has previously been used to characterize the affinity and capacity of plasma and cytosolic steroid hormone binding proteins, as well as steroid hormone receptors (Jennings *et al.*, 2000, Orchinik *et al.*, 2000). Since steroid and thyroid hormones share many physiological features, and bind to similar receptors, this technique should be effective in analyzing CTHBPs and TRs as well.

Initial Assays: Frozen Tissue

Our initial assays using frozen tissue samples did not result in strong specific binding, despite attempting a range of modifications to assay protocols. To

determine whether TH binding characteristics of CTHBPs and TRs vary among tissues and developmental stages we began with an assay to assess specific binding. Both non-specific and total binding were assessed from cytosolic fractions containing CTHBPs and nuclear fractions containing TRs. Our initial assay examined which filter type (GF/B or GF/C) would result in the highest levels of specific binding. However, there was no difference between total binding vs. non-specific binding with any tissue or filter type.

The lack of differences in specific binding between filter types prompted us to test the effects of protein concentration on binding. Total binding was higher than non-specific binding for bothTRs and CTHBPs across all protein levels examined. Thus protein level did not seem to dramatically impact specific binding. Low protein levels resulted in a slightly stronger signal, suggesting that samples with high protein levels may contain TH binding proteins that do not adhere to filters.

Subsequent binding assays tested the effects of incubation time, charcoal removal of endogenous hormones, and PEI treatment of filters in an effort to maximize specific binding while minimizing tissue breakdown (Figure 9). Overall, TR binding gave a higher signal than CTHBPs under all conditions. There was no difference between TB and NSB with any of the assay conditions and specific binding was minimal or non-existent in all trials. Finally, frozen tail and gut tissue samples were compared to determine if binding parameters varied among tissue types. Overall, there was no difference between total binding and non-specific binding with either tissue sampled, and specific binding was again minimal or non-existent in all trials.

Fresh tissue assays

Since frozen tissue samples did not yield robust results, subsequent assays employed only fresh tissue samples. In addition, sample preparations were modified to follow methods used by Galton and Schaafsma (1983) to examine liver TR expression by dialysis based binding assays. Specific modifications included a new buffer system and nylon mesh (215 μm and 64μm) for preparation of cleaner nuclear fractions. Lastly Sephadex G-25 columns were tested as an alternative technique for bound vs. free separation because this method had recently been used to characterize the affinity and capacity of TR binding in cell cultures (Cunha-Lima and Rodrigues, 2011). However, in our assays Sephadex G-25 columns were not effective in separating bound vs. free radiolabel and gave a weak signal for both TRs and CTHBPs in all tissue samples. In contrast, GF/C filters consistently had higher binding for both TRs and CTHBPs in all samples.

A trend seen throughout our previous radioligand assays were nuclear fractions containing TRs continually gave a higher signal. In addition, tissue specific responses induced by TH during amphibian metamorphosis are thought to be mainly controlled by TRs, although non-genomic effects of TH are known to occur (Buchholz *et al.*, 2006). Accordingly, thyroid receptors became the main focus in our research. To determine a difference in the maximum binding sites (B-max) within different tissues a specific binding assay was conducted. Fresh tissues using the label at 0.85 nM were tested to assess the potential for tissue specific differences in capacity. These initial B-max estimates showed a slight difference in binding capacity for TRs in each tissue; tail tissue had the lowest binding at 15,000 DPM

next was hindlimb tissue at 25,000 DPM, and the highest binding capacity was seen in liver tissue at 34,000 DPM.

Since the initial B-max estimates run at 0.85nM suggested a difference in tissue capacity, we ran a full saturation binding assay. Fresh tissue from *Hyla* species was assayed through a saturation curve using five concentrations of radiolabeled [I^{125}] thyroid hormone ranging from 0-0.4nM. While the slopes of the curves generated were relatively close, binding capacity seemed to differ between tissues. Both tail and hindlimb tissues were relatively equal at 5000 DPM, while radioligand binding to liver tissue was highest at 12,000 DPM. Even at the highest level of radioligand there was no indication of reaching saturation.

Therefore in the subsequent assay the highest concentration of [I^{125}] thyroid hormone was increased from 0.4nM to 5nM. Nine concentrations of radiolabel ranging from 0-5nM were used in this assay. Once again the slopes were relatively equal however the binding capacity seemed to differ between tissues. The capacity values for limb tissues had the lowest signal at 100,000 DPM with tail tissue being slightly higher at 140,000 DPM. Liver tissue was the highest at 225,000 DPM and was the only tissue that began to show saturation. Overall, this data continues to suggest a difference among binding capacity for tissue competence but accurate B-max estimates require full saturation.

In the final assay radiolabeled [I^{125}] thyroid hormone concentration was again increased and ranged from 0-10.5nM. The slopes were relatively close; however the binding capacity of each tissue seemed to differ and had an increased signal. Tail tissue was the lowest at 150,000 DPM. Next the liver in metamorphosed frogs

(240,000 DPM) and hindlimb tissue (241,000 DPM) were relatively equivalent. Liver from late stage tadpoles exhibited the highest at 280,000 DPM. Overall, our saturation assays consistently resulted in differences in TR binding capacity between tissues within a species. A trend seen throughout these saturation binding assays was that liver tissue consistently had the highest binding and was the only tissue to show signs of saturation (Figure 14). Tail and hindlimb tissues did not yield as consistent a result; in one assay (Figure 14) hindlimb capacity was the lowest of tissues examined while tail tissue was lowest in a second assay (Figure 15). One potential explanation for the mixed results is that pooled samples did not always contain the same range of stages. In the future there needs to be a more consistent and equal distribution among the stages of tadpoles collected.

Conclusions/future studies

In summary, I hypothesized that tissue specific responses in affinity and/or capacity of TRs and CTHBPs to bind TH throughout metamorphosis underlie the timing and extent of tissue remodeling. Unfortunately, the cytosolic fraction containing CTHBPs continually gave a weak signal throughout all assays. In contrast the nuclear fraction containing TRs gave a strong signal and suggested a difference in binding capacity among tissues as predicted. Overall the separation technique used throughout these radioligand assays was successful in separating bound vs. free ligand, but still needs to be further refined. Once a robust protocol has been developed these preliminary assays need to be replicated in order to ensure consistency in results and to permit statistical analyses.

There are three major issues with the saturation assays conducted thus far: (1) lack of full saturation even at the highest amount of radiolabeled [I125] TH tested, (2) affinity differences between TR α vs. β isoforms, (3) specificity of TH binding. While our assays did not show saturation with radioligand concentrations up to 10.5nM, other nuclear receptors have also been shown to require a high concentration of ligand to reach saturation. For example, recent assays using similar techniques to assess binding of corticosteroids in house sparrows suggest that affinity measures (Kd) require label concentrations up to 20nM in order to reach saturation (Lattin *et al.*, 2012). If our assays techniques are valid for TR characterization we expect to reach saturation using similar levels (20nM) of radioligand.

Our last assay suggested the possibility of two binding sites, given that the saturation curve seemed to level off at ligand concentrations between 3-6nM (Figure 15). To address the binding parameters of TRα vs. TRβ saturation assays that include a specific inhibitor of TRβ binding of TH are planned. The most effective TRβ specific TH competitor (GC-1) has a 10-30 fold affinity for TRβ over TRα (Grover *et al.*, 2007). The inclusion of a receptor specific competitor in saturation assays has been used to differentiate between mineralocorticoid and glucocorticoid receptors in birds (Lattin *et al.*, 2012) and plasma steroid binding globulins in lizards (Jennings *et al.*, 2000). Techniques that separate TRα vs. TRβ binding are critical for understanding developmental changes in TR function since different tissue types are known to have differences in expression levels of TR isoforms during development (Buchholz *et al.*, 2006). TRα is expressed relatively early in metamorphosis and in

tissue that proliferate, whereas TRβ is expressed later in metamorphosis in tissues that resorb such as the tail (Buchholz *et al.*, 2006; Cossette and Drysdale, 2004; Furlow and Neff, 2006; Tata, 2006). Finally a full characterization of TR requires a competition assay to demonstrate specificity for binding of radiolabeled [I^{125}] TH. The competition assay is predicted to show differences in binding specificity among thyroid hormones (T3, T4, T2 and rT3). In this competition assay unlabeled T3 is predicted to be the most powerful competitor for [I^{125}] binding, followed by unlabeled T4. Unlabeled T2 and rT3 are predicted to be less effective competitors.

Our results showed a potential binding capacity difference in tissues sampled. In addition, our results potentially showed a difference in binding capacity among developmental stages (metamorphosed vs. juvenile, Figure 15). While preliminary our findings suggest that TR binding capacity is directly related to tissue remodeling. This opens the possibility to use filtration assays to address ontogenetic and phylogenetic changes in the roles of TH in amphibian development and metamorphosis. Similar binding assays have demonstrated that individual tissues within a species vary in receptor affinity and capacity (Lattin *et al.*, 2012), but little information is available for developmental changes in tissue specific receptor characteristics. If TR are central to metamorphic changes affinity and/or capacity should increase during stages when tissues are undergoing remodeling. In addition affinity and/or capacity are predicted to be higher in species with rapid development times than in species with more prolonged larval periods. For instance, *Sc. couchii* with a larval period of 12 days should have higher TR affinity or capacity than its close relative *Sp. multiplicata* with a larval period of 16 days (Hollar *et al.*, 2011).

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