Disruption of thyroid hormone action by environmental contaminants in vertebrates

By

Ashley Hinther

B.Sc., University of Saskatchewan, 2007

A Dissertation Submitted in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

In the Department of Biochemistry and Microbiology

Supervisory Committee

Disruption of thyroid hormone action by environmental contaminants in vertebrates

By
Ashley Hinther
B.Sc., University of Saskatchewan, 2007

Supervisory Committee

Dr. Caren C. Helbing, (Department of Biochemistry and Microbiology)
Supervisor

Dr. Caroline Cameron, (Department of Biochemistry and Microbiology)
Departmental Member

Dr. John Taylor, (Department of Biology)
Outside Member

Abstract

Supervisory Committee:

Dr. Caren C. Helbing, (Department of Microbiology and Biochemistry)
Supervisor

Dr. Caroline Cameron, (Department of Microbiology and Biochemistry)
Departmental Member

Dr. John Taylor, (Department of Biology)
Outside Member

Thyroid hormones (THs) are important hormones involved in developmental processes, including foetal brain maturation. THs are also involved in the maintenance of homeostasis. One in three people in Canada are considered to have some form of thyroid disorder. One reason for the high level of thyroid disorders may be the increasing amount of anthropogenic chemicals released into the environment that affect normal hormone action.

Amphibian metamorphosis is completely dependent on TH and provides a model to study such chemicals. This thesis uses the *Rana catesbeiana* tadpole as a model to study potential TH disrupting chemicals by developing a novel screening assay called the cultured tail fin biopsy assay, or the “C-fin” assay. The C-fin assay uses tail biopsies from premetamorphic tadpoles, Taylor-Kollros stage VI-VIII. The biopsies are cultured in serum-free media along with the test chemical for 48 hours.

QPCR is used to measure the mRNA steady-state levels of selected gene transcripts. Two TH-responsive gene transcripts were measured: the up-regulated gene transcript, thyroid hormone receptor β (*TRβ*) and the down-

regulated gene transcript, *Rana* larval keratin type I (*RLKI*). Heat-shock protein 30 (*HSP30*) and catalase (*CAT*) were used as indicators of cellular stress. Another model system used in this thesis is rat pituitary cells, or GH3 cells. QPCR was used to measure the mRNA steady-state levels of three TH-responsive genes growth hormone (*GH*), deiodinase I (*DIOI*), and prolactin (*PRL*); heat-shock protein 70 (*HSP70*) was used as an indicator of cellular stress.

Nanoparticles, used in various consumer products, were one class of chemicals examined. Using the C-fin assay, nanosilver and quantum dots (QDs) caused perturbations in TH-signalling and also showed signs of cellular stress. There was no overt toxicity observed as was determined by the normalizer, house-keeping gene transcript, ribosomal protein L8. The GH3 cells also detected TH disrupting effects by both nanosilver and QDs; however, nanosilver did not appear to cause cellular stress whereas QDs did.

Nitrate and nitrite, major waterway contaminants, were also examined and there were no TH-perturbations observed using the C-fin assay.

Finally, two antimicrobials used in many consumer products, triclocarban (TCC), triclosan (TCS) and its metabolite, methyl-TCS (mTCS) were examined using both the C-fin assay and GH3 cells. Both the C-fin assay and the GH3 cells determined mTCS to be more potent than TCS in disrupting TH action. TCC also caused perturbations in TH-signalling as well as causing a significant amount of cellular stress.

Overall the C-fin assay and the GH3 cells proved to be excellent models in studying the potential disruptors of the TH axis. The C-fin assay and GH3 cells

detected novel TH disruptors and gave further insight into already known disruptors of the TH axis.

Table of Contents

Supervisory Committee ..... **ii**
Abstract ..... **iii**
Table of Contents ..... **vi**
List of Tables ..... **viii**
List of Figures ..... **ix**
Acknowledgements ..... **xii**
Chapter 1: Introduction **1**
1.1 Significance of Research 1
1.2 Thyroid Hormone Importance 5
1.3 Anuran Model and Metamorphic Program 6
1.4 Thyroid Hormone Metabolism 13
1.5 Thyroid Hormone Receptors 18
1.6 Thyroid Hormone Non-Genomic Actions 23
1.7 Regulation of Metamorphosis 25
1.8 Environmental Contaminants - Disruptors of Thyroid Hormone Action 28
1.9 Determination of Thyroid Hormone Disrupting Chemicals 36
1.10 Thesis Overview 39
Chapter 2: Materials and Methods **42**
2.1 Experimental Animals 42
2.2 Organ culture of tail fin biopsies 42
2.3 Cell Culture 45
2.4 Isolation of RNA, quantification of gene expression 47
2.5 Statistical Analyses 49
Chapter 3: C-fin: A cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals **51**
3.1 Introduction 51
3.2 Results 54
3.2.1 The C-fin assay *54*
3.2.2 Location of biopsy is independent of the T_3-induced response *57*
3.2.3 Detection of disruption of TH action with the C-fin assay *60*
3.3 Discussion 64
Chapter 4: Nanometals induce stress and alter thyroid hormone action in amphibia and mammalian cells at or below North American water quality guidelines **69**
4.1 Introduction 69
4.2 Materials and Methods 73
4.2.1 Particle Characterization *73*
4.3 Results 73
4.3.1 NP Characterization *73*
4.3.2 TH-Response Gene Transcript Levels upon Exposure to Nanosilver, Quantum Dots, and Nanozinc Oxide *74*
4.3.3 Stress Response Gene Transcript Levels upon Exposure to Nanosilver, Quantum Dots, and Nanozinc Oxide *78*

4.3.4 GH3 cell TH- and Stress Response Gene Transcript Levels upon Exposure to Nanosilver and QDs *79*
4.3.5 Context within Water Quality Guidelines *84*
4.3.6 TH- and Stress Response Gene Transcript Levels upon Exposure to Low Concentrations of Nanosilver *87*
4.3.7 TH- and Stress Response Gene Transcript Levels upon Exposure to Low Concentrations of QDs *89*
4.4 Discussion 92
Chapter 5: Nitrite and nitrate do not induce stress or disrupt thyroid hormone action in *Rana catesbeiana* cultured tadpole tail fin tissue 101
5.1 Introduction 101
5.2 Results 105
5.3 Discussion 108
Chapter 6: Evaluation of triclocarban, triclosan, and its metabolite, methyl-triclosan for thyroid hormone disruptive effects in frog and mammalian systems 111
6.1 Introduction 111
6.2 Results 115
6.2.1 Mammalian cell exposures *115*
6.2.2 Amphibian organ culture exposures *122*
6.3 Discussion 126
Chapter 7: Discussion and Future Directions 131
Bibliography 136
Appendix 1: Abbreviations 168
Appendix 2: Primer Information 172

List of Tables

Table 2.1. QPCR Primer Information.....**49**

List of Figures

Figure 1.1. Structure of thyroxine, triiodothyronine, reverse-triiodothyronine, diiodothyronine, and the deiodinase enzymes involved in the conversion of the thyroid hormone molecules**.....2**

Figure 1.2. Hypothalamus-pituitary-thyroid signalling axis**.....3**

Figure 1.3. Stages of metamorphosis (A) and a graph outlining the increase in TH levels that take place at birth (B)**.....8**

Figure 1.4. Schematic representation of the synthesis of thyroid hormone (TH; T_3 and T_4) in thyroid gland follicular cells, thyrocytes**.....15**

Figure 1.5. Various isoforms encoded by the thyroid hormone receptor alpha (TRα) (A) and thyroid hormone receptor beta (TRβ) (B) genes**.....20**

Figure 1.6. Structure of tetrabromobisphenol A (TBBPA) (A), acetochlor (B), triclosan (C), methyl-triclosan (D), triclocarban (E), roscovitine (F), and genistein (G)**.....34**

Figure 3.1. Overview of the C-fin assay**.....55**

Figure 3.2. Sensitivity of the C-fin assay to T_3 as assessed by *TRβ* and *RLKI* transcript levels**……..……...56**

Figure 3.3. Examination of uniformity of response to T_3 across the tail fin tissue as analyzed by *TRβ* and *RLKI* transcript levels**.....58**

Figure 3.4. Assessment of the effects of Triac and roscovitine on *TRβ* and *RLKI* transcripts in the C-fin assay**.....59**

Figure 3.5. Analysis of TBBPA and acetochlor for TH disrupting activity through assessment of *TRβ* and *RLKI* transcript levels**.....61**

Figure 4.1. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to nanosilver, quantum dots (QD), and nanozinc oxide in the absence (A) or presence (B) of 10 nM T_3**……..……...75**

Figure 4.2. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nanosilver, quantum dots (QD), and nanozinc oxide in the absence (A) or presence (B) of 10 nM T_3**……...77**

Figure 4.3. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to nanosilver in the absence and presence of 10 nM T_3**.....81**

Figure 4.4. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to quantum dots (QD) in the absence and presence of 10 nM T_3**.....83**

Figure 4.5. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to nanosilver, micron-silver, and silver nitrate in the absence (A) or presence (B) of 10 nM T_3**.....86**

Figure 4.6. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nanosilver, micron-silver, and silver nitrate in the absence (A) or presence (B) of 10 nM T_3**.....88**

Figure 4.7. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to quantum dots (QDs) and micron-cadmium telluride (micron-CdTe) in the absence (A) or presence (B) of 10 nM T_3**.....90**

Figure 4.8. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to quantum dots (QD) and micron-cadmium telluride (micron-CdTe) in the absence (A) or presence (B) of 10 nM T_3**.....91**

Figure 5.1. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to nitrite and nitrate in the absence (A) or presence (B) of 10 nM T_3**.....106**

Figure 5.2. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nitrite and nitrate in the absence (A) or presence (B) of 10 nM T_3**.....107**

Figure 6.1. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to TCS**.....116**

Figure 6.2. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to mTCS**.....118**

Figure 6.3. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to TCC**.....120**

Figure 6.4. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to triclosan (TCS), methyl-triclosan (mTCS), and triclocarban (TCC) in the absence (A) or presence (B) of 10 nM T_3**.....123**

Figure 6.5. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to triclosan (TCS), methyl-triclosan (mTCS), and triclocarban (TCC) in the absence (A) or presence (B) of 10 nM T_3**.....125**

Acknowledgements

I would like to thank my supervisor, Dr. Caren Helbing for her continuous support, commitment, guidance, and encouragement throughout this entire project. I would also like to thank my committee members, Dr. Caroline Cameron and Dr. John Taylor, for their support and helpful suggestions.

I would like to thank Jeremy Wulff for supplying me with testing chemicals; thank-you to Dr. Juan Ausio, Dr. Robert Burke, and Dr. Chris Nelson for use of their QPCR machines.

I am grateful to have been a part of the Helbing lab, and had the chance to work with and learn from the past and current lab members. I would like to thank Nik Veldhoen for constant insight and knowledge. I am grateful for Saadia Vawda's hardwork, focus, and patience. Thank-you to Stacey Maher and Amanda Carew for their excellent proofreading skills and helpful suggestions. I would also like to thank all past and previous lab members for helpful discussions, assistance, and support: Stacey Maher, Amanda Carew, Vicki Rehaume, Pola Wojnarowicz, Melissa Cabecinha, Sabrina Pittroff, Jay Jordan, Sara Horne, Kurtis Vallee, Austin Hammond, and Dannika Bakker.

Thank-you to my family for their continual love, support, and encouragement throughout this entire project.

Chapter 1: Introduction

1.1 Significance of Research

The thyroid hormones (THs), 3,5,3',5'-tetraiodothyronine (thyroxine; T_4) (Figure 1.1) and 3,5,3'-triiodothyronine (T_3) (Figure 1.1), are important signalling molecules in the endocrine system (Shi, 2000). THs are important throughout all stages of life and act on nearly every cell in the body; THs are important for developmental processes and the maintenance of homeostasis. Their release is controlled by the neuroendocrine system and is tightly regulated through feedback mechanisms (Figure 1.2). THs are released from the thyroid gland, a butterfly shaped endocrine organ located in the anterior side of the neck. The hypothalamus releases thyrotropin-releasing hormone (TRH) in humans, and corticotropin releasing factor (CRF) in amphibians, which stimulates the anterior pituitary gland to release thyroid-stimulating hormone (TSH), also known as thyrotropin. TSH stimulates the thyroid gland to release T_3 and T_4, T_4 being the major hormone produced by the thyroid gland. T_4, the transport form, is converted to T_3, the effector or more biologically active form, in peripheral tissues by the deiodinase enzymes (Figure 1.1). Once TH is released, it will not only act on its target tissues but it will also signal the pituitary gland and hypothalamus to stop releasing TRH or CRF, and TSH, respectively (Figure 1.2). This negative feedback ensures the levels of TH are tightly regulated

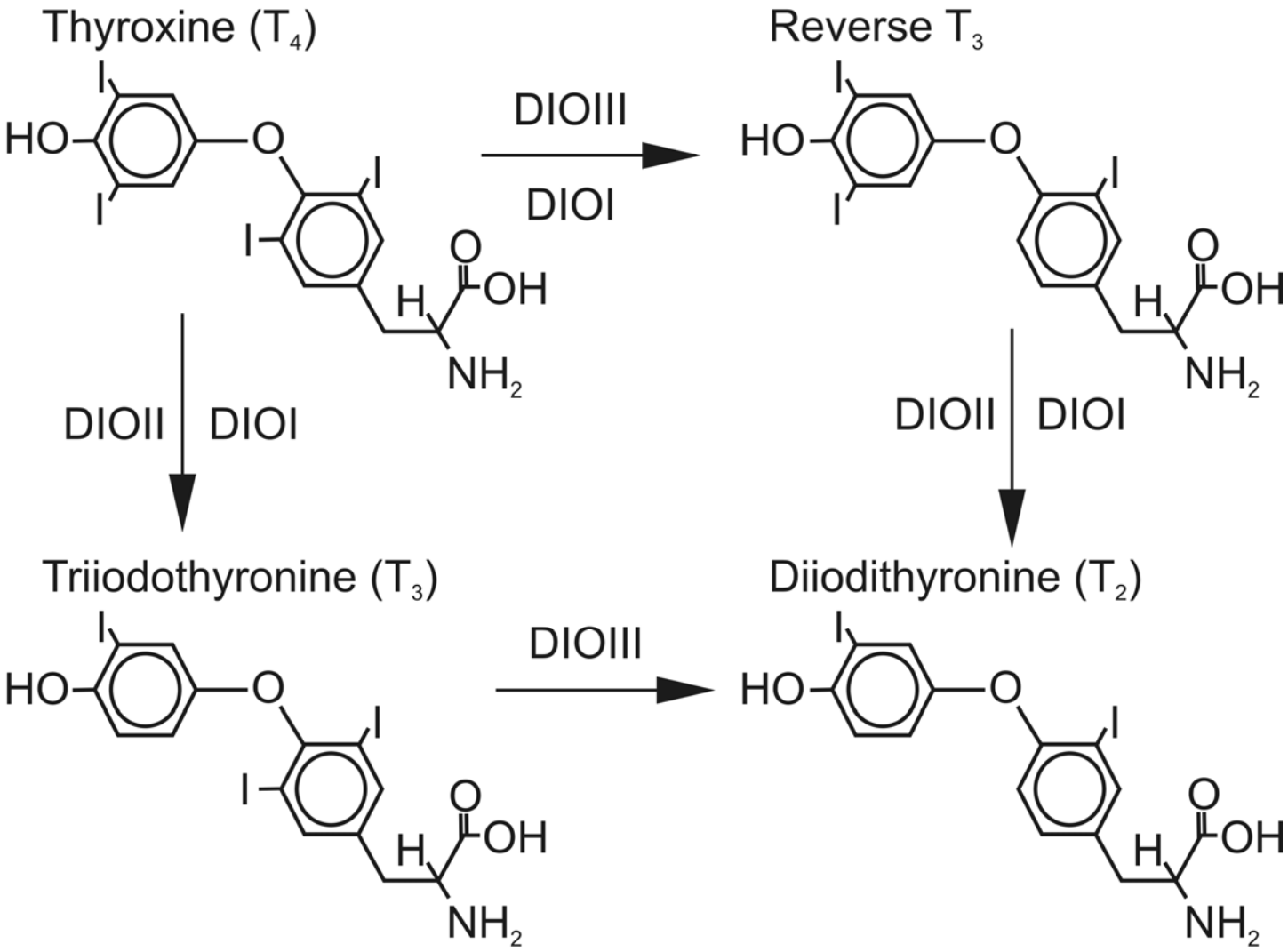


Figure 1.1. Structure of thyroxine, triiodothyronine, reverse-triiodothyronine, diiodothyronine, and the deiodinase enzymes involved in the conversion of the thyroid hormone molecules. Deiodinase I (DIOI) can remove iodine from both the inner and outer rings. Deiodinase II (DIOII) removes iodine from the outer ring and deiodinase III (DIOIII) deiodinates the inner ring. Adapted from (Bianco and Kim, 2006).

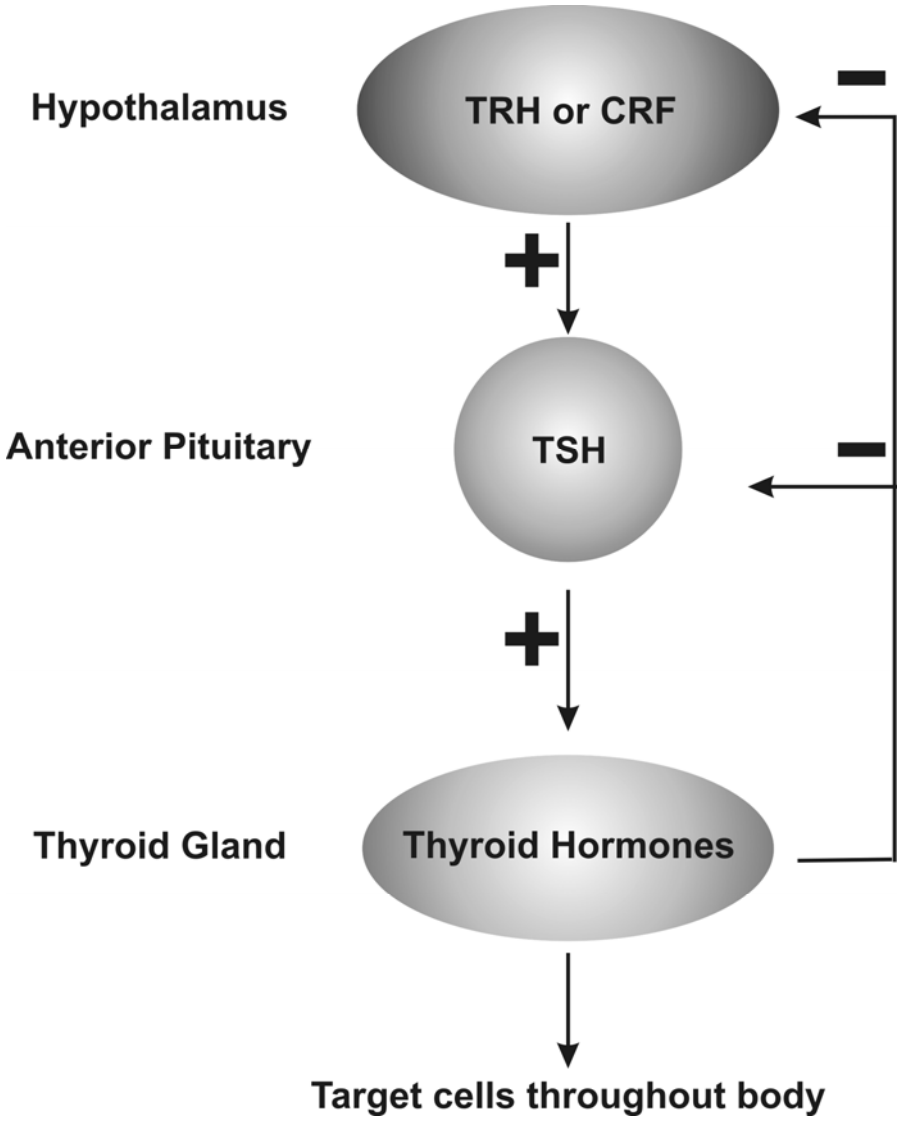


Figure 1.2. Hypothalamus-pituitary-thyroid signalling axis. Release of thyrotropin releasing hormone (TRH; mammals) or corticotrophin releasing factor (CRF; amphibians) from the hypothalamus stimulates the release of thyroid stimulating hormone (TSH), also known as thyrotropin, from the anterior pituitary gland. TSH causes the thyroid gland to release thyroid hormone (TH). The control of thyroid hormone secretion is exerted by negative feedback where TH feeds back onto the hypothalamus and anterior pituitary to inhibit the release of TRH or CRF, and TSH, respectively. Adapted from http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/thyroid/physio.html, 2010.

(Dodd and Dodd, 1976; White and Nicoll, 1981; Kikuyama *et al.*, 1993; Kaltenbach, 1996; Denver, 1997).

Currently over 100,000 manufactured chemicals are produced in the marketplace (Commission, 2006). Some of these chemicals are released into the environment intentionally, such as through agricultural processes, and some are released inadvertently through waste and from products in use. Beyond their toxic effects on organisms when present in high amounts, a number of these chemicals have adverse effects at sublethal concentrations such as acting as endocrine disrupting compounds (EDCs). Close to a hundred compounds have been classified as endocrine disruptors of the TH axis and many new chemicals are hypothesized to act as EDCs of TH action (Boas *et al.*, 2006).

The growing concern that polluting anthropogenic chemicals are potential disruptors of the TH axis has prompted organizations such as the U.S. Environmental Protection Agency (EPA), Environment Canada, and the Organisation for Economic Co-operation and Development (OECD) to develop assays to assess the safety of newly created chemicals and those already present in the environment. The total dependence of amphibian metamorphosis on TH and the high degree of conservation of TH signalling pathway in vertebrates has led to the suggestion that metamorphosis can be used as a model for the detection of chemicals affecting the TH axis in vertebrates (Tata, 2007). This thesis develops a cultured tail fin biopsy or “C-fin” assay based upon the sensitivity of frog tissues to TH and identifies novel disruptors of TH action. By taking multiple tail fin biopsies per animal and then exposing each biopsy to a different treatment condition, the C-fin assay enables the screening of multiple chemicals

simultaneously while maintaining complex tissue structure and enabling the determination of biological variation of a response. In addition to the C-fin assay, this thesis uses cell culture to look at the effects some of these chemicals have on the mammalian system.

1.2 Thyroid Hormone Importance

The TH plays a critical role in maintaining homeostasis and metabolic processes. It regulates the metabolism of fats, proteins, and carbohydrates as well as body temperature (Tata, 2007). TH is important for the normal growth and development of many organs, including the brain and heart, plays an important role in puberty, and is needed for the proper development of the gonads. Perhaps the most important role TH plays is during embryo development and the few critical months after birth (Demonacos *et al.*, 1996; Dorshkind and Horseman, 2000; Mariash, 2003; Silva, 2005; Calamandrei *et al.*, 2006).

The postembryonic period is the few months before birth and several months after birth and TH plays a critical role in the development that takes place during this time (Tata, 1993). The thyroid gland, which synthesizes and secretes THs to the rest of the body, is developed 12 weeks after gestation; however, it is not until 20 weeks after gestation that significant TH production takes place. TH levels begin to increase 4 months before birth and reach a maximum peak at birth and will remain high for several months after birth (Figure 1.3B). This surge in TH is critical for the proper brain development of the foetus and a deficiency in TH can lead to irreversible neurological defects and mental retardation (Hetzel and Dunn, 1989; Hetzel and

Mano, 1989; Porterfield and Hendrich, 1993; Tata, 1993; Hsu and Brent, 1998; Morreale de Escobar *et al.*, 2004; de Escobar *et al.*, 2007).

There are many changes that occur during foetal development and these changes all occur in the presence of high plasma levels of TH. The intestines change from a tubular structure in the foetus to a structure with extensive epithelial folding (Shi, 1996), the haemoglobin genes are changed from a foetal type to an adult type and there is significant increase in serum albumin levels. The skin undergoes keritinization; there is induction of the urea cycle enzymes as well as complete extensive development and restructuring of the central nervous system (CNS) and peripheral nervous system (PNS). The maturation of lungs is essential because the foetus changes its living habitat from aquatic to terrestrial (Tata, 1993; Atkinson *et al.*, 1994; Shi, 2000).

1.3 Anuran Model and Metamorphic Program

Another developmental process completely regulated by TH is amphibian metamorphosis. The anuran amphibian, the frog, undergoes extensive changes to metamorphose from a larval, aquatic, herbivorous tadpole into a juvenile, terrestrial, carnivorous frog. Metamorphosis affects nearly every tissue or organ in the tadpole and many of these changes are similar to the changes foetuses undergo during postembryogenesis (Shi, 1996; Shi, 2000).

This present study used the North American bullfrog*, Rana catesbeiana* (*R. catesbeiana*) tadpoles to study the effects chemicals have on the TH signalling pathway. *R. catesbeiana* is found on every continent, with the exception of Antarctica,

which makes using them as a model organism relevant to most parts of the world (AmphibiaWeb, 2010; GlobalInvasiveSpeciesDatabase, 2010). *R. catesbeiana* tadpoles are also large and make working with them easier, as there is more tissue available, and therefore, more genomic material available to work with. Currently there is a decline of *R. catesbeiana* species in some parts of the world. Anurans are also considered to be sentinel species because of their wide global distribution, their close proximity to potentially contaminated water, as well as their sensitivity to environmental pollutants (Kloas *et al.*, 1999; Kloas, 2002).

There are three major changes that take place during metamorphosis and these include: i) the death and resorption of larval type organs and tissues used only by the tadpole (eg. tail) ii) remodelling of the larval organs to adult form and function (eg. intestine), which involves the coordinated apoptosis of larval cells with the proliferation and differentiation of adult precursor cells, and iii) *de novo* development of new organs and tissues for adult use (eg. hindlimbs).

Metamorphosis can be separated into 3 distinct periods: premetamorphosis, prometamorphosis, and metamorphic climax (Shi, 2000) (Figure 1.3A). There are staging systems based on a tadpoles' morphology used to distinguish the different developmental stages a tadpole undergoes. This manuscript uses the Taylor and Kollros (TK) system to refer to *R. catesbeiana* tadpole stages (Taylor and Kollros, 1946). Premetamorphosis (TK stages I-IX) is primarily for tadpole growth and takes

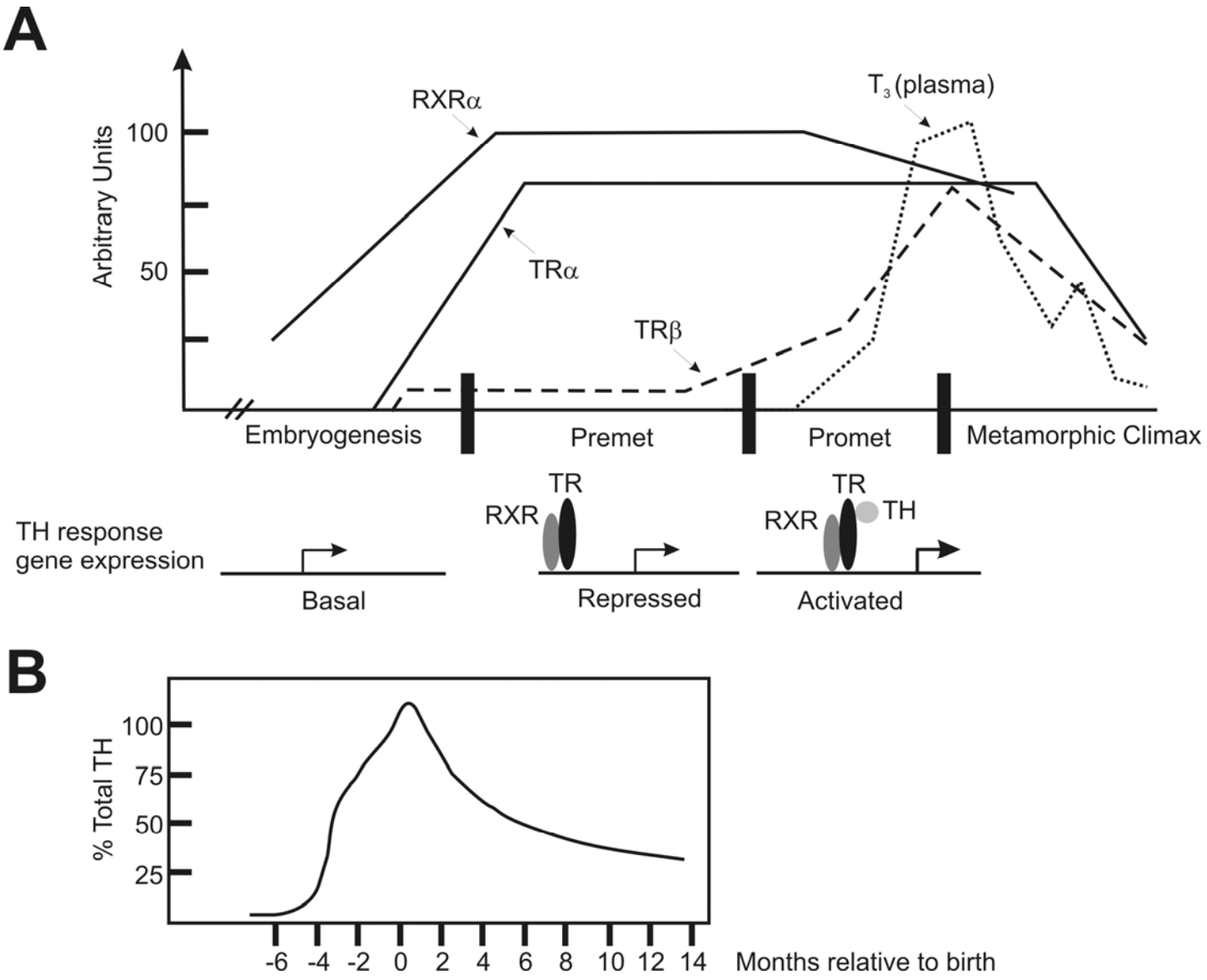


Figure 1.3. Stages of metamorphosis (A) and a graph outlining the increase in TH levels that take place at birth (B). (A) These are the stages a tadpole undergoes when progressing through metamorphosis. The levels of T_3 increase in amount until metamorphic climax. TRβ levels also increase to maximum levels during metamorphic climax. RXRα and TRα are present during premetamorphosis; however, transcription is repressed due to the absence of TH. Once TH is present, TH-responsive genes can undergo transcription. Adapted from (Das *et al.*, 2010). (B) The surge in the TH levels that takes place around birth is very similar to the surge in TH levels that occurs in amphibians around the time of metamorphic climax. Adapted from (Tata, 1993).

place in the absence of TH, as the thyroid gland is immature at this point; the tadpole is functionally athyroid. There is also limited development of the hindlimbs, from undifferentiated mesenchyme cells, that occurs during premetamorphosis and the hindlimb buds continue to grow with the tadpole. The next stage in development is prometamorphosis (TK X to XIX) and is characterized by the rising endogenous concentrations of TH due to the increase in growth of the thyroid gland. The hindlimbs undergo morphogenesis at this stage and includes toe differentiation and rapid and extensive growth of the limbs. The final stage in metamorphosis is metamorphic climax (TK stage XX to XXIV) and is the stage where TH reaches peak endogenous levels. The surge in TH levels that takes place during this period is similar to the surge in TH levels that take place in humans just before birth (Figure 1.3) (Tata, 1993). The peak plasma concentrations reached for either T_4 and T_3 in *R. catesbeiana* are approximately 10 nM (White and Nicoll, 1981). Many morphological changes take place during this period but perhaps the most striking is the apoptosis and complete resorption of the tail. The gills also undergo resorption and the beginning of gill resorption takes place around the same stage or slightly earlier than tail resorption. The intestine changes from a tubular structure to a structure with many epithelial folds, which increases the surface area of the intestine to allow for more nutrient absorption. The intestine begins to undergo metamorphosis at around the onset of metamorphic climax and continues until the end of metamorphosis. The end of the tail resorption marks the end of metamorphosis at TK stage XXV and TH levels begin to decrease to suprabasal levels in the juvenile at this point. There are many other organs, which undergo changes during metamorphosis; the nervous system undergoes extensive

reorganization, which includes degeneration, *de novo* development, and differentiation (Dodd and Dodd, 1976; Shi, 2000).

The process of metamorphosis is completely dependent on TH and can be inhibited by blocking the synthesis of TH or by removing the thyroid gland. Conversely, exposing premetamorphic tadpoles to exogenous TH, through injection or through exposure in the water, can precociously induce metamorphosis; removal of the thyroid gland or exposure to TH inhibitors can inhibit metamorphosis from occurring. Not only can a tadpole be induced to undergo metamorphosis but the organs, such as the tail, of a tadpole can be exposed to TH in culture *in vivo* and undergo metamorphosis the same as it would naturally (Tata, 1968; Ji *et al.*, 2007).

As in humans, the secretion of TH is neuroendocrine control through the hypothalamus-pituitary-thyroid (HPT) axis (Figure 1.2). In tadpoles, the hypothalamus responds to environmental stimuli and releases corticotropin releasing factor (CRF). CRF is the larval equivalent to mammalian TRH and acts directly on the pituitary thyrotropes and corticotropes to stimulate the release of TSH and adrenocorticotropin, respectively (Denver, 1998). Once TSH is released, it acts on the thyrotropes of the thyroid gland to synthesize and release T_4 and T_3. TSH is present in prometamorphs and increases in concentration at early metamorphic climax and then the levels drop, at the end of metamorphosis, to levels lower than found in the prometamorphic period. The drop in TSH levels coincides with the peak TH levels. The tadpole feedback system to control the levels of TH is similar to the humans HPT axis feedback system; TH inhibits both the release of TSH and CRF (Denver, 1997; Okada *et al.*, 2000).

There are other hormones in the tadpole endocrine system, which are also involved in metamorphosis. CRF stimulates the release of adrenocorticotropin releasing hormone (ACTH) from the corticotropes of the pituitary, and ACTH acts on the interrenal glands to produce the corticoid steroids, cortisone and aldosterone; both cortisone and aldosterone have binding sites present in the tail. Corticoids act through their nuclear receptor, glucocorticoid receptor (GR), and are TH-inducible in the tail but repressed in the brain. Cortisone and aldosterone concentrations have been shown to increase simultaneously with TH levels (Jaffe, 1981; Krug *et al.*, 1983; Jolivet Jaudet and Leloup Hatey, 1984; Kikuyama *et al.*, 1986; Tonon *et al.*, 1986; Kikuyama *et al.*, 1993; Hayes, 1997). Corticoids accelerate metamorphosis at high TH concentrations and antagonize metamorphosis at low TH levels (Hayes *et al.*, 1993; Kikuyama *et al.*, 1993; Krain and Denver, 2004).

Gonadal steroids, testosterone and estradiol, are thought to inhibit metamorphosis. It is believed gonadal steroids act on the HPT axis, since these effects have only been observed in whole animals (Gray and Janssens, 1990; Hayes, 1997). In *Xenopus laevis* estrogen treated embryos, there was suppression of nervous system development suggesting that estrogen exposure inhibited brain development (Nishimura *et al.*, 1997). Estrogen has been shown to accelerate metamorphosis in *Bufo bufo* (Frieden and Naile, 1955). In *Silurana (Xenopus) tropicalis* estrogen receptor (ER) transcript levels change throughout development with *ERα* low in the brain, liver, and gonad/kidney at Nieuwkoop and Faber (NF) stage 51 (premetamorphosis) (Nieuwkoop, 1994) and *ERβ* high in the brain, low in the liver and gonad/kidney at NF stage 51. At NF stage 60 (metamorphic climax) both *ERα*

and *ERβ* transcript levels were high in brain, liver, and gonad/kidney and their levels remained high throughout metamorphosis (Takase and Iguchi, 2007). Also in *S. tropicalis*, sex steroid synthesis enzymes are active in embryos and exposure to an aromatase inhibitor affected expression of TR and DIO during early development (Langlois *et al.*). Androgen receptor gene transcripts are positively regulated by T_3 in *S. tropicalis* (Duarte-Guterman *et al.*) and blocking TH production with ammonium perchlorate during *X. laevis* development resulted in a female-biased sex ratio (Goleman *et al.*, 2002) suggesting TH is involved in male development. In the brain and gonad-mesonephros complex (GMC) of *S. tropicalis*, sex steroid related genes are responsive to TH, suggesting there is crosstalk between TH and sex steroids in the brain and the GMC (Duarte-Guterman and Trudeau; Duarte-Guterman and Trudeau).

Prolactin (PRL) has been shown to be antimetamorphic. TH indirectly causes the pituitary lactotropes to release PRL. In the tadpole tail, the PRL receptor increased in expression at metamorphic climax. Exogenous PRL prevented TH-induced expression of TH receptors which inhibited the regression of the tadpole tail (Tata *et al.*, 1991; Baker and Tata, 1992; Hasunuma *et al.*, 2004).

These additional hormones involved in metamorphosis play an important role in coordinating the metamorphosis of the different tissues and organs but only TH is capable of inducing the metamorphic process. These additional hormones have specific actions at different concentrations of TH, and different metamorphic stages. Their control is dependent on the timing of metamorphosis, which is dependent on environmental stressors. Therefore, at high levels of TH, a hormone may have

inhibitory or stimulatory effects on a specific tissue, but not have any effect on another tissue. These specific actions of the hormone are dependent on the presence and level of receptors and downstream effectors.

1.4 Thyroid Hormone Metabolism

The actions of TH must be tightly regulated so the tadpole can undergo proper metamorphosis. The levels and actions of TH are controlled at multiple levels. There are 3 ways in which TH is regulated: i) synthesis and secretion of TH in the thyroid gland, ii) proteins that bind TH and control the levels of free TH, and iii) metabolic enzymes which act on TH to either activate or inactivate it (Shi, 2000).

THs are synthesized in the follicles of the thyroid gland (Figure 1.4). The follicles are made up of thyrocytes, which surround a lumen that contains colloid which contains TH precursor, thyroglobulin. Thyroglobulin contains many tyrosine residues. The base of the thyrocytes is exposed to the circulating plasma and contains a sodium-iodine symporter pump; inorganic iodine (I^-) is pumped into the thyrocytes from the plasma and diffuses across the apical side into the lumen. The integral membrane enzyme, thyroid peroxidase, converts the inorganic iodine into active iodide and then iodinates the tyrosine residues on thyroglobulin. The iodination of thyroglobulin produces 3-monoiodinated (MIT) and 3,5-diiodinated (DIT) tyrosine residues. Thyroid peroxidase then couples MIT and DIT to form TH. MIT and DIT combine to produce T_3; DIT and DIT combine to produce T_4. TH remains linked to the thyroglobulin protein to form the colloid. TSH stimulates pinocytosis of the iodinated thyroglobulin protein from the colloid, inclusion, and fusion with the lysosomes. T_4 and T_3 are released from

thyroglobulin through hydrolysis and T_4 and T_3 enter the blood stream. Currently the exact mechanism of TH secretion from the follicular cells into blood is unknown; however, a recent study demonstrated that monocarboxylate transporter 8 (MCT8), a TH transmembrane transporter is localized at the basolateral membrane of thyrocytes and is involved in the secretion of TH from the thyroid gland in mice (Di Cosmo, 2010).

TSH up regulates the sodium-iodine symporter and the thyroglobulin protein within the thyrocytes. The release of TH has a negative feedback effect on the pituitary to inhibit the release of TSH (Figure 1.2) (Denver, 1997; Levy *et al.*, 1997; Fort *et al.*, 2007). TH synthesis is highly conserved between mammals and anurans. Currently the exact mechanisms whereby the developmental levels of plasma TH are regulated are unclear but it is believed to be a combination of growth and maturation of the thyroid gland, and hormonal cues.

T_4 is the predominant form that is released from the thyroid gland; however, there is a small amount of T_3, the more biologically active form that is released from the thyroid gland as well (Figure 1.4). T_3 can bind to its receptors at a 5-10 fold higher affinity. About 30% of the T_3 is released from the thyroid gland and the other 70% is produced in the target tissues' cells from T_4 (Utiger, 1995; Fort *et al.*, 2007). T_4 can be converted to T_3 by the enzyme 5'-deiodinases, type I (DIOI) and type II (DIOII) (Figure 1.1). DIOI is the only deiodinase able to remove iodine from both the inner and outer rings (Fekkes *et al.*, 1982). In rat, DIOI is expressed in the liver, kidney, central nervous system (CNS), pituitary gland, thyroid gland, intestine, and placenta. In humans, DIOI is absent from the CNS but is present in the liver, kidney, thyroid, and pituitary (Campos-Barros *et al.*, 1996; Nishikawa *et al.*, 1998). TH can increase the activity and transcript

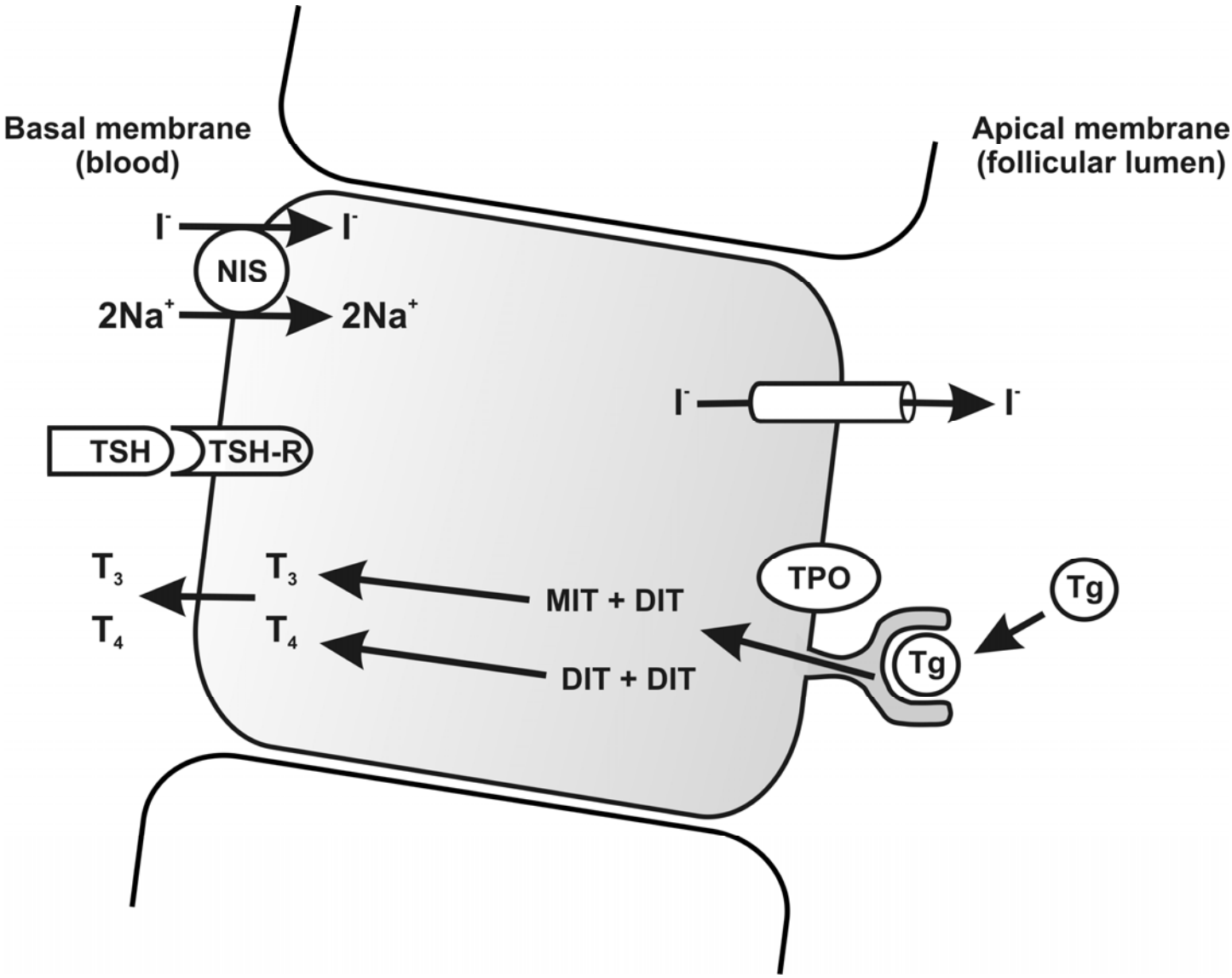


Figure 1.4. Schematic representation of the synthesis of thyroid hormone (TH; T_3 and T_4) in thyroid gland follicular cells, thyrocytes. The thyrocytes surround the follicular lumen that contains colloid, which contains the TH precursor, thyroglobulin (Tg). The base of the thyrocytes is exposed to the circulating plasma and contains a sodium-iodine symporter pump (NIS); inorganic iodine (I^-) is pumped into the thyrocytes from the plasma and diffuses across the apical side into the lumen. The integral membrane enzyme, thyroid peroxidase (TPO), converts the inorganic iodine into active iodide and then iodinates thyroglobulin. Thyroid stimulating hormone (TSH) binds to its receptor (TSH-R) and stimulates pinocytosis of the iodinated thyroglobulin protein from the colloid. TSH also up regulates the sodium-iodine symporter and the thyroglobulin protein within the thyrocytes. The iodination of thyroglobulin produces 3-monoiodinated (MIT) and 3,5-diiodinated (DIT) tyrosine residues. TPO then couples MIT and DIT to form TH. T_4 and T_3 diffuse into the plasma. Adapted from (Boas *et al.*, 2006).

levels of DIOI in rats, mice, and humans (Berry *et al.*, 1990; Berry *et al.*, 1991); in humans, the DIOI gene contains two TH response elements (TREs) in the 5'-flanking region of the gene (Toyoda *et al.*, 1995; Jakobs *et al.*, 1997; Jakobs *et al.*, 1997; Zhang *et al.*, 1998). 5-deiodinase (type III; DIOIII) inactivates T_4 and T_3 by the removal of an iodine molecule on the inner ring to produce reverse-T3 (rT_3) and T_2, respectively (Figure 1.1). Reverse-T_3 and T_2 can be further deiodinated to produce T_0 by both DIOII and DIOIII. The kidneys excrete T_0 (Davey *et al.*, 1995; St Germain and Galton, 1997). In *R. catesbeiana* 5'-deiodinase activity is regulated in a tissue- and stage-dependent manner. The enzyme activity is not detected in the liver, kidney, or red blood cells, and low levels of activity are detected in the brain and heart. The activity of 5'-deiodinase is highest during metamorphic climax, with the highest amount of activity found in tissues undergoing metamorphosis, and little activity present during pre- and prometamorphosis. As mentioned above, 5'-deiodinase makes a more active form of TH by converting T_4 into T_3 and 5-deiodinase inactivates THs; therefore, it is important to regulate and balance the levels of both these enzymes.

5-deiodinase activity is also regulated in a tissue- and stage-dependent manner. 5-deiodinase levels are high in the tail before resorption takes place, which is just prior to the peak activity levels reached by 5'-deiodinase (St Germain *et al.*, 1994; Becker *et al.*, 1997).

TH is removed from the plasma by uridine disphosphate glucoronyltransferase (UDP-GT) (DeVito *et al.*, 1999). UDP-GT is present in the liver and glucorodinates THs and causes them to be eliminated in bile. UDP-GT is a major phase II biotransformation family of enzymes and is under control of the pregnane X receptor

(PXR). PXR is a nuclear receptor that forms a heterodimer with 9-cis-retinoic acid receptors (RXRs) when binding to DNA (Chen *et al.*, 2003). There is very little free circulating TH in the blood plasma. Most TH is bound by binding proteins (THBP). The THBPs include serum albumin, thyronine binding globulin (TBG), transthyretin (TTR), and lipoproteins (Shi, 2000; Fort *et al.*, 2007). THBPs play many important roles in regulating TH. They act to increase the lifespan of TH, as a buffer for TH levels, and a reserve for TH (Shi, 2000). TBG is the main THBP in humans and carries both T_3 and T_4, while in rodents THBPs are serum albumin and TTR, which only carries T_4. During metamorphosis, the major THBP in tadpoles is TTR and carries both T_3 and T_4; however, T_3 binds TTR at a much higher affinity than T_4.

The lipophilic THs are thought to enter the cells through multiple ways, including: diffusion of free TH, diffusion as a complex with a THBP, or through active transport. Recently there have been studies to suggest that TH enters the cell through active transport using amino acid transporters (Ritchie *et al.*, 2003). This was shown in *R. catesbeiana* blood cells where the aromatic amino acid transporter (system-T)-linked transporter actively imported TH into the cell.

Once TH enters the cell, they are bound to cytoplasmic TH binding proteins (CTHBP). CTHBP also play an important role in regulating TH. CTHBPs have multifunctional roles (eg. Pyruvate kinases, myosin light chain kinase, and disulfide isomerase) and it is thought there are 4 roles CTHBPs have with respect to regulating TH signal transduction: i) import TH from the extracellular matrix to inside the cell, ii) intracellular TH metabolism ,iii) transport TH to the nucleus, and iv) buffer to modulate the free TH concentration (Shi, 2000).

1.5 Thyroid Hormone Receptors

The actions of TH are largely facilitated through the nuclear thyroid hormone receptor (TR), a member of the nuclear hormone superfamily (Tata and Widnell, 1966; Tata, 1967; Oppenheimer *et al.*, 1979; Buchholz *et al.*, 2006; Yen *et al.*, 2006). THs interact with the TRs to cause changes in gene expression. THs are also known to act through non-genomic actions (Ji *et al.*, 2007; Davis *et al.*, 2008; Skirrow *et al.*, 2008); however, the majority of the actions of TH are through the TRs. The TR is located in the nucleus and binds chromatin in both the absence and presence of TH. T_3 binds TR with 5-10 fold higher affinity than T_4 (Shi, 2000). T_4 is thought to only have biological effects once it is converted into T_3 by DIOII (Figure 1.1), because inhibition of DIOII has been shown to also inhibit the biological effects of T_4. However, there is evidence that T_4 has its own unique effects independent of conversion to T_3 in the brain (Helbing *et al.*, 2007).

TRs were first cloned in 1986 in chicken and humans (Sap *et al.*, 1986; Weinberger *et al.*, 1986). Vertebrates, including *R. catesbeiana*, have two TR genes (Davey *et al.*, 1994; Bassett *et al.*, 2003) on chromosome 17 and 3 that encode for TRα and TRβ, respectively. TRα and TRβ bind T_3 with similar affinities. In humans and mice, the TRα locus encodes four proteins but only one, TRα1, is the true nuclear receptor (Figure 1.5). TRα2, TR$\Delta\alpha$1, TR$\Delta\alpha$2 are antagonists of TRα1 because these isoforms bind to DNA but not to TH, which is a mechanism to suppress the expression of genes containing the thyroid hormone response element (TRE). (Koenig *et al.*, 1989; Chassande *et al.*, 1997; Plateroti *et al.*, 2001). The TRβ locus encodes 2 receptors: TRβ1 and TRβ2. TRβ1 and TRβ2 differ in length in the amino-termini

(Flamant and Samarut, 2003). The rat TRβ locus also encodes TRβ3 and TRΔβ3 isoforms (Williams, 2000). *R. catesbeiana* is known to have one isoform of each gene (Helbing *et al.*, 1992; Schneider *et al.*, 1993; Davey *et al.*, 1994). Anurans express TRα during embryogenesis and it remains present throughout postembyronic development and metamorphosis. TRβ is not present during embryogenesis and the levels have been found to increase with the increasing concentrations of TH (Figure 1.3A) (Eliceiri and Brown, 1994).

The TRs have five domains, A/B, C, D, E, and F, as listed from N-terminal to C-terminal end (Figure 1.5). There is a high-degree of conservation between the TR and other members of the nuclear hormone superfamily with differences in the hormone-binding domain (Zhang and Lazar, 2000)). The A/B domain, also known as the activation function (AF-1) domain, is variable in sequence (comparing TRα) and length (comparing TRβ isoforms). The AF-1 can function to recruit coactivators independent of T_3 (Yaoita *et al.*, 1990). The C domain is the DNA binding domain (DBD) and is the most highly conserved domain among other nuclear hormone receptors. The DBD, which also serves as the dimerization domain, consists of 2 adjacent zinc fingers, which contains 2 histidine and 2 cysteine residues. The DBD binds to TH response element (TRE) in the promoter or enhancer regions of TH-responsive genes (Yen, 2001). The D domain is the variable hinge region but also carries the nuclear localization signal as wells as transactivation and DNA binding functions. The E domain, along with the F domain, is the ligand, or hormone, binding domain (LBD) and is highly conserved among TRs in different species but has a low

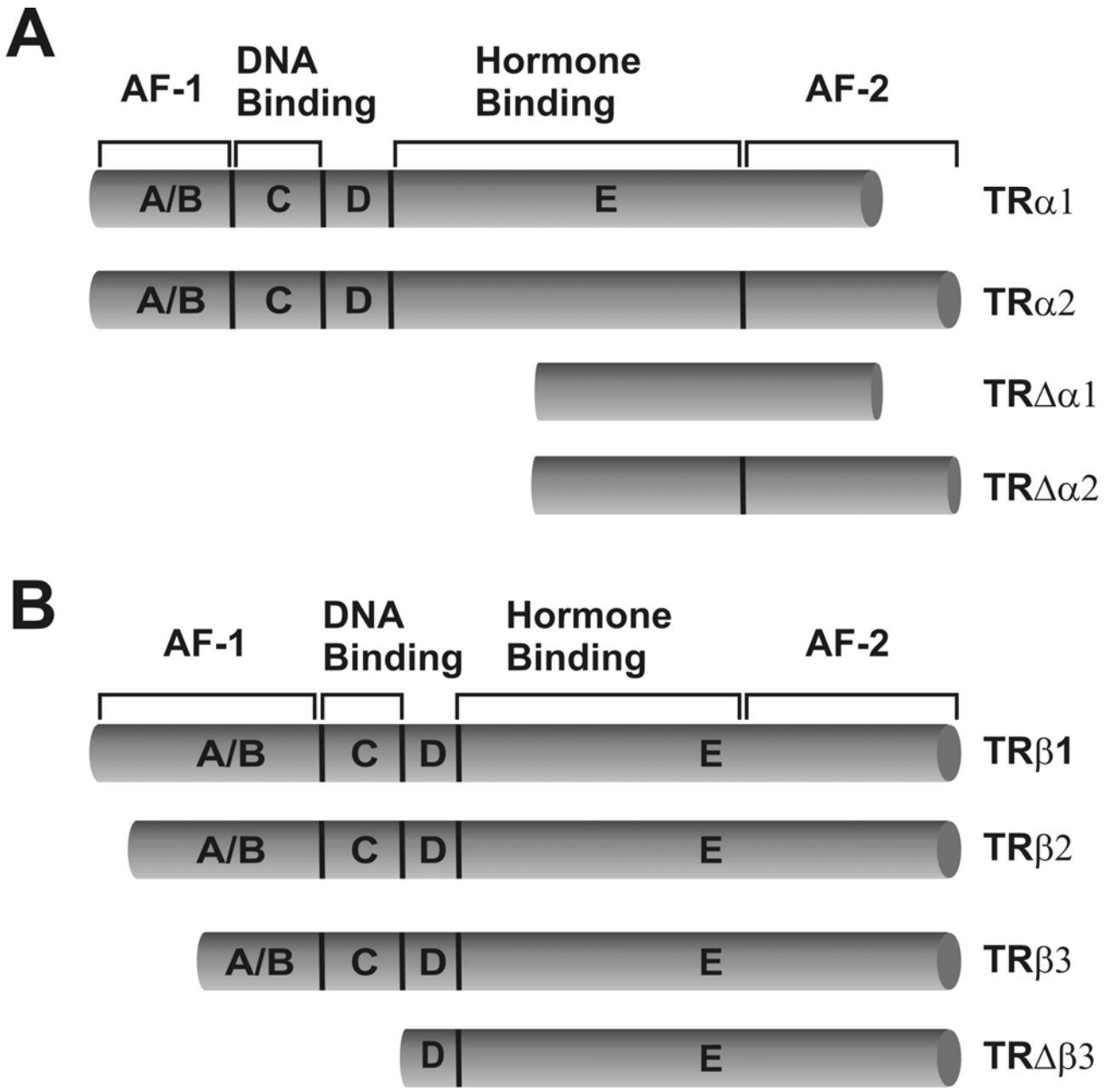


Figure 1.5. Various isoforms encoded by the thyroid hormone receptor alpha (TRα) (A) and thyroid hormone receptor beta (TRβ) (B) genes. The isoforms are generated through alternative splicing or use of different promoters. This schematic represents the different domains involved TR function which include the DNA-binding domain (DBD; C) and hormone-binding, or ligand-binding domain (LBD; E); these are specifically present in TRα1, TRβ1, TRβ2, and TRβ3 proteins, which act as the true T_3 nuclear receptors. TRα2 and truncated TRΔ lack either one of both of the DBD and LBD. Co-factor binding domains are located in A/B, D, and E; dimerization domains are located in domains C and E. AF-1 and AF-2 domains are important for transcriptional activation. Adapted from (Kress *et al.*, 2009).

level of homology among other members of the nuclear hormone receptor superfamily. The E domain functions in transcriptional activation and repression (Yen, 2001).

TRs can bind to DNA as monomers, homodimers with each other, or heterodimers with RXRs. There are three RXRs: RXRα, RXRβ, and RXRγ. The TR-RXR heterodimer is considered to be the most stable and the facilitators of TH genomic action. TR-RXR specifically recognizes the TRE and interacts with transcription factors (Wong *et al.*, 1995). TRs bind DNA in both the presence and absence of ligand and, unlike some other members of the steroid/TH nuclear receptor family, possess a dual function (Das *et al.*) in the activation and repression of genes depending upon TH status (Figure 1.3A). There is little or no binding of TR-RXR heterodimer to endogenous TREs during embryogenesis, but TRs are present from maternal stores in the oocyte and play an important role in conferring competence of tadpoles to respond to TH and in coordinating metamorphosis. This will be discussed later in the chapter.

TH can both up-regulate and down-regulate genes. Up-regulated TH-responsive genes are repressed in the absence of TH, and activated upon the addition of TH. The binding of TR-RXR to TRE is independent of the presence of TH (Figure 1.3A). In the absence of TH, the TR-RXR heterodimer is bound to the TRE and also interacts with corepressors such as silencing mediators of receptors of TH (SMRT) and nuclear receptor corepressor (N-CoR). SMRT and N-CoR interact with the LBD of both the TR and RXR and have been shown to form a complex binding to transcriptional repressor Sin3A, which in turns interacts with histone deacetylases (HDACs). The

activity of the HDACs leads to a more compact chromatin structure, which can inhibit transcription (Yen, 2001; Yen *et al.*, 2006; Yen *et al.*, 2006).

When TH is present, it binds to the TR and induces a conformational change in the protein. Upon TH binding, corepressors are released and coactivators such as cAMP response element binding protein (CBP/p300), steroid receptor coactivators (SRC/p160), p300/CBP-associated factor (P/CAF) are recruited (Yen, 2001; Bassett *et al.*, 2003)). The AF-2 domain plays a critical role in interacting with the coactivators. These coactivators have intrinsic histone acetyltransferase (HAT) activity and can increase gene transcription. Other coactivators that do not have intrinsic HAT activity are TR associated proteins (TRAPs) and Vitamin D receptor interacting proteins (DRIPs). TRAP and DRIP form a mediator complex that is associated with the recruitment and activation of RNA polymerase II (Bassett *et al.*, 2003).

The mechanism of TH-down-regulated genes is not well understood. In terms of TH-downregulated genes, the TR-RXR heterodimer complex is bound to the TRE in the absence of TH yet transcription is stimulated. When TH is present and binds TR, transcription is inhibited. It is thought the position of the TRE, creating a negative-TRE (nTRE), and different coregulators play a role in TH-downregulated genes. nTREs are usually located close to the transcription start site or downstream of the TATA box. These have been found in TH-down-regulated genes such as TSH and TRH (Shibusawa *et al.*, 2003). The absence of TH causes a different conformation of the TR on a nTRE than on a TRE associated with up-regulated genes, and still causes recruitment of corepressors such as SMRT and N-Cor even though there is high level of gene transcription taking place (Eckey *et al.*, 2003).

cTH binding to TRs on nTREs causes HDAC recruitment followed by inhibition of transcription. The majority of the mechanism of TR gene regulation is based on *in vitro* studies (Buchholz *et al.*, 2006). Recently Wang *et al* (2009) demonstrated TH-up-regulated and down-regulated gene mechanisms have more in common than previously thought. In rat pituitary cells, T_3 decreased transcription and increased HAT activity of the TSHα promoter, a known TH-down-regulated gene. Histone H3K9 and H3K18, whose acetylations are associated with transcriptional activation, were acetylated in the negatively regulated TSHα promoter. Chromatin immunoprecipitation assays showed the addition of T_3 caused the release of a corepressor complex composed of HDAC3, transducin b-like protein (TBL1), N-CoR, and SMRT. Interestingly, an overexpression of the corepressors N-CoR and HDAC3 caused an increase of the T_3-independent basal transcription. This demonstrates there are many similarities between TH-up-regulated and –down-regulated genetic mechanisms (Wang *et al.*, 2009).

1.6 Thyroid Hormone Non-Genomic Actions

As mentioned above, TH also has nongenomic actions, which are largely, mediated through signal transduction pathways, causing an effect without first affecting transription. In mammalian cells, nongenomic actions are considered to be rapid and in the order of seconds to minutes; there is no requirement for protein synthesis to take place, and it is independent of nuclear TRs (Shi *et al.*, 1996; Bassett *et al.*, 2003; Davis *et al.*, 2005; Davis *et al.*, 2008). The TH nongenomic mechanisms are equally responsive to T_4 and T_3 and in some cases more responsive to T_4. Some

of TH nongenomic actions include changes in cell morphology, respiration (mitochondrial function), and ion homeostasis. TH exerts these effects through multiple pathways. TH can bind to cell surface proteins, for example the cell surface protein integrin αvβ3 binds strongly to T_4 (Davis *et al.*, 2005). Binding of T_4 to integrin aVb3 affects cell-extracellular matrix interactions and triggers intracellular signalling processes (Davis *et al.*, 2005). TH also binds to cytosolic proteins and often these proteins have different functions, such as enzymes. Binding of TH to these cytosolic proteins may have an effect on the additional functions of these proteins (Parkison *et al.*, 1991; Shi *et al.*, 1994). Also, a small amount of the TRβ can exist in the cytoplasm and in TH-treated cells TRβ can form a complex with MAPK. Unliganded TRβ can also interact with PI3K in the cytoplasm (Davis *et al.*, 2005; Storey *et al.*, 2006; Guigon and Cheng, 2009).

Nongenomic actions and genomic actions of TH have been shown to crosstalk and there has been evidence to show the importance of interaction between kinase signaling cascades and TH signaling pathways in determination of cell and tissue fate (Skirrow *et al.*, 2008). Skirrow et al. (2008) showed roscovitine, a Cdk inhibitor, to prevent T_3-induced regression of cultured *R. catesbeiana* tail tips suggesting phosphorylation is important in establishing the T_3-dependent proapoptotic gene expression program. Cyclin C, was also shown to be a novel T_3-responsive gene, suggesting Cdk8 to be the most likely candidate involved in tail regression (Skirrow *et al.*, 2008). Another protein, which appears to play an important role in the regression of the tadpole tail, is protein kinase C. Genistein, a tyrosine kinase inhibitor, prevented T_3-induced regression in *R. catesbeiana* tadpole tails and T_3-induced up-

regulation of TRβ transcript levels, suggesting T_3-induced tail regression is dependent on tyrosine kinase signaling. Genistein effects were more potent within the first 24 hours after T_3-injection and less so 48 hours after injection, suggesting tyrosine kinase signaling may be important in the genetic program transition and phosphorylation events are also important in T_3-induced tail tip regression (Ji *et al.*, 2007). Therefore, it appears both nongenomic and genomic actions play a role in mediating TH action and there is also evidence for crosstalk between the two actions.

1.7 Regulation of Metamorphosis

Metamorphosis is a complex program that requires different tadpole tissue and organs to develop *de novo* or resorb at specific time points throughout the metamorphic program. THs control the diverse cellular processes that take place during metamorphosis and they also control the balance between cell proliferation and differentiation. The diverse actions of THs on organ targets depend on: specific TR expression in the target organ, local transport and metabolism of T_3 and T_4, cell type, the developmental stage (progenitor or differentiated), pathophysiological state (normal or tumour cell), and cellular context (Kress *et al.*, 2009). THs and TRs play their roles by interacting with other signalling pathways in a cell-specific manner (Kress *et al.*, 2009). THs and TRs modulate cell proliferation through modification of expression of different genes/proteins involved in the cell cycle control: growth factors, cell surface receptors, proteins acting on cell membrane, transcription factors, and cyclins (Kress *et al.*, 2009).

It is important for the *de novo* synthesis of the fore- and hindlimbs to be complete before the tadpole completely resorbs the tail. Also, a tadpole must have functioning lungs before it loses function and resorbs the gills. It is because of this critical sequence of events that the development of the hindlimbs begins to take place in premetamorphosis and the tail is not resorbed until metamorphic climax, where the complete resorption of the tail marks the end of metamorphosis (Shi, 2000). Both TH and TR play key roles in regulating the timing of metamorphosis, with TR controlling the development of the tadpole in both the absence and presence of TH. During embryogenesis, TR, RXR, and TH are not present. The TH and TR do not regulate TH-inducible genes when the hormone and receptor levels are absent or low, allowing basal transcription (Figure 1.3A) to take place, which in turn allows for proper embryonic organ development (Eliceiri and Brown, 1994; Wong and Shi, 1995). After tadpole hatching, TRα expression increases and reaches maximum levels around the time the tadpole begins to feed; RXR levels follow a similar pattern to the levels of TRα. The TRα - RXRα heterodimers, when bound to TH-up-regulated genes, promote recruitment of corepressors and repression of gene transcription (Figure 1.3A). Unliganded TR-RXR in premetamorphic tadpoles regulates the timing for the initiation of metamorphosis to ensure the tadpole undergoes proper growth and prevents early metamorphosis (Puzianowska-Kuznicka *et al.*, 1997; Sato *et al.*, 2007). When TH levels begin to increase, the TR-RXR complex can activate TH-responsive genes, and metamorphosis begins to take place (Figure 1.3A).

TRs control anuran growth and development before the onset of metamorphosis is to take place; however, once TH is present, TH levels play an important role in

controlling the timing of morphogenesis in different tissues at different stages throughout metamorphosis. For example, the hindlimbs begin to develop *de novo* in premetamorphosis but the tail does not undergo tail resorption until late in metamorphosis. The hindlimb development begins when T_4 levels are at 1-2 nM and T_3 is nondetectable (Leloup and Buscaglia, 1977). CTHBP and 5-deiodinase are repressed and 5'-deiodinase activity is high (Shi *et al.*, 1994; Becker *et al.*, 1997). TRα, RXRα, and RXRγ are highly expressed followed by a reduction in their expression (Figure 1.3) (Wong and Shi, 1995). Recently Trudeau's group developed a complete developmental profile for TRs and DIOs (DIOI, DIOII, and DIOIII) during embryogenesis and early larval stages in *S. tropicalis*. Whole embryos and larvae contain *TRβ* and all three *DIO* transcripts and *TRα* transcript only appear after gastrulation. *TRβ* and *TRα* transcripts significantly increase before hatching. *TRs*, *DIOII* and *DIOIII* transcripts were able to respond to T_3 exposure in a dose dependent manner as early as NF stage 46 (feeding tadpole stage) (Duarte-Guterman and Trudeau).

The TH-TR complex has a low K_d (0.1 nM), and therefore, only a small amount of T_3 is needed to carry out this action. Conversely, tail resorption begins to take place immediately following the point when peak TH levels are reached, which are approximately 10 nM. CTHBP and 5-deiodinase levels are high and decrease following metamorphic climax when the tail resorbs. 5'-deiodinase activity increases after metamorphic climax is reached. TRβ, RXRα, RXRγ are also increased in the tail during this time (Shi *et al.*, 1994; St Germain *et al.*, 1994; Becker *et al.*, 1997; Veldhoen *et al.*, 2002). *TRα* transcript levels increase in *R. catesbeiana*

metamorphosing tadpole tails. Veldhoen et al. (2006) measured *TRα* and *TRβ* transcript levels in *R. catesbeiana* tadpole tail during natural metamorphosis and saw an increase of the transcript levels of both receptors. Recent studies have shown TRα primarily plays a role in proliferating tissues, such as the limbs, where as TRβ is associated with regressing tissues such as the tail (Denver *et al.*, 2008).

Cofactors also play a role in regulating specific tissue responses. Tissue content and promoter context can affect cofactor specificity. For example, both the basic leucine-zipper motif-containing transcription factor (TH/bZIP) and the TRβ genes recruit the coactivator SRC3 in the intestine but only TH/bZIP recruits SRC3 in the tail (Paul *et al.*, 2005).

TH controls gene expression by activating and repressing genes within metamorphosing organs. The direct response genes of TH, then in turn, affect the expression of down-stream genes, which have an effect to the cell fate and in the end lead to metamorphosis. It is believed that some of TH direct response genes are ubiquitous throughout all tissues whereas other TH direct response genes are tissue specific (Shi, 2000).

1.8 Environmental Contaminants - Disruptors of Thyroid Hormone Action

Over the past 5 decades there has been increasing concern for human and wildlife exposure to the increasing amount of chemicals released into the environment. There is growing evidence to suggest many of these chemicals can act as endocrine disrupting chemicals (EDCs). An EDC is a chemical, which has the potential to interfere with the endocrine system of wildlife and humans at ecologically relevant

concentrations. They are exogenous substances that act like or on hormones in the endocrine system and disrupt the physiologic function of endogenous hormones. Traditional studies on environmental contaminants focused on the study of high-level concentrations of chemicals and their toxicity with respect to the causation of overt damage, cytotoxicity, mutagenesis, and genotoxicity. Recently, the field of EDCs has grown and now a multitude of studies researched the consequences of low-dose exposures as well as the effects of mixtures of EDCs.

The majority of EDC research has focused on the disruption of the hypothalamus-pituitary-gonadal axis and the reproductive functions in wildlife and humans; however, there is growing concern about chemicals, which may disrupt the hypothalamus-pituitary-thyroid axis. It is estimated that 200 million people in the world and recent studies indicate that as many as 30% of Canadians have some form of thyroid disease (Thyroid Foundation of Canada, 2010); however, as many as 50% of people in Canada with thyroid disease are undiagnosed. The most common abnormality is the development of goitre, an enlargement of the thyroid gland, due to a TH deficiency because of a lack of iodine. Cretinism is also another disease associated with TH deficiency (Hetzel and Dunn, 1989; Hetzel and Mano, 1989). Cretinism is a mental deficiency and skeletal defects due to a lack of TH during foetal development (Hetzel and Dunn, 1989; Hetzel and Mano, 1989). Hypothyroidism can also be caused by an autoimmune defect, which targets TH producing cells of the thyroid gland. This type of hypothyroidism is known as Hashimoto's disease (McConahey, 1972). Conversely, hyperthyroidism is known as Grave's disease (Thyroid Foundation of Canada, 2010).

TH deficiency can be caused for many reasons, some of that include a lack of iodine, removal of TH gland, or absence of the gland because of congenital defects (Thyroid Foundation of Canada, 2010); however, there is reason to believe there are chemicals humans and wildlife are exposed to which may disrupt the normal actions of the TH axis. The Great Lakes are known to contain a large amount of EDCs and many top-level predator fish found have goitre (Leatherland, 1992). Currently there are now over 100 naturally and synthetic substances that have been reported to have thyroid disrupting effects (Boas, Feldt-Rasmussen et al. 2006).

TH disrupting chemicals can more specifically be defined as xenobiotics that alter the structure or function of the thyroid gland, alter regulatory enzymes associated TH homeostasis, or change circulating tissue concentrations of THs (Crofton *et al.*, 2005). There are a wide range of EDCs that act through a variety of mechanisms and these include (known or suspected mechanisms of actions): substances known to inhibit thyroidal iodine uptake such as perchlorate, nitrates, and thiocyanate; compounds with direct actions on the TR such as polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs), bisphenol-A (BPA), triclosan (TCS); compounds that displace T_4 from the serum TTR such as PBDEs, hydroxylated PCBs; compounds that inhibit TPO activity such as isoflavones; compounds that decrease T_4 half-life by activating hepatic clearance enzymes such as organochlorine pesticides, dioxins and furans. Compounds that have direct actions on the TR or interact with THBP or CTHBP may have structural similarities to TH. TH structure (Figure 1.1) resembles some polyhalogenated aromatic hydrocarbons (PHAHs) such as PCBs and brominated flame retardants (BFRs) and this could explain why PCB displaces T_4 from

TTR and TBG, causing an increase in T_4 excretion, as well as cause direct actions on the TR (Boas *et al.*, 2006). PCBs have also been correlated with decreased serum T_4 levels and increased TSH levels in numerous wildlife species as well as in rats and humans (Brucker-Davis, 1998; Leatherland, 2000; Zoeller, 2005; Boas *et al.*, 2006).

Several chemicals with known TH disrupting effects were examined in this thesis. Tetrabromobisphenol-A (TBBPA) (Figure 1.6A) is a type of BFR, which acts as a flame retardant which is commonly found in plastics, building materials, paints, and electronic equipment; it has structural similarity to T_4 (Figure 1.1). The use of TBBPA has been on the rise and between 1990 and 2000 the use of BFRs, and mostly TBBPA, has doubled (Law *et al.*, 2006). Similarly to PCBs, TBBPA decreased serum T_4 levels in rats, which may be due to an increase in the activity of UDP-GT causing an increase in the glucoronidation of T_4 and, therefore, increasing its removal through the bile (Legler and Brouwer, 2003). TBBPA bound human TTRs with almost a 10 times higher affinity than T_4; TBBPA also has been shown to compete for human TR *in vitro* (Meerts *et al.*, 2000; Kitamura *et al.*, 2005; Kitamura *et al.*, 2005). TBBPA had an effect on metamorphosis inhibiting tail fin shortening and limb development in tadpoles (Kitamura *et al.*, 2005; Veldhoen *et al.*, 2006; Fini *et al.*, 2007). TBBPA has been detected in human plasma and foetal tissue (Thomsen *et al.*, 2001; Ikezuki *et al.*, 2002; Schonfelder *et al.*, 2002) indicating the developing foetus is exposed to TBBPA. Given the evidence that TBBPA disrupts the normal actions of TH and the important role TH plays during development, it is of concern TBBPA has been detected in foetuses.

Acetochlor (Figure 1.6B) is another chemical with detected endocrine disrupting abilities. Acetochlor is an herbicide used at a rate of over 10 million kilograms per year primarily on corn crops (Barbash *et al.*, 2001). Although acetochlor does not have structural similarity to TH, it accelerated TH-induced precocious metamorphosis in *Rana pipiens* and *Xenopus laevis* (Cheek *et al.*, 1999; Crump *et al.*, 2002). This chemical was shown to increase TRβ transcript levels in the tail fins of both *Xenopus laevis* and *R. catesbeiana* tadpoles (Veldhoen and Helbing, 2001; Crump *et al.*, 2002). A cDNA array-based study showed that acetochlor increased TH-induced genes and attenuated the response of TH-responsive down-regulated genes (Helbing *et al.*, 2006). In premetamorphic *R. catesbeiana* tadpoles exposed to acetochlor and TH, there was an increase in the transcript levels of both TRα and TRβ in the brain (Helbing *et al.*, 2006). The mechanism whereby acetochlor acts is presently unknown; however, studies have shown that acetochlor does not bind TRβ but can bind to the estrogen receptor (Cheek *et al.*, 1999; Rollerova *et al.*, 2000).

Triclosan (TCS) (Figure 1.6C) is an antimicrobial agent found in numerous pharmaceuticals and personal care products (PPCPs). TCS works by blocking the active site of enoyl-acyl carrier protein reductase enzyme (ENR), which is the essential enzyme in fatty acid synthesis (Heath *et al.*, 2000). TCS blocks ENR, an enzyme humans do not have, therefore, preventing the synthesis of bacterial cell membranes and division. TCS can bioaccumulate in the tissues of wildlife and humans (McMurry *et al.*, 1998; Levy *et al.*, 1999; Heath and Rock, 2000). TCS has a structural similarity to TH (Figure 1.1) and has been shown to have TH disrupting effects. In *R. catesbeiana* premetamorphic tadpoles TCS accelerated T_3-induced

metamorphic hindlimb development as well as decreased the transcription levels of the *TR*α and *TR*β in tail fins (Veldhoen *et al.*, 2006). TCS also increased TH-induced *TR*β transcript levels in *X. laevis* XTC-2 and tail cells (Fort *et al.*; Veldhoen *et al.*, 2006; Fort *et al.*, 2010). Not only has TCS been shown to have effects in anurans but it has also been shown to have TH disrupting affects in rats by decreasing serum T_4 levels (Paul *et al.*). The mechanism by which TCS causes these adverse affects is currently unknown. Not only has TCS been found in the environment but its biological metabolite, methyl-TCS (mTCS; Figure 1.6D), has also been found in the environment. TCS and mTCS were detected in lakes and in a river in Switzerland at concentrations up to 74 and 2 ng/L, respectively (Lindstrom *et al.*, 2002). However, there is very little research done on the endocrine disrupting abilities of mTCS.

Also, another commonly used antimicrobial related to TCS is triclocarban (TCC; Figure 1.6E). TCC is also used in high amounts in PPCPs since 1957 and its use is estimated to be 1 million pounds per year (Sapkota *et al.*, 2007). With a half-life of 108 days in aerobic soil, TCC can be inadvertently used as an agriculture pesticide (Ying *et al.*, 2007). Very little research has been done on TCC and its potential as an EDC, and no studies have investigated potential effects of TCC on the HPT axis. Recently, TCC increased the transcription of testosterone-regulated genes in human embryonic kidney 293 cells that lack critical steroid metabolizing enzymes (designated as 2933Y cells) (Chen *et al.*, 2008). TCC exposure also increased the size of testosterone-dependent organs, such as the prostate gland, when fed to rats (Chen *et al.*, 2008). Both TCS and TCC are readily absorbed from the gastrointestinal tract

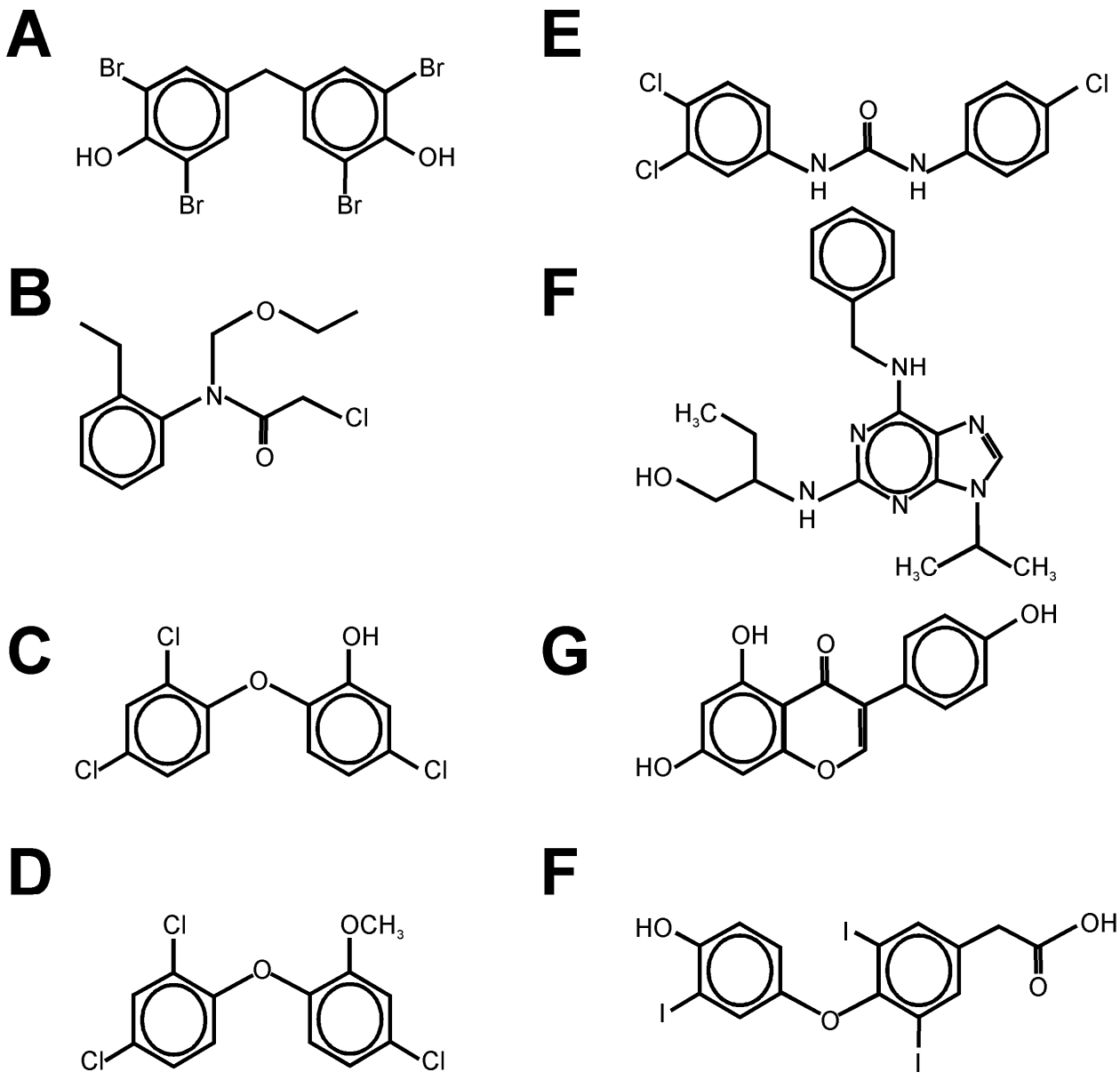


Figure 1.6. Structure of tetrabromobisphenol A (TBBPA) (A), **acetochlor** (Ace) (B), **triclosan** (TCS) (C), **methyl-TCS** (mTCS) (D), **triclocarban** (TCC), (E) **roscovitine** (F), **genistein** (G), and **Triac** (F).

and oral mucosa (Maibach *et al.*, 1971; Scharpf *et al.*, 1975; Sandborgh-Englund *et al.*, 2006).

Roscovitine (Figure 1.6F) is a cyclin-dependent kinase (Cdk) inhibitor. It has been shown to induce cell cycle arrest both in late G1 and late G2 phase in tobacco cells (Planchais *et al.*, 1997). It has also been shown to specifically inhibit the activity of Cdk1, Cdk2, and Cdk5 of animal cells and is a competitive inhibitor of ATP binding (DeAzevedo *et al.*, 1997; Meijer *et al.*, 1997). When cultured tail fins were exposed to both T_3 and roscovitine, the tail fins did not undergo tail regression and the T_3-induced increase of the *TR*β transcript was prevented (Skirrow *et al.*, 2008). The mechanism is thought to occur through the inhibition of the transcription modulatory Cdk8/Cyclin C activity, possibly through negative effects on RNA Polymerase II (RNA Pol II) (Bregman *et al.*, 2000; Skirrow *et al.*, 2008). The effect of roscovitine was found to occur at the level of initiation rather than during tail regression where it prevented the establishment of the genetic program at the commitment point (i.e. 24-48 h of T_3 treatment) required for tail regression (Skirrow *et al.*, 2008).

Genistein (Figure 1.6G) is one of several known isoflavones found in a number of plants with soybeans and soy products like tofu and textured vegetable protein being the primary food source. Genistein is a tyrosine kinase inhibitor, which has been shown to prevent T_3-dependent tail regression likely through inhibition of protein kinase C activity and TRβ phosphorylation (Ji *et al.*, 2007).

Triac (3,3'-5-triiodothyroacetic acid; Figure 1.6F) is a T_3 analogue that was also used in this study. Triac has a 8.8-fold higher affinity for TRβ than T_3 (Messier *et al.*, 2001).

1.9 Determination of Thyroid Hormone Disrupting Chemicals

In 2002, the World Health Organization (WHO), published criteria which outline whether an EDC can be considered a disruptor of the thyroid hormone action as follows: demonstrating a temporal relationship between exposures to the contaminant and changes in the HPT endpoints; demonstrating a strong association between exposure to the contaminant and effects on the HPT axis; demonstrating a consistent HPT response across multiple studies; determining the biological plausibility of the response; determining whether recovery of the HPT axis occurs following removal of the contaminant (Damstra *et al.*, 2002). There are numerous different endpoints that can be used to assess if a chemical is acting as a disruptor of TH action and these include: thyroid histopathology, measurements of plasma THBPs and THs, measurement of TH deiodination and metabolism, target tissue and receptor level endpoints. This thesis focuses on the molecular level endpoints through the measurement of the steady state mRNA levels of TH-responsive genes using the method of real time quantitative polymerase chain reaction (QPCR).

I studied the effects of known and potential TH-disrupting chemicals on the transcript levels of two different TH-response genes in the tail fin of *R. catesbeiana* using the C-fin assay developed in the lab. TRβ is an up-regulated TH early response gene, regulated within 24 hours of TH treatment; *Rana* larval keratin type I (RLKI) is a

down-regulated TH early response gene. RLKI was recently discovered in our lab. In *R. catesbeiana* tadpole tail fins exposed to TH, *RLKI* transcript levels decreased and its N-terminal caspase protein cleavage fragment increased (Domanski and Helbing, 2007). Two other gene transcript levels were measured, catalase (*CAT*) and heat shock protein 30 (*HSP30*). Both *CAT* and *HSP30* are indicators of cellular stress and they are both TH-responsive (Helbing *et al.*, 1996; Kashiwagi *et al.*, 1999; Valko *et al.*, 2006; Woolfson and Heikkila, 2009). CAT is an enzyme, which is involved in the removal of reactive oxygen species (ROS), such as hydrogen peroxide from the cell by converting it into oxygen and hydrogen. CAT activity has been shown to decrease just prior to the onset of tail apoptosis (Kashiwagi *et al.*, 1999; Valko *et al.*, 2006). As discussed earlier, tail apoptosis is a key step in the metamorphic program and it can be triggered by the presence of ROS; a decrease in the activity of CAT would lead to an increase in ROS and therefore suggests CAT may play an important role in the mechanism of programmed cell death. TH not only decreased the activity of CAT but also decreased the transcript levels (Kashiwagi *et al.*, 1999). HSP30 is a member of the heat shock protein family. Heat shock proteins protect thermally- or chemically-stressed cells and act as molecular chaperones (Fernando and Heikkila, 2000). They restore cellular functions subjected to environmental stress, act as housekeeping proteins for proper cell growth, and they are often transient but necessary in the development and differentiation of specific tissues (Lindquist and Craig, 1988; Arrigo and Tanguay, 1991; Atkinson *et al.*, 1993; Gernold *et al.*, 1993; Tanguay *et al.*, 1993). *HSP30* transcript levels have been shown to increase in the liver in response to TH exposure (Helbing *et al.*, 1996).

The concern for the increasing presence of TH disrupting chemicals in the environment has prompted research in this area and a need for screening assays, which can assess the safety of the chemicals being tested. Anurans present an excellent model to study TH disruptors for many reasons.

Tadpole metamorphosis provides observable and measurable morphological outcomes. Anurans have aquatic and terrestrial life stages, permeable skin, fewer active detoxifying enzyme systems than humans, and lack protective membranes (such as egg shells or amniotic membranes) during the critical stages of embryonic and larval development (Waring and Harris, 2005). Premetamorphic tadpoles can respond to exogenous TH and have no endogenous production of TH, providing a true zero base line without inducing a disease state, as is the case for rodent models that require chemical or physical manipulation of the thyroid gland (Tata, 1968; Tata, 1993; Shi, 2000). Premetamorphosis is the simplest stage to assess TH disruptors because the tadpole is not producing TH at this stage; however, the tadpole is still able to respond to TH. It is the period when the tadpole is the most sensitive to TH. Not only can whole tadpoles respond to exogenous TH, but also its organs can be cultured and exposed to TH and undergo the metamorphosis process. Tail fins have previously been cultured in our lab and after exposure to TH, the tail tips underwent regression (Ji *et al.*, 2007).

Although anurans present an excellent model to study potential TH disruptors, it is also important to look at the effects these chemicals can cause in a mammalian system. Another model used in this thesis is the GH3 cell line. The GH3 cell line is a widely used rat pituitary somatolactrophic cell line originally isolated from the MtT/W5

pituitary tumour (Tashjian *et al.*, 1970). TH is critical for proper brain development and is responsible for neuronal proliferation, cell migration and differentiation in the developing mammalian brain (Oppenheimer and Schwartz, 1997; Bernal *et al.*, 2003). As such, GH3 cells provide an excellent model to study potential TH disrupting chemicals, which may have a direct impact on TH actions on the brain. The GH3 cell line possesses unique characteristics of the original differentiated tissue and these include: the ability to produce growth hormone (GH) and prolactin (PRL), ability to express intracellular TR in high amounts, and the ability to proliferate in response to TH (Samuels *et al.*, 1988; Hohenwarter *et al.*, 1996). TH has been shown to up-regulate *GH* mRNA levels in GH3 cells (Williams *et al.*, 1991). Recently Gutleb *et al*. developed (2005) an *in vitro* T-screen assay to identify potential TH disrupting chemicals using GH3 cells. This assay is based on TH-dependent cell growth of the GH3 cells. A cell proliferation-based assay doesn't provide indications of molecular mechanisms of TH disruption so this thesis used QPCR to measure the steady state mRNA levels of TH-responsive genes. The genes studied were TH-responsive up-regulated genes, *GH* and deiodinase I (*DIOI*), and TH-responsive down-regulated gene, *PRL*. Heat shock protein 70 (*HSP70*) transcript levels were also measured as a marker of cellular stress.

1.10 Thesis Overview

The amount of chemicals released into the environment is increasing and, therefore, there is a pressing need to study the potential effects of environmental contaminants. This thesis takes advantage of the anuran metamorphosis system and

utilizes a novel assay, the cultured tail-fin biopsy (C-fin) assay. The C-fin assay uses cultured premetamorphic *R. catesbeiana* tadpole tail fin biopsies and exposes these biopsies to chemicals of interest in the absence and presence of TH. This study begins with the refinement of the C-fin assay and uses *TR*β, *RLKI*, *CAT*, and *HSP30* mRNA as endpoints.

After the establishment of the C-fin assay using previously studied chemicals, the thesis next studies the potential effects of nanoparticles. Nanoparticles (NPs) are materials with at least one dimension between 1 and 100 nm. NPs are found in a diverse range of products including electronics, optics, textiles, medical devices, cosmetics, food packaging, water treatment technologies, fuel cells, catalysts, biosensors, and agents for environmental remediation (Project on emerging nanotechnologies, 2009). The use of NPs is greatly increasing and because of their small size, they exert different chemical and physical properties compared to their larger counterparts; however, there is little research looking at the different properties these materials possess. This thesis uses the C-fin assay to study the effects nanosilver, cadmium-telluride quantum dots (QD), and nanozinc oxide has on TH action as well as on cellular stress. GH3 cells were also used to measure the effects these NPs have on TH-mediated actions in a mammalian system.

Finally, this thesis uses the C-fin assay to further examine into the thyroid disrupting effects of TCS, as well, examine the potential effects mTCS and TCC have on TH-mediated processes. Again, the GH3 cells were exposed to all three of these chemicals to determine if these chemicals have an effect on a mammalian model.

This study demonstrates the benefits of a novel assay, which can detect chemical disruption on the TH-signalling pathway. Using, this assay and a cell culture exposure assay, can help provide mechanistic insight into the actions of chemicals which disrupt the normal actions of the TH. Not only does this thesis look into previously characterized disruptors of TH action but it also studies the TH disrupting effects of chemicals, which have previously not been studied in this context.

Chapter 2: Materials and Methods

The following is a compilation of materials and methods used throughout the thesis. Additional materials and methods specific to a chapter will be found in the appropriate chapter.

2.1 Experimental Animals

Premetamorphic *Rana catesbeiana* tadpoles were caught locally (Victoria, BC) or purchased from Ward's Natural Science Ltd. (St. Catherines, ON). Taylor and Kollros (TK) (Taylor and Kollros, 1946) stage VI–VIII animals were used . Animals were housed in the University of Victoria aquatics facility and maintained in 100 gallon fiberglass tanks containing recirculating water at 12°C with exposure to natural daylight. Tadpoles were fed daily with spirulina (Aquatic ELO-Systems, Inc., FL). Animals used in this study were treated and maintained in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Organ culture of tail fin biopsies

Preparation of the tail fin biopsy cultures was adapted from conditions described previously (Veldhoen *et al.*, 2006; Ji *et al.*, 2007). Premetamorphic (Taylor and Kollros (TK) stage VI-VIII (Taylor and Kollros, 1946)) *R. catesbeiana* tadpoles were euthanized in 0.1% tricaine methanesulfonate (Syndel Laboratories, Vancouver, BC, Canada) in 25 mM sodium bicarbonate, and subsequently washed four times in 100 ml per tadpole in sterile magnesium-free (MFM) solution (7.5 mM Tris-HCl pH 7.6, 88 mM NaCl, 1 mM

KCl, 2.4 mM $NaHCO_3$, 0.88 mM $CaCl_2$). Six to ten biopsies were obtained per animal, from the dorsal and ventral tail fins using a six mm dermal biopsy punch (Miltex Inc., York PA USA), to allow the assessment of six-ten treatments per animal. Biopsies were cultured in individual wells at 25ºC in air for 48 h in 24-well multi-well culture plates (Primaria, BD Biosciences) in 1 ml per biopsy of 70% strength Leibovitz's L15 medium (Gibco, Invitrogen) supplemented with 10 mM HEPES pH 7.5, 50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate (Gibco, Invitrogen), and 50 μg/ml neomycin (Sigma-Aldrich).

For the chemical treatments, the media additionally contained the vehicle control, 10 nM 3,3',5-triiodothyronine (T_3), or 10 nM T_3 in combination with the test chemicals at different concentrations or an equal volume of the test chemical solvent. T_3 was prepared as a 10^{-5} M stock in 400 μM NaOH and was applied at 1 μl/ml of media, giving a final concentration of 10 nM T_3 (400 nM NaOH final). An equal volume of vehicle was applied to those treatments lacking T_3. The vehicle concentration was kept constant throughout. All test chemical stocks, with the exception of nanoparticles, were prepared as 100X concentrates through serial dilutions in the indicated solvents (solvent concentration was kept constant) and stored at -20°C.

Chemicals tested were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON) unless noted otherwise. Test chemicals and their respective solvents included: acetochlor (2-chloro-*N*-(ethoxy-methyl)-*N*-(2-ethyl-6-methylphenyl) acetamide; CAS 34256-82-1; 99.8% purity; AccuStandard) in methanol (CAS 67-56-1; 99.8% purity (0.05% H_2O); Caledon, Edmonton AB Canada), genistein (CAS 446-72-0; 98% purity) in dimethyl sulfoxide (DMSO; CAS 67-68-5; 99.9% purity (0.1% H_2O); ACP Chemicals

Inc.), roscovitine (CAS 186692-46-6; ≥98% purity) in DMSO, tetrabromobisphenol A (2,2',6,6'-Tetrabromo-4,4'-isopropylidendiphenol; TBBPA; CAS 79-94-7; 99.8% purity; AccuStandard, New Haven CT USA) in DMSO, and Triac (3, 3',5-Triiodothyroacetic acid; CAS 51-24-1) in DMSO.

Chemicals prepared in water included: micron-cadmium telluride (micron-CdTe; <250μm; CAS 1306-25-8; 99.99% purity), micron-silver (particle size: 5-8 microns; CAS 7440-22-4; 99.9% purity), silver nitrate ($AgNO_3$; CAS 7761-88-8), sodium nitrate ($NaNO_3$; CAS BP360-500g; Fisher), sodium nitrite ($NaNO_2$; CAS S2252-500g; > 99.5% purity), and sodium gluconate ($C_6H_{11}NaO_7$; CAS S-2054).

NPs [nanosilver (CAS 7440-22-4; 99.1% purity), nanozinc oxide (CAS 8051-03-4; 99.1% purity), and green quantum dots (QDs; CAS 1306-25-8; 99.1% purity)] manufactured to the specifications of 90% particles within the 1-10 nm diameter with the majority within the 3-5 nm range were purchased from Northern Nanotechnologies (now known as ViveNano, Toronto, ON) at a concentration of 0.8 μM. These particles were water soluble due to a proprietary carboxyl-functionalized coating common to all three. They were stored at 4ºC.

Triclosan (5-chloro-2-[2, 4-dichloro-phenoxy]-phenol; TCS; CAS 3380-34-5; ≥97% pure) and Triclocarban (3-(4-chlorophenyl)-1-(3,4-dichlorophenyl)-urea; TCC; CAS 101-20-10; 99% pure) were prepared in 400 μM NaOH and in DMSO, respectively. Methyl-triclosan was a gift from Jeremy Wulff, Department of Chemistry, University of Victoria, and was prepared in DMSO.

The biopsies were pretreated with 0.5 ml of the appropriate concentration of the test chemical in culture media for 2 h prior to the addition of T_3. After the 2 h

incubation, 0.5 ml of the appropriate concentration of the test chemical plus 20 nM T_3 (in 800 µM NaOH) were added into the wells giving a final concentration of 10 nM T_3 (in 400 nM NaOH). For the wells not containing T_3, 0.5 ml of the appropriate concentration of the test chemical plus 800 µM NaOH were added. Each test chemical stock was applied at 1 µl/ml medium giving final nominal concentrations as indicated in the text with a solvent dilution of 1:1,000. At the end of the 48 h incubation period for each treatment, the biopsy was stored in 100 µl of RNA*later* (Ambion Inc., Austin TX USA) for 24 hours at 4°C and then transferred to -20ºC until processed for RNA.

2.3 Cell Culture

Rat pituitary cells originating from the GH3 cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in F-12 Kaighn's medium (Gibco, Invitrogen) supplemented with 50 units/ml penicillin G sodium, 50 µg/ml streptomycin sulfate (Gibco, Invitrogen), 1 mM sodium pyruvate (Gibco,Invitrogen), and 10% foetal bovine serum (FBS; Gibco-Invitrogen). FBS was inactivated prior to use by heating at 60°C for 20 minutes. All cell culture work was performed in a model 1106 Laminar Flow Hood (Forma Scientific, Waltham, MA, USA). Cells were initially grown on a 10 cm diameter sterile culture dish (Sarstedt, Inc., Montreal, PQ, Canada) at 37°C in air and 5% CO_2. At confluence, the cells were harvested by the addition of 0.05% (w/v) trypsin (Gibco-Invitrogen) in 0.5 nM ethylenediaminetetraacetic acid (EDTA) to each 10 cm dish and plated on sterile Falcon 6-well plates (VWR International) at 2×10^5 cells/well (1×10^5 cells/mL) in modified F-12

Kaighn's media supplemented with penicillin streptomycin, sodium pyruvate and 10% heat-shocked FBS in 37°C in air and 5% CO2. Following 24 h incubation, the media was subsequently removed and the GH3 cells were washed three times with 2 mL/well sterile phosphate buffer saline solution (PBS). F-12 Kaighn's media supplemented with penicillin streptomycin, sodium pyruvate, and also containing 10% heat-inactivated hormone-stripped (charcoal/dextran stripped) FBS (Fisher, SH 30068.03) was then added (2 mL) and cells were incubated for a further 48 h at 37°C in air and 5% CO_2. After this time, treatment of GH3 cells in the absence and presence of 10 nM T_3 took place.

For the chemical treatments, the media additionally contained the vehicle control, 10 nM 3,3',5-triiodothyronine (T_3), or 10 nM T_3 in combination with the test chemicals at different concentrations or an equal volume of the test chemical solvent. T_3 was prepared as a 10^{-5} M stock in 400 µM NaOH and was applied at 1 µl/ml of media, giving a final concentration of 10 nM T_3 (400 nM NaOH final). An equal volume of vehicle was applied to those treatments lacking T_3. The vehicle concentration was kept constant throughout. All test chemical stocks, with the exception of the nanoparticles, were prepared as 100X concentrates through serial dilutions in the indicated solvents (solvent concentration was kept constant) and stored at -20^{o}C.

Chemicals tested were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON) unless noted otherwise. NPs [nanosilver (CAS 7440-22-4; 99.1% purity), and green quantum dots (QDs; CAS 1306-25-8; 99.1% purity)] manufactured to the specifications of 90% particles within the 1-10 nm diameter with the majority within the 3-5 nm range were purchased from Northern Nanotechnologies (now known as ViveNano, Toronto,

ON) at a concentration of 0.8 μM. These particles were water soluble due to a proprietary carboxyl-functionalized coating common to all three. They were stored at 4ºC. The appropriate amounts of NPs were added to the media to obtain the test concentrations.

Triclosan (5-chloro-2-[2, 4-dichloro-phenoxy]-phenol; TCS; CAS 3380-34-5; ≥97% pure) and Triclocarban (3-(4-chlorophenyl)-1-(3,4-dichlorophenyl)-urea; TCC; CAS 101-20-10; 99% pure) were prepared in 400 μM NaOH and dimethyl sulfoxide (DMSO), respectively. Methyl-triclosan was a gift from Jeremy Wulff, Department of Chemistry, University of Victoria, and was prepared in DMSO. Each test chemical stock was applied at 1 μl/ml medium giving final nominal concentrations as indicated in the text with a solvent dilution of 1:1,000. 2mL of media containing the appropriate test chemical was applied to the cells. Cells were incubated in the test chemicals for 48 h followed by removal of the growth medium and addition of 1mL TRIzol to each well for isolation of total RNA as described below.

2.4 Isolation of RNA, quantification of gene expression

RNA was isolated using TRIzol reagent as described by the manufacturer (Invitrogen). Mechanical disruption of the tail biopsies utilized 300 μl TRIzol reagent, a 1 mm diameter tungsten-carbide bead, and safe-lock Eppendorf 0.5 ml microcentrifuge tubes in a Retsch MM301 Mixer Mill (Fisher Scientific) at 20 Hz two times for 1.5 min with the chambers being rotated in between the cycles. Twenty μg of glycogen (Roche Diagnostics, Laval PQ Canada) were added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 10 μl diethyl

pyrocarbonate (DEPC)-treated RNase-free water and stored at -70ºC. cDNA was synthesized from 5 μl (~0.5 μg) total RNA as per manufacturer's protocol using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) with minor modifications: RNA was first annealed with 200 ng random hexamer primer in the presence of dNTPs and then cDNA was synthesized by adding the mixture of reaction buffer, ribonuclease inhibitor and RevertAid H Minus M-MuLV reverse transcriptase and incubating at 25ºC for 10 min and then at 42ºC for 1.5 h. The tail biopsies cDNA products were diluted five-fold and the GH3 cell cDNA products were diluted 20-fold, prior to PCR amplification and stored at -20ºC. Selected transcripts, designed using the computer software Primer Premier (version 5.00; Table 2.1) were analyzed using a MX3005P real-time quantitative PCR system (Stratagene, La Jolla CA USA). Each 15 μl amplification reaction contained 10 mM Tris-HCl (pH 8.3 at 20ºC), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene OR USA), 200 μM dNTPs, 69.4 nM ROX reference dye (Invitrogen), the appropriate concentration of the test primer as listed in Table 2.1, 2 μl of diluted cDNA, and one unit of Hot Start Taq DNA polymerase (Fermentas). The thermocycle program for both gene targets included an initial enzyme activation step at 95ºC (4 min) followed by 40 cycles of 95ºC denaturation (15 sec), appropriate annealing for each primer as listed in Table 2.1 (30 sec), and 72ºC elongation (45 sec). Controls lacking cDNA template and an inter-plate standard containing a mixture of cDNA were included to determine the specificity of target cDNA amplification as well as the quality of each QPCR run. Quadruplicate reactions were performed for each sample and data were averaged and normalized to the expression of the invariant control gene encoding

the ribosomal protein L8 using the comparative Ct method (Livak and Schmittgen, 2001). The integrity of amplification reactions was confirmed by the presence of a single DNA product following gel electrophoresis and by amplicon sequencing. Additionally, the efficiency of the target amplifications was validated to be approximately equal allowing the use of the comparative Ct method.

2.5 Statistical Analyses

The C-fin data was non-parametric and statistical analyses were performed using SPSS Ver. 12.0 (Chicago, IL) software using the Kendall's W and paired Wilcoxon tests since these data were generated from a repeated—measures type of experimental design. The types of comparisons made were as follows: First, the test chemical results were examined relative to the vehicle control and, second, once it was verified that an expected TH-dependent response was obtained, the test chemicals in combination with T_3 results were compared relative to T_3 alone. In the latter case, the response to a test chemical in the presence of T_3 was expressed as a fold change relative to the response to T_3 alone of each individual to eliminate inter-animal variation thus enabling us to better identify chemical-induced perturbations relative to each individual's ability to respond to T_3 Therefore, the T_3 values in this comparison were given a value of one and the graphs show the fold change relative to the T_3-induced response.

The GH3 cell data was non-parametric and statistical analyses were performed using SPSS Ver. 12.0 (Chicago, IL) software using the Kruskal Wallace and Mann-Whitney U tests.

Gene	Species	GenBank Accession Number	Primer Name	Primer Sequence (5' → 3')	Amplicon Size (bp)	PCR Temperature (°C)	Picomoles of primers/reaction
Ribosomal Protein L8	*Rana catesbeiana*	AY452063	L82up	AGGGGACAGAGAAAAGGTG	270	55	5
			UL8dn	TGAGCTTTCTTGCCACAG			
TRβ	*Rana catesbeiana*	M95194	FE022up	AGCAGCATGTCAGGGTAC	538	55	10
			FE022dn	TGAAGGCTTCTAAGTCCA			
RLKI	*Rana catesbeiana*	EF156435	DDKerF3	GTTGGCGTTGGTGTTAGCGG	336	55	5
			DDKerRQ	GGCACTGCTTCTTGCAACTTG			
Catalase	*Rana catesbeiana*	GQ222411	Q556a	GAATGGTTACGGCTCACA	174	60	5
			Q556b	GCAATGGCTTCATACAGA			
Heat Shock Protein 30	*Rana catesbeiana*	U44894	FN028up	GCCTCCACCAGACT	198	61	5
			RCHSP30dn	GTCTCCTTCCTTCCG			
Ribosomal Protein L8	*Rattus norvegicus*	NM_001034916	UL8-up	GGTGTGGCTATGAATCCTGT	126	55	5
			L82-dn	ACGACGAGCAGCAATAAGAC			
Growth Hormone	*Rattus norvegicus*	NM_001034848	NOR1	TTGATCCAGCAGACCTACATT	305	60	5
			NOR2	CTCCCTGGCTCCTGACCTT			
Deiodinase-I	*Rattus norvegicus*	NM_021653	NOR3	GGACTCTGCTACAAGGGTAA	304	60	5
			NOR4	GCTCGGTATTGCTTTATCT			
Prolactin	*Rattus norvegicus*	NM_012629	NOR7	CCTCCTGCTGATGATGTC	498	60	5
			NOR8	TGTGACCAAACCAAGTAGAT			
Heat Shock Protein 70	*Rattus norvegicus*	NM_031971	NOR9	GGCTTCCACTAGACCACG	117	60	5
			NOR12	AACTGGATCGAAGGCGTA			

Table 2.1. QPCR Primer Information. A list of the QPCR primers that are used in this thesis to measure the mRNA steady-state levels of selected transcripts. Primers were designed using the computer software program Primer Premier (version 5.00).

Chapter 3: C-fin: A cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals

Adapted for the thesis from: Hinther, A., Domanski, D., Vawda, S., and Helbing, CC. C-fin: A cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals. *Environmental Toxicology and Chemistry* **29** (2010), pp. 380–388.

3.1 Introduction

The world's development and industrial progress is increasingly adding anthropogenic chemicals into the environment. The number of chemicals currently released into the environment has been estimated at over 100,000 (Commission, 2006). Some of these chemicals are released into the environment intentionally, such as through agricultural processes, and some are released inadvertently through waste and from products in use. Beyond their toxic effects on organisms when present in high amounts, a number of these chemicals have adverse effects at sublethal concentrations such as acting as EDCs at sublethal concentrations. The focus of research thus far has been mainly on EDCs of gonadal steroids with (anti-) estrogenic and (anti-)androgenic capabilities with relatively limited information regarding disruptors of TH action (Zoeller and Tan, 2007). Nevertheless, close to a hundred compounds have been classified as endocrine disruptors of the TH axis and many new chemicals are hypothesized to acts as EDCs of TH action (Boas *et al.*, 2006).

The growing concern that polluting anthropogenic chemicals are potential disruptors of the TH axis has prompted organizations such as the U.S. Environmental Protection Agency (EPA), Environment Canada, and the Organisation for Economic Co-operation and Development (OECD) to look at screening assays to assess the safety of newly created chemicals and those already present in the environment. The total dependence of amphibian metamorphosis on TH and the high degree of conservation of TH signalling pathway in vertebrates has led to the suggestion that metamorphosis can be used as a model for the detection of chemicals affecting the TH axis in vertebrates (Tata, 2006). Indeed, several metamorphosis-based assays using whole animal exposures are currently in the validation phase such as the amphibian metamorphosis assay (AMA) and the *Xenopus* metamorphosis assay (XEMA) (Fort *et al.*, 2007) that are based upon developmental rate and thyroid histology, and the native species-based *Rana catesbeiana* metamorphosis assay that is based upon morphological and molecular endpoints (Veldhoen *et al.*, 2006). The OECD in their report on the AMA for the detection of thyroid active substances expressed the need for additional *in vivo/in vitro* assays to expand the battery of screening assays (OECD, 2006). Moreover, there is a substantial drive through the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to develop methods to reduce the use of animals in screening assays (The NICEATM-ICCVAM Five Year Plan (2008-2012) http://iccvam.niehs.nih.gov/docs/5yearplan.htm).

We have previously developed a tail fin biopsy assay using *Rana catesbeiana* tadpoles which enables non-lethal tissue sampling from live animals and the assessment of exposure to thyroid active agents by examining mRNA transcript levels

of known TH-responsive genes such as *TR*β (Veldhoen and Helbing, 2001; Veldhoen and Helbing, 2005). This method enables a repeated measures experimental design where a tail fin biopsy can be obtained from the same tadpole at multiple time points. Despite the advantages of this experimental design, only a single chemical treatment can be tested per animal, which is not conducive to a high throughput screening approach for multiple chemicals or conditions.

This current study builds on the tail fin biopsy approach on a native amphibian tadpole by combining this concept with the fact that tadpole tissues can be successfully cultured (Veldhoen *et al.*, 2006; Ji *et al.*, 2007) to develop a cultured tail fin biopsy or "C-fin" assay. By taking multiple tail fin biopsies per animal and then exposing each biopsy to a different treatment condition, the C-fin assay enables the screening of multiple chemicals simultaneously while maintaining complex tissue structure and enabling the determination of biological variation of a response. The C-fin assay is designed to determine direct effects on target tissue TH signalling by examining TH-responsive gene transcript levels. Not only is the C-fin assay designed to study chemicals that are potential TH disruptors, it also allows us to look at the direct tissue effects a chemical has in the absence of TH thus providing further mechanistic insight.

Herein, we examined a variety of known or potential TH agonists and antagonists including Triac (a TR agonist) (Messier and Langlois, 2000), genistein (an isoflavone) (Ji *et al.*, 2007), roscovitine (a cyclin-dependent kinase (Cdk) inhibitor) (Skirrow *et al.*, 2008), the brominated flame retardant tetrabromobisphenol A (TBBPA) (Veldhoen *et al.*, 2006), and the herbicide acetochlor (Crump *et al.*, 2002; Veldhoen *et al.*, 2002) to determine the direct tissue effects of these chemicals using the C-fin assay. We used

quantitative real time polymerase chain reaction (QPCR) to assess TH-dependent transcript levels, TH-induced TH receptor β (*TRβ*) and *RLKI*, a TH-repressed *Rana* larval keratin type I transcript whose N-terminal protein fragment increases upon TH-treatment *in vivo* (Domanski and Helbing, 2007). The C-fin assay was able to detect perturbations in TH-signalling within 48h of exposure demonstrating that this method has utility as a novel screen for TH disrupting chemicals.

3.2 Results

3.2.1 The C-fin assay

Previous studies have shown the tail tissue to be a robust indicator of TH action and that premetamorphic tadpoles are more sensitive than prometamorphic tadpoles to TH disruption (Opitz *et al.*, 2006; Zhang *et al.*, 2006). The C-fin assay thus uses tail fin tissue from premetamorphic (TK stage VI-VIII) *R. catesbeiana* tadpoles, which are competent to respond to T_3 but have not yet been exposed to the high levels of the endogenous TH that would occur in prometamorphosis. Up to eight 6 mm biopsies were taken from the dorsal and ventral tail fins of each tadpole and incubated in serum-free media in individual wells in a multiwell-plate (Figure 3.1). This allowed for the tissue from an individual animal to be exposed to multiple treatments. For example, as illustrated in Figure 3.1, eight biopsies from one animal enabled the testing of vehicle control, T_3 alone, three concentrations of the test chemical and T_3 in combination with the test chemical at the three concentrations. This experimental design permits the assessment of biological variation within a population and the detection of TH agonists and antagonists. Moreover, the individual response to TH can be normalized to the

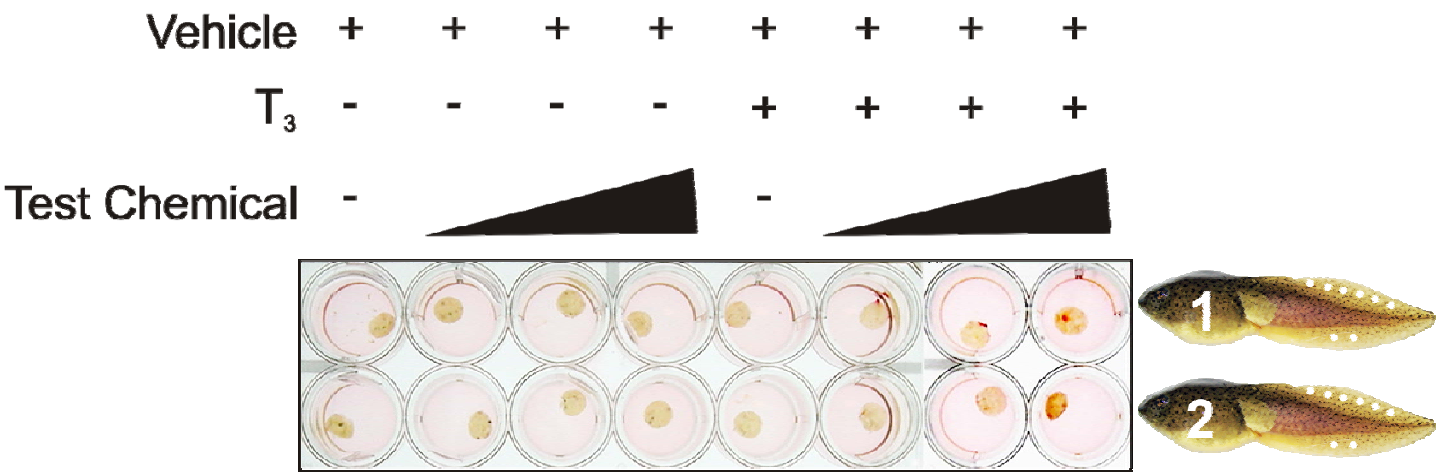


Figure 3.1. Overview of the C-fin assay. Eight biopsies of 6 mm in diameter are taken per individual premetamorphic *Rana catesbeiana* tadpole tail fin and placed into separate wells of a 24-well plate containing serum-free medium. These biopsies are then exposed to a total of eight different conditions. A typical experiment consists of exposure to three different concentrations of test chemicals in the presence and absence of 10 nM T_3 plus vehicle and T_3 alone controls. This experimental design enables the identification of individuals that do not respond to TH and focus upon those animals that demonstrated inherent TH responsiveness for assessing TH disruptive effects of a test chemical. Moreover, this experimental design maintains biological variation and tissue structure while reducing the number of animals by a factor of eight. Adapted from (Hinther *et al.*, 2010A).

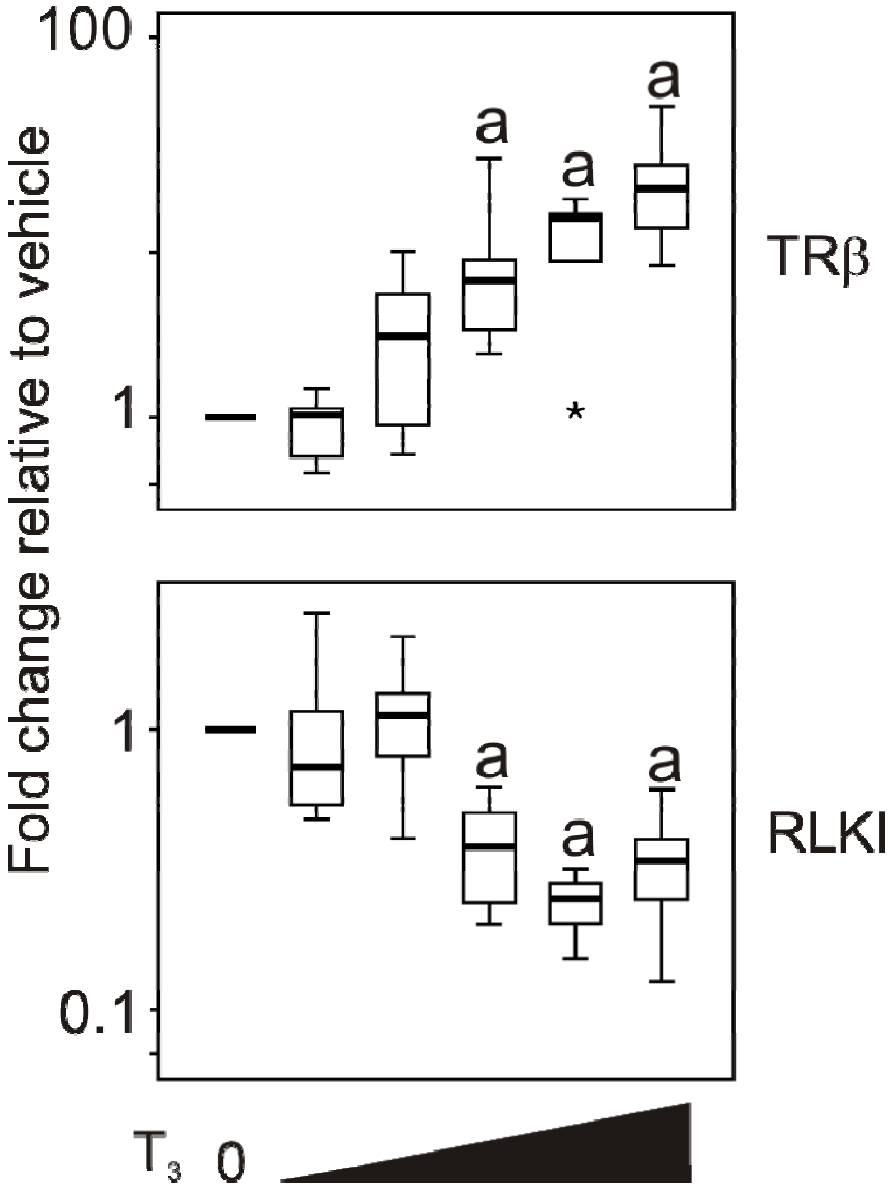


Figure 3.2. Sensitivity of the C-fin assay to T_3 as assessed by *TRβ* and *RLKI* transcript levels. Premetamorphic *R. catesbeiana* tadpole tail fin tissue biopsies were exposed to 0.01, 0.1, 1, 10, or 100 nM T_3 or vehicle control ("0") for 48 h and assessed for *TRβ* and *RLKI* transcript levels. The bar graphs show the fold change in transcript levels relative to vehicle control as determined by QPCR (n=8). The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Outlier and extreme values are indicated by an open circle and asterix, respectively. Increasing concentration of T_3 is represented by the bevel. Significance is indicated by "a" for $p \leq 0.05$ (Wilcoxon). Adapted from (Hinther *et al.*, 2010A).

individual's baseline value. At the end of the incubation period, the tissue was used to evaluate gene transcript or protein levels by QPCR or Western blot (Hinther *et al.*, 2010A), respectively.

We first determined the relative responses of the tail fin biopsies to different concentrations of the thyroid hormone, T_3 (Figure 3.2). We examined the well-studied direct early response gene *TR*β transcript, which has been used as a candidate biomarker for TH action in a number of studies (Veldhoen and Helbing, 2001; Helbing *et al.*, 2006; Opitz *et al.*, 2006; Zhang *et al.*, 2006). In addition, we also examined a novel gene transcript encoding RLKI whose transcript and N-terminal protein fragment we have previously shown to be decreased and increased, respectively, *in vivo* by this hormone (Domanski and Helbing, 2007). Figure 3.2 demonstrates the change in transcript levels with significant median elevation up to 20-fold in *TR*β and a 3-fold reduction in *RLKI* transcript levels occurring at 1 nM T_3 or higher after 48 h exposure. The results with *RLKI* represent one of very few examples of a transcript whose levels are substantially reduced upon TH exposure.

3.2.2 Location of biopsy is independent of the T_3-induced response

We chose the 10 nM T_3 concentration which represents the level measured previously in metamorphic climax tadpoles (Regard *et al.*, 1978). The C-fin assay maximizes the use of the tail fin from an individual animal by taking multiple biopsies from a single tail. However it was necessary to evaluate whether there were any differences in TH-dependent responses between biopsies taken from these different locations. As shown in Figure 3.3, seven different locations that covered the usual

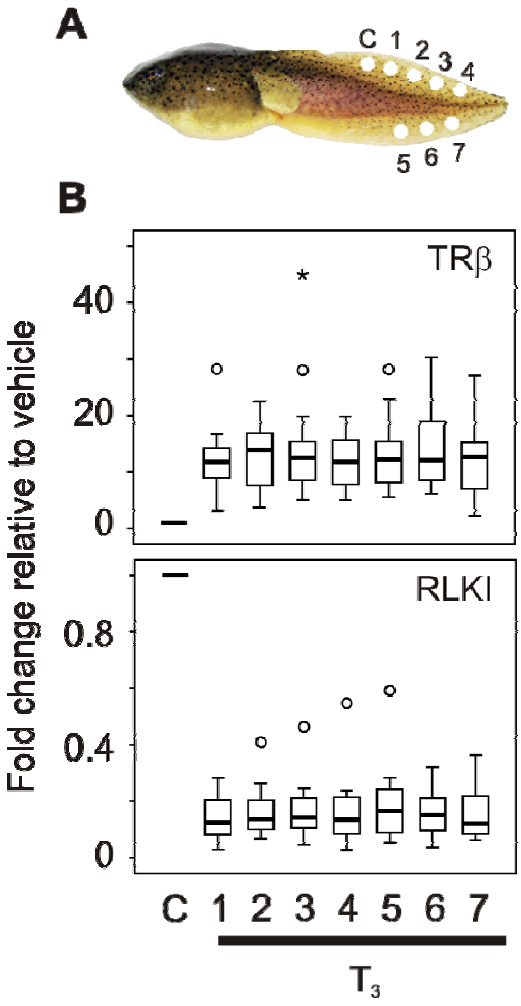


Figure 3.3. Examination of uniformity of response to T_3 across the tail fin tissue as analyzed by *TRβ* and *RLKI* transcript levels. **(A)** Sampled locations within the tail fin used to test for a uniform response to T_3. Biopsies (6 mm) "1" to "7" were exposed to 10 nM T_3 for 48 h and compared to biopsy "C" exposed to vehicle control. Biopsies were exposed individually in serum-free organ culture medium in multi-well plates. **(B)** The bar graphs show the fold change in *TRβ* and *RLKI* transcript levels in the 10 nM T_3- exposed biopsies ("1" to "7") compared to vehicle control ("C") as determined by QPCR. The data from two independent experiments are shown (n=16). The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Outlier values are indicated by an open circle. All T_3-treated biopsies showed a significant increase (*TRβ*) or decrease (*RLKI*) in transcript levels relative to the vehicle control ($p \leq 0.05$) irrespective of sampling location, whereas no significant differences in response levels were observed between sampling locations (Wilcoxon). Adapted from (Hinther *et al.*, 2010A).

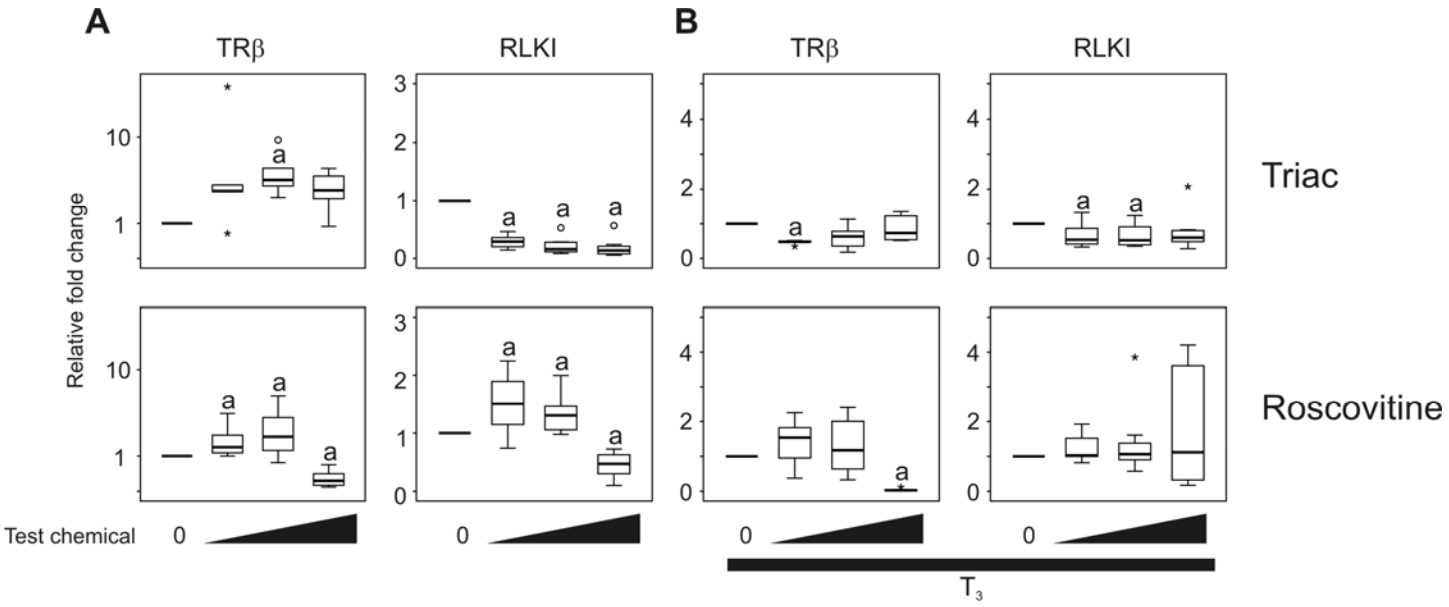


Figure 3.4. Assessment of the effects of Triac and roscovitine on *TRβ* and *RLKI* transcripts in the C-fin assay. Fold change in steady-state levels of the *TRβ* and *RLKI* transcripts relative to vehicle control as determined by QPCR in the absence (A), or presence (B) of 10 nM T_3. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Methods section. Tail fin biopsy samples were exposed to vehicle control ("0"), 10 nM T_3 ("T_3"), and the indicated test chemicals for 48 h before QPCR evaluation of *TRβ* and *RLKI* transcript levels. Test chemical concentrations were as follows: 1, 10, and 100 nM Triac; and 0.6, 6, and 60 μM roscovitine. Only those individuals that demonstrated a T_3-dependent increase or decrease in *TRβ* or *RLKI* transcript levels, respectively, were examined in this graph. Significance is indicated by "a" ($p \leq 0.05$; Wilcoxon). Adapted from (Hinther *et al.*, 2010A).

sampling region were assessed for changes in indicator transcript levels after the biopsies were exposed to 10 nM T_3 for 48 h. An eighth biopsy was exposed to vehicle solution only. Uniform responses were observed with no statistical difference in response to T_3 between locations for either transcript tested. A similar response throughout the *R. catesbeiana* tail fin after TH induction has been shown before in biopsies taken from live tadpoles (Veldhoen and Helbing, 2001).

3.2.3 Detection of disruption of TH action with the C-fin assay

Numerous chemicals were tested that were previously implicated as disruptors of TH action using the C-fin assay. Because C-fin is a repeated measure type of experiment, we are able to identify individual animals' responses to the test conditions. Since we were interested in evaluating TH-mediated responses, we first established whether or not a set of biopsies from an individual showed the ability to respond to T_3 treatment. Seven to nine percent of animals were defined as failing to respond to T_3 treatment (< 2-fold increase in *TRβ* transcript or < 1.25-fold decrease in *RLKI* transcript; n=165 and 164, respectively) and were not considered for evaluation of TH-disrupting activity. Of the remaining 91-93% of TH-responsive animals, two comparisons were made: first, the test chemical results were examined relative to the vehicle control and, second, the test chemicals in combination with T_3 results were compared relative to T_3 alone. In the latter case, the response to a test chemical in the presence of T_3 were expressed as a fold change relative to the response to T_3 alone of each individual to better identify perturbations relative to the individual's ability to respond to T_3. Therefore the T_3 values in Figures 3.4B and 3.5B are given a value of one.

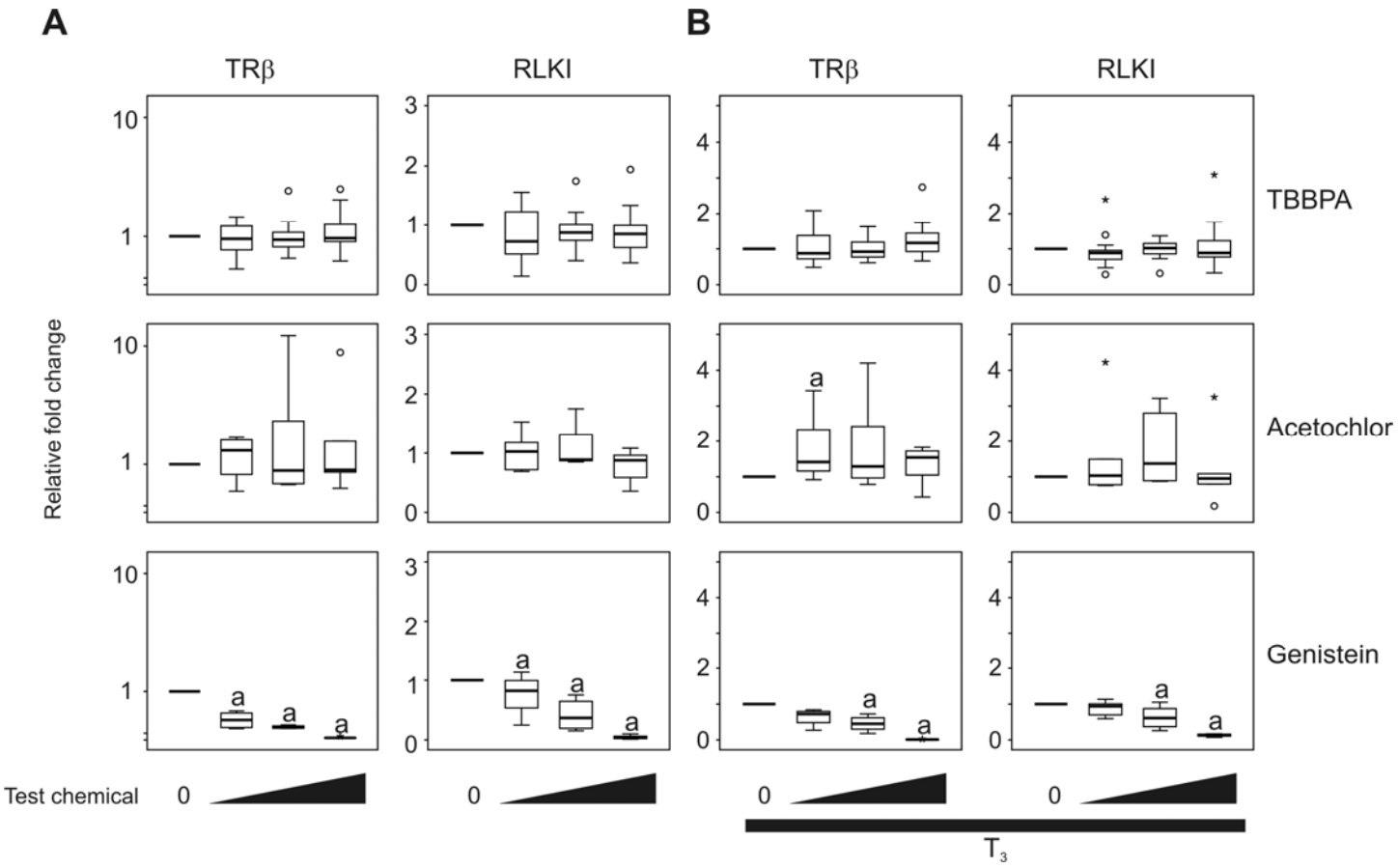


Figure 3.5. Analysis of TBBPA and acetochlor for TH disrupting activity through assessment of *TRβ* and *RLKI* transcript levels. Fold change in steady-state levels of the *TRβ* and *RLKI* transcripts relative to vehicle control as determined by QPCR in the absence (A), or presence (B) of 10 nM T_3. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Methods section. C-fin samples were exposed to vehicle control ("0"), 10 nM T_3 ("T_3"), and the indicated chemicals alone or in combination with 10 nM T_3 for 48 h. Biopsies were pre-treated with test chemical for 2 hours before the addition of 10 nM T_3 or solvent. The chemical treatments were: TBBPA: 10, 100, and 1,000 nM; acetochlor or genistein: 1, 10, and 100 nM. Significance is indicated by "a" ($p \leq 0.05$; Wilcoxon). Adapted from (Hinther *et al.*, 2010A).

We compared the baseline levels of *TRβ* and *RLKI* transcripts within each animal and found a strong positive correlation (Correlation coefficient=0.311, p=0.0001, n=134) between these two transcripts. We also found a strong negative correlation between baseline level of each transcript compared to its T_3-induced level (TRβ: Correlation coefficient=-0.391, p=0.0001, n=151; RLKI: Correlation coefficient=-0.211, p=0.005, n=145).

Triac is a T_3 analogue that can bind to TRs (Messier and Langlois, 2000). At 1, 10, and 100 nM, Triac increased *TRβ* transcript levels by a median 4-fold compared to vehicle control (10 nM, p=0.043) and reduced *RLKI* transcript levels between 3- to 8-fold (1 nM, p=0.018; 10 nM, p=0.012; 100 nM, p=0.012) (Figure 3.4A). The T_3-dependent reduction in *RLKI* transcript levels were further reduced by 2-fold (T_3 + 1 nM, p=0.050; T_3 + 10 nM, p=0.036) (Figure 3.4A) whereas Triac prevented full T_3-dependent induction of *TRβ* transcript by 2-fold at the lowest concentration examined (p=0.043; Figure 3.4B).

Roscovitine inhibits T_3-dependent tail tip regression in culture at 60 μM and reduces *TRβ* transcript levels (Skirrow *et al.*, 2008). We found that roscovitine decreases steady state levels of *TRβ* and *RLKI* transcripts by 5- and 2-fold, respectively, at this concentration (p=0.018 and 0.012; Figure 3.4A). Lower concentrations of roscovitine increased the levels of both transcripts up to 1.9-fold (*TRβ*; 0.6 μM, p=0.028; 6 μM, p=0.043) and 1.5-fold (*RLKI*; 0.6 μM, p=0.036; 6 μM, p=0.036). The T_3-dependent induction of *TRβ* transcript was eliminated (34-fold decrease; p=0.018) when 60 μM roscovitine was present, but not at lower roscovitine concentrations (Figure 3.4B). The T_3-dependent reduction of *RLKI* transcript levels was

unaffected at any concentration although there was a marked increase in variation observed at 60 μM roscovitine (Figure 3.4B).

We then tested the effects of three environmentally-relevant chemicals that have previously been implicated in disruption of TH action. TBBPA had no effect at any of the concentrations tested (10-1,000 nM; Figure 3.5) on *TRβ* and *RLKI* transcript levels. Acetochlor was tested at three concentrations of 1, 10, and 100 nM which span median and maximum concentrations measured in streams in the Midwestern United States (Scribner *et al.*, 2000). Acetochlor enhanced the T_3-dependent increase in *TRβ* transcript levels by 1.5-fold (1 nM, p=0.028; Figure 3.5B), but had no effect on its own (Figure 3.5A). *RLKI* transcripts were not affected (Figure 3.5).

In contrast to TBBPA and acetochlor, genistein (1-100 nM) had pronounced effects on the steady state levels of both transcripts. Genistein reduced the levels of *TRβ* transcripts by 3- to 34-fold (p=0.013, Kendall's W) and *RLKI* transcripts by 1.2- to 25-fold (p=0.003, Kendall's W; Figure 3.5A). The T_3-dependent induction of *TRβ* transcript levels was reduced by 2-fold upon administration of 10 nM genistein (p=0.043) and was strikingly reduced over 90-fold in the presence of 100 nM genistein (p=0.034; Figure 3.5B). The T_3-dependent reduction in *RLKI* transcripts at 10 nM genistein (p=0.046) was also augmented to 8-fold upon co administration of T_3 with 100 nM genistein (p=0.043; Figure 3.5B).

3.3 Discussion

There is a growing concern regarding the presence of pharmaceuticals and personal care products in the environment and their possible deleterious effects on wildlife and humans and there is a great need for the development of effective assays to detect disruption of TH action. The C-fin assay uses the organ culture of multiple tail fin biopsies from a native frog species as the basis of a novel screening tool which enables the assessment of effects on a highly responsive target tissue. By taking multiple biopsies from each tadpole, every tadpole in essence is exposed to all treatment conditions, allowing the researcher to see the biological variation taking place within each experiment, while evaluating direct tissue effects of a chemical alone or in the presence of TH. C-fin is responsive to TH agonists and antagonists as indicated by exposure profiles upon administration of Triac and roscovitine (Figure 3.4) (Opitz *et al.*, 2006; Ji *et al.*, 2007; Skirrow *et al.*, 2008) each with different, yet possibly overlapping, mechanisms (Messier and Langlois, 2000; Skirrow *et al.*, 2008).

Previous studies on the flame retardant, TBBPA, demonstrated that this chemical had varied effects upon TH-mediated responses and a number of studies have reported agonist or antagonist activities that were hypothesized to be through binding to carriers such as transthyretin and/or the TRs (Meerts *et al.*, 2000; Kitamura *et al.*, 2005; Kitamura *et al.*, 2005; Hamers *et al.*, 2006; Veldhoen *et al.*, 2006). TBBPA could potentiate certain TH-dependent responses in the Pacific tree frog and in rat pituitary GH3 cells (Hamers *et al.*, 2006; Veldhoen *et al.*, 2006). However, in the Pacific tree frog, TBBPA only caused a significant change in the levels of the gelatinase A transcript, but not the *TR*β transcript in the tail (Veldhoen *et al.*, 2006). Other studies

also did not observe potentiation of the TH-dependent response (Kitamura *et al.*, 2002; Kitamura *et al.*, 2005; Schriks *et al.*, 2006). It should be noted that in most of these studies, far higher (μM) concentrations were used than in the present study. The majority of the literature, however, appears to indicate TBBPA has antagonistic effects on TH action possibly by interacting with the TRs, but again these studies used application concentrations in the μM range. For example, using a reporter gene assay with a TRE-luciferase reporter construct in PC12 mammalian cells expressing avian TRα1, TBBPA between 10 and 60 μM induced luciferase expression, but lost any effect above 60 μM (Jugan *et al.*, 2007). In addition, when 0.3 nM T_3 was added, TBBPA reduced the hormone-dependent induction of luciferase with an IC_{50} of 50 μM (Jugan *et al.*, 2007). These findings indicate that TBBPA at high concentrations can probably bind to TRs; however, the absence of an effect of TBBPA on tail fins in this study suggests that a direct mode of action on the tissue is unlikely at lower concentrations. Additional factor(s) such as the hypothalamus-pituitary-thyroid axis, serum carrier proteins or deiodinases present in the whole animal might be more important.

Acetochlor acted as a weak agonist with respect to the *TRβ* transcript level in the presence of T_3; however, no other significant effects were observed for acetochlor under the conditions used in the C-fin assay. Previous lines of evidence using intact tadpoles demonstrated that acetochlor disrupted TH-dependent processes. Acetochlor accelerated T_3-induced precocious metamorphosis in the northern leopard frog tadpole, *Rana pipiens* (Cheek *et al.*, 1999) and in *Xenopus laevis* premetamorphic tadpoles (Crump *et al.*, 2002) and enhanced the steady state levels of TR transcripts in the tail of

precociously induced premetamorphic tadpoles (Crump *et al.*, 2002) or naturally developing prometamorphic tadpoles (with elevated endogenous TH) (Helbing *et al.*, 2006). Turque et al (2005) showed a modest increase in green fluorescence in the brains of transgenic premetamorphic *X. laevis* tadpole carrying a TRE-containing TH/bZIP-EGFP reporter construct (Turque *et al.*, 2005). The general weak to lack of response of acetochlor exposure in the C-fin context suggests that a direct tissue effect is most likely not the primary mechanism for this pesticide. The modest enhancement of *TR*β transcript levels however is consistent, albeit weaker than, the whole animal observations. The lack of response of the *RLKI* transcript, the observation that acetochlor does not affect the levels of either transcript in the absence of hormone, and the lack of binding of human recombinant TR to acetochlor (Cheek *et al.*, 1999) suggest that acetochlor's effects are unlikely to be modulated through TRs, rather may be through alternate mechanisms requiring the intact animal.

Genistein is a major isoflavone in soy products and is ingested in very high amounts by infants exclusively fed soy-based formulas. The plasma isoflavone concentration of these infants is approximately 7 μM, approximately 300 times higher than in milk formula- or breast-fed infants (Setchell *et al.*, 1998; Badger *et al.*, 2002). Genistein has previously been associated with disruption of TH metabolism (Doerge and Sheehan, 2002) and we have recently shown that similar concentrations could influence T_3 action directly on frog tadpole tissues possibly through targeting protein kinase C and the TR (Ji *et al.*, 2007). The C-fin assay clearly demonstrated that genistein had profound effects on T_3 signalling acting to accentuate TH-dependent repression events likely through these pathways (Ji *et al.*, 2007).

The dependence of amphibian metamorphosis on TH has led to the suggestion that metamorphosis can be used to assess TH disruption. A number of research groups have used this premise to develop assays for the detection of TH-modulating compounds ranging from whole-animal morphological assays to assays based on germinal transgenic tadpoles with TRE-driven reporters. A central goal of this study included establishing a rapid, sensitive and amenable to high-throughput method for assaying TH agonists and antagonists. The C-fin assay fulfilled a number of important criteria such as low background with no interference from endogenous hormone, robust and statistically significant responses, dose dependence, and low threshold. The use of a single tadpole for multiple exposures reduces animal use and the multi-well format reduces reagent costs. Furthermore, having multiple animals all exposed to the same set of different treatments will provide information on biological variation in response to the individual treatments within the animal population. In this way, we were able to determine that approximately 7% of tadpoles are unable to respond TH treatment. This is in contrast to most metamorphic assays such as the *Xenopus* metamorphosis assay (XEMA) and the AMA where individual animal responses are not tracked (Fort ***et al.***, 2007). The C-fin assay also requires a much shorter time frame than morphological evaluation.

Despite its advantages, the C-fin assay based solely on the tail fin tissue is unable to determine TH disrupting effects that occur on the thyroid gland or the HPT-axis; however the assay can still encompass a large range of potential interferences. These include interferences with TH degradation, with TH transport across the cell membrane, with binding to cellular proteins, and interferences with binding to the

receptors or coregulator associations. The limitation of this assay to certain levels of the TH pathway can also be helpful at suggesting possible modes of action. We have largely focused upon QPCR-based analyses in this study. However, it is important to note that C-fin is amenable to the use of protein biomarkers as well. Additional protein analysis, such as with the RLKI cleavage fragment (Hinther *et al.*, 2010A) could provide a fuller profile of mechanistic effects of a test chemical. The appearance of the N-term RLK I protein fragment occurred as early as 12 h with a 10 nM T_3 dose in this organ-culture setting (Hinther *et al.*, 2010A). The amount of fragment increased with time, with the 48 h time point having a substantially high level. This fragment also appeared at significantly elevated levels at 1 nM T_3 within 48 h (Hinther *et al.*, 2010A). Incorporation of protein analyses would not be difficult as half of the biopsy could be used to study transcript levels and the other half to study protein levels. With further refinements and the potential for expanding the transcript and protein endpoints, C-fin has the potential to become a significant addition to the battery of EDC tests needed to handle the ever-increasing challenge of chemical risk assessment and monitoring.

Chapter 4: Nanometals induce stress and alter thyroid hormone action in amphibia and mammalian cells at or below North American water quality guidelines

The material in this chapter was adapted from and published in part in: Hinther, A., Vawda, S., Skirrow, R. C., Veldhoen, N., Collins, P., Cullen, J. T., van Aggelen, G., and Helbing, CC. Nanometals induce stress and alter thyroid hormone action in amphibia at or below North American water quality guidelines. *Environmental Science and Technology,* **44** (2010), pp. 8314-21.

4.1 Introduction

Nanoparticles (NPs) are materials with at least one dimension of nanometer size. NPs are found in a diverse range of products including electronics, optics, textiles, medical devices, cosmetics, food packaging, water treatment technologies, fuel cells, catalysts, biosensors, and agents for environmental remediation. Nanosilver has strong antibacterial properties and has a wide range of medical applications. It is the most commonly used NP in consumer products representing approximately one quarter of NP-enhanced products on the market (Project on emerging nanotechnologies, 2009) including wound dressings, contraceptive devices, surgical instruments, and prostheses (Chen and Schluesener, 2008). Quantum dots (QDs) are semiconductor NPs comprised of heavy metal compounds and are used in medical research and diagnostics as markers for selective imaging of tumour cells in living animals. QDs are

also used for optical applications and in electronics (Gao *et al.*, 2004). Nanozinc oxide is used in a variety of applications in electronics and personal care products, such as sunscreen (Jingxia *et al.*, 2009).

NPs are released into the environment from manufactured goods, manufacturing effluent, spillage during the shipping and handling of products, or through deliberate disposal (Handy *et al.*, 2008; Handy *et al.*, 2008). The biological activity of NPs is dependent on many factors, some of which include size, shape, and surface properties (Oberdorster *et al.*, 2005). At the lower end of the nanoscale (1-10 nm), NPs exhibit unusual chemical and physical properties different from their larger sized counterparts. The small size of the NPs facilitates their cellular uptake and their high surface to volume ratio allows association with other molecules that could be carried into cells (Klein, 2007). The inventory of NP-enabled consumer products has expanded from ~200 in 2006 to over 1,000 in 2009 with an estimated 1,600 products by 2011 (Project on emerging nanotechnologies, 2009). An intermediate estimate of the amount of NPs released into the aquatic environment per year is 65 tonnes (Blaser *et al.*, 2008). Despite the prevalence of NPs, information related to their biological effects is limited.

Recent research in fish has revealed nanometals possess distinctive toxicity profiles, including transcriptome changes, relative to materials in larger or ionic forms (Griffitt *et al.*, 2007; Chen and Schluesener, 2008; Chae *et al.*, 2009; Griffitt *et al.*, 2009). Despite the current information, there is no known information regarding the effects of NPs on amphibians. Being sensitive to toxic contaminants, amphibians are regarded as environmental sentinels (Houlahan *et al.*, 2000). Given the increasing amounts of NPs released into the environment and the previously published effects of NPs on fish, there

is reason to believe frogs may be affected by NPs. Not only are frogs sentinel species but they also undergo a postembryonic metamorphosis from a tadpole into a frog in a process that is directly dependent on THs (Tata, 2006). Premetamorphic tadpoles are free living and fully formed, but do not produce measurable levels of THs, while prometamorphic tadpoles synthesize increasing amounts of THs leading to metamorphic changes (Shi, 2000). Premetamorphs are responsive to exogenous administration of TH inducing a precocious metamorphosis (Shi, 2000).

The TH signalling pathway is highly conserved in vertebrates. THs are important in brain, nervous system, heart, and lung development in humans and regulate the postembryonic development of certain species of echinoderms, fish, and birds (reviewed in (Miller *et al.*, 2009)). The TH-dependent changes occurring in the tissues of a tadpole metamorphosing into a frog is an excellent model for detecting chemicals affecting the TH axis in vertebrates (Tata, 2006). Not only is it essential to assess the toxicity of NPs on frogs but it is also vital to examine their potential TH-disrupting capability. We have recently developed an *in vitro* cultured tail fin biopsy or "C-fin" assay that allows direct tissue assessment of chemicals of concern on premetamorphic tadpole tail fin biopsies (Helbing *et al.*, 2010; Hinther *et al.*, 2010A). Herein, we use this C-fin assay in combination with quantitative real time polymerase chain reaction (QPCR) to evaluate the exposure effects of three NPs: nanosilver, QDs, and nanozinc oxide, both in the absence and presence of TH-mediated gene regulation. None of these particles have been examined previously for impact on frog tissues or as potential disruptors of TH action. Comparison of the biological effects of NPs to other forms of

elemental or ionic metals was also performed. Nanosilver effects were compared to ionic silver in the form of silver nitrate.

Disruption of TH-action in the C-fin assay was assessed by measurement of mRNA abundance of transcripts encoding TH-induced TH receptor β (*TRβ*) and TH-repressed *Rana* larval keratin type I (*RLKI*) (Domanski and Helbing, 2007). We also determined the effects these chemicals had on cellular stress by measuring transcript levels of heat shock protein 30 (*HSP30*) (Helbing *et al.*, 1996; Woolfson and Heikkila, 2009) and catalase (*CAT*) (Kashiwagi *et al.*, 1999; Valko *et al.*, 2006).

We also used another model system to assess the effects NPs have on TH action. We used GH3, or rat pituitary cells, to determine if the NPs had any TH disrupting activity to the cells. GH3 cells are derived from rat pituitary and have been suggested for use as a TH-dependent cell proliferation-based T screen for TH disrupting chemicals. GH3 cells are a useful comparator to the C-fin assay because not only is it a mammalian model but they also represent a simpler level of organization.

In the GH3 cells, we measured the levels of TH-responsive transcripts, growth hormone (*GH*), deiodinase I (*DIOI*), and prolactin (*PRL*) and we examined the effects NPs had on stress by measuring the transcript, heat shock protein 70 (*HSP70*). Alterations in the steady-state levels of these transcripts corresponded to the specific type of NP and are suggestive of the potential for perturbing hormone-dependent postembryonic development, cell function, and inducing cellular stress.

4.2 Materials and Methods

4.2.1 Particle Characterization

NPs [nanosilver (CAS 7440-22-4; 99.1% purity), nanozinc oxide (CAS 8051-03-4; 99.1% purity), and green QDs comprised of cadmium telluride (CAS 1306-25-8; 99.1% purity)] manufactured to the specifications of 90% particles within the 1-10 nm diameter range were purchased from Northern Nanotechnologies (now known as ViveNano, Toronto, ON) at a NP concentration of 0.8 µM (1.5 mg/mL for nanosilver and nanozinc oxide; 20 mg/mL for QDs). These particles were water soluble due to a proprietary carboxyl-functionalized coating common to all three. They were stored at 4ºC. The zeta potential of each NP was determined using a PALS Zeta Potential Analyzer Ver. 3.57 (Brookhaven Instruments Corporation, Holtsville, NY). Particle size and distribution information was provided by J. Dinglasan (ViveNano) using transmission electron microscopy and dynamic light scattering (DLS) measurements. NP metal content was measured for the specific lots by ViveNano using inductively coupled plasma (ICP) analysis.

4.3 Results

4.3.1 NP Characterization

The characteristics of the NPs used in this study were determined using a variety of methods. For nanosilver, TEM and DLS approaches revealed particle sizes of 2-6 nm and 10 nm respectively. Silver comprised 29.29% of the mass of the NP as determined by ICP analysis. The zeta potential of nanosilver was -41.14 mV ± 1.99. TEM and DLS determined the QDs to have a particle size range of 2-10 nm and 10-15 nm,

respectively. Cadmium comprised 8.98% (for the C-fin studies) and 10.62% (for the acute toxicity studies) of the total NP mass as measured by ICP analysis. The zeta potential of the QDs was -54.10 mV ± 0.69. Nanozinc oxide in the present study had a particle size range of 2-10 nM and 9 nM as determined by TEM and DLS, respectively. Zinc comprised 12.29% of the NP mass as determined by ICP analysis. The zeta potential of nanozinc oxide was -67.34 ± 2.74. The size of these metal particles were confirmed to be <10 nm, using TEM, when prepared in the culture medium used for the tail fin biopsies (Hinther *et al.*, 2010B).

4.3.2 TH-Response Gene Transcript Levels upon Exposure to Nanosilver, Quantum Dots, and Nanozinc Oxide

In the first experimental set, we asked if equimolar amounts (0.1 to 10 nM) of the three NPs tested had differential effects on cultured tadpole tail fin tissue. *TRβ* transcript levels were decreased 2-fold by exposure to 10 nM nanosilver (p=0.012) compared to the control. *RLKI* transcript levels were decreased up to 2-fold at the two highest concentrations tested (5 nM, p=0.028; 10 nM, p=0.018) compared to the control (Figure 4.1A).

To determine if NP exposure altered normal amphibian TH-dependent responses, cultured tail biopsies were exposed to T_3 in the presence and absence of the NP. In the presence of 10 nM T_3 alone, *TRβ* transcript levels increased by 10 ± 1.4-fold (n=50; data not shown) and *RLKI* transcript levels decreased by 3 ± 0.2-fold (n=54; data not shown); results consistent with those reported previously (Hinther *et al.*, 2010). The results for the T_3-treated animals are expressed relative to the individual animal's

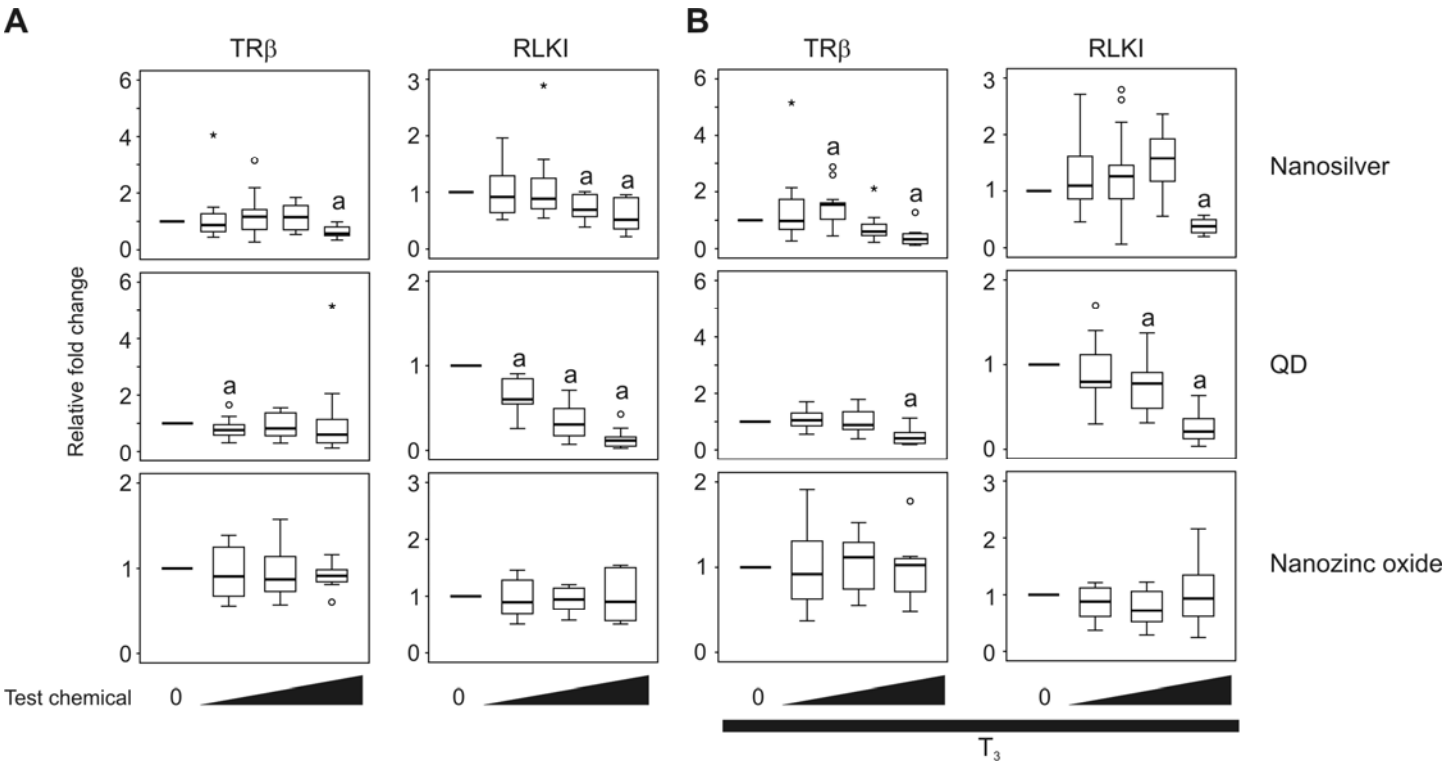


Figure 4.1. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to nanosilver, quantum dots (QD), and nanozinc oxide in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=6-14) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: 0.1, 1.0, 5.0, and 10 nM nanosilver and 0.1, 1.0, and 10 nM quantum dots or nanozinc oxide particles. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Methods section. The medians are shown as solid black lines within the box, and the box indicates the first and third quartiles. The whiskers indicate minimum and maximum values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Increasing concentrations of test chemicals are represented by bevels. Significance is indicated by an 'a' (p< 0.05 relative to control; Wilcoxon). Adapted from (Hinther *et al.*, 2010B).

response to T_3 alone to better discern the responses to NP exposure within this context. Therefore, although animals responded to T_3 treatment, the transcript levels were set to one (Figure 4.1B).

Two distinct nanosilver concentration-dependent responses were observed. *TRβ* transcript levels increased (1.4-fold) when exposed to 0.1 and 1 nM nanosilver compared to T_3 alone (1 nM, p=0.022) while the highest nanosilver concentration (10 nM) attenuated the T_3-induced increase in *TRβ* transcript levels (3-fold, *p*=0.012) compared to T_3 alone (Figure 4.1B). In contrast, the T_3-dependent decrease in *RLKI* transcripts was magnified 3-fold (*p*=0.018) by co-exposure with 10 nM nanosilver compared to T_3 alone (Figure 4.1B).

Exposure to QDs alone decreased *TRβ* levels at 0.1 nM (1.3 fold, *p*=0.030) compared to the control and reduced *RLKI* transcript levels ~9-fold (*p*=0.0001, Kendall's W; 0.1 nM, *p*=0.001; 1.0 nM, *p*=0.0001; 10 nM, *p*=0.0001, Wilcoxon) (Figure 4.1A). QDs also reduced the T_3-dependent induction of *TR*β transcript levels by 3-fold (*p*=0.0001, Kendall's W; 10 nM, *p*=0.03, Wilcoxon) and further magnified the T_3-dependent reduction in *RLKI* transcript levels up to 5-fold (*p*=0.0001, Kendall's W; 1.0 nM, *p*=0.006, 10 nM, *p*=0.0001, Wilcoxon) (Figure 4.1B).

Nanozinc oxide had no effect on *TRβ* and *RLKI* transcript levels in the presence and absence of T_3 (Figure 4.1).

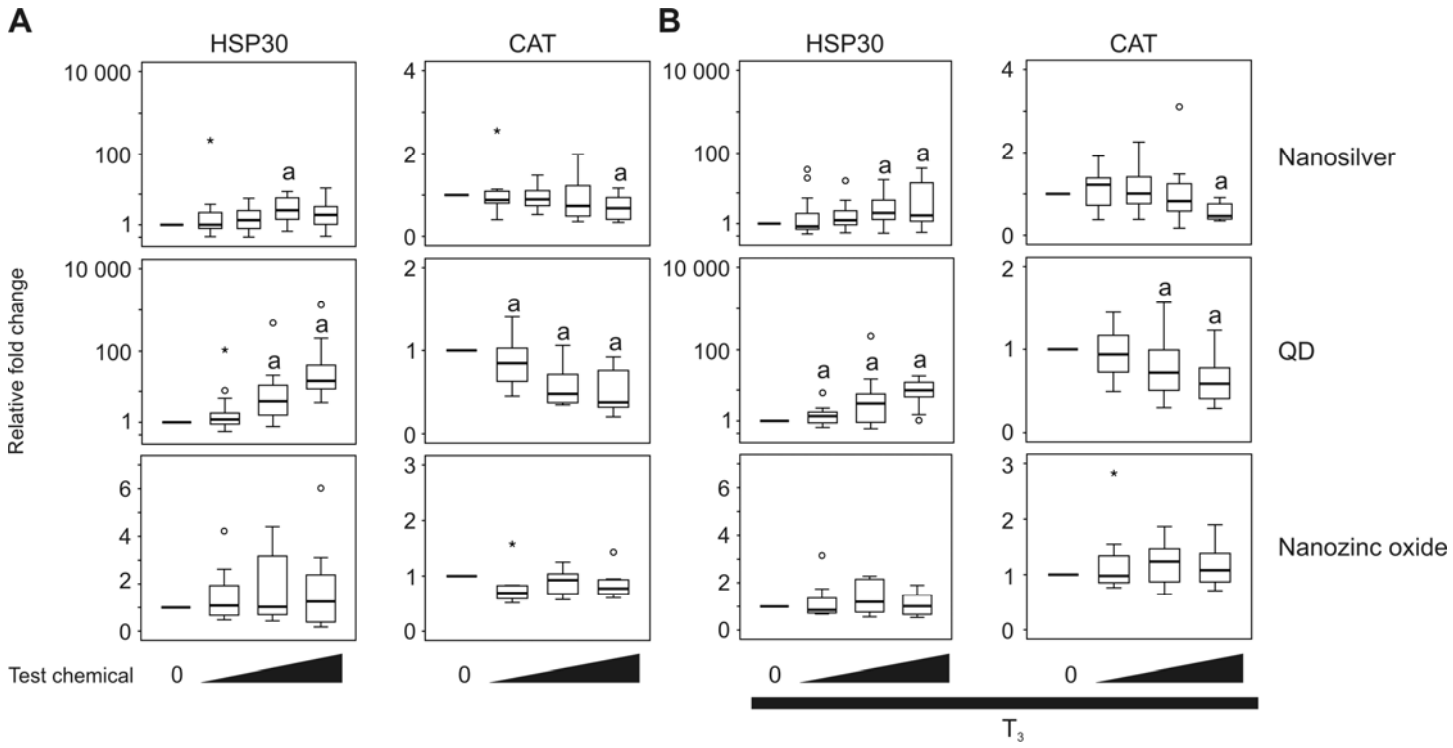


Figure 4.2. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nanosilver, quantum dots (QD), and nanozinc oxide in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=6-14) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: 0.1, 1.0, 5.0, and 10 nM nanosilver and 0.1, 1.0, and 10 nM quantum dots or nanozinc oxide particles. See Figure 1 legend for more graph details. Adapted from (Hinther *et al.*, 2010B).

4.3.3 Stress Response Gene Transcript Levels upon Exposure to Nanosilver, Quantum Dots, and Nanozinc Oxide

To determine if the response to NP exposure included a cellular stress component, we examined *HSP30* expression. Tail fin biopsies exposed to nanosilver showed a trend toward increased *HSP30* transcript levels up to 3-fold (5 nM, p=0.025; Figure 4.2A). QDs induced a 38- and 123-fold increase in *HSP30* transcript levels at 1.0 and 10 nM, respectively (p=0.001, Wilcoxon for both; p=0.0001, Kendall's-W; Figure 4.2A). In contrast, nanozinc oxide had no effect on *HSP30* transcript levels (Figure 4.2A).

Not only do the *HSP30* transcript levels increase when exposed to stressors, but TH also increases *HSP30* transcript levels (Helbing *et al.*, 1996). Treatment of biopsies with T_3 resulted in a 7 ± 1.6-fold (n=78; data not shown) induction of *HSP30* transcript levels relative to the vehicle control consistent with previously reported data (Helbing *et al.*, 1996). Nanosilver and QDs both affected the T_3-mediated *HSP30* response (Figure 4.2B). Nanosilver increased the T_3-induced response up to 11-fold (p=0.05, Kendall's-W; 5.0 and 10 nM, p=0.036, Wilcoxon) while QDs increased the T_3-induced response by 10 to 18-fold (p=0.0001, Kendall's-W; p=0.030, 0.011, 0.0001, Wilcoxon for 0.1, 1.0, and 10 nM, respectively; Figure 4.2B). Nanozinc oxide showed no significant changes in *HSP30* transcript levels in the presence of T_3 (Figure 4.2B).

Metals also induce oxidative cellular stress (Kashiwagi *et al.*, 1999; Valko *et al.*, 2006). Catalase (*CAT*) is an enzyme involved in the removal of H_2O_2 and a decrease in the expression or activity of this enzyme allows for the accumulation of reactive oxygen species, which could be deleterious to cells. Nanosilver exposure had a marginally

significant 1.5-fold reduction in *CAT* transcript levels at 10 nM (p=0.05, Wilcoxon) whereas QD exposure resulted in a significant reduction of *CAT* transcripts up to 2-fold (p=0.0001, Kendall's W; p = 0.039, 0.001, 0.0001, Wilcoxon for 0.1, 1.0, and 10 nM, respectively; Figure 4.2A). Nanozinc oxide had no effect on *CAT* transcripts (Figure 4.2A).

TH induces oxidative stress and previous results have shown *CAT* transcript levels to decrease in response to TH treatment (Kashiwagi *et al.*, 1999; Valko *et al.*, 2006). T_3 treatment alone resulted in a 1.3 ± 0.1-fold (n=78; data not shown) decrease in *CAT* transcript levels relative to the vehicle control which is consistent with previous reported observations (Kashiwagi *et al.*, 1999).

Nanosilver and QDs strengthened the T_3-induced reduction of *CAT* transcripts by up to 2-fold (nanosilver: p=0.001, Kendall's-W; 10 nM, p=0.012, Wilcoxon; QDs: p=0.002, Kendall's-W; p=0.026, 0.001, Wilcoxon for 1.0, and 10 nM, respectively; Figure 4.2B). Nanozinc oxide did not affect *CAT* transcript levels (Figure 4.2B).

4.3.4 GH3 cell TH- and Stress Response Gene Transcript Levels upon Exposure to Nanosilver and QDs

The effects of nanosilver and QDs were tested using GH3 cells, to see if the effects were similar in a mammalian system. We tested the effects of nanosilver and QDs in both the absence and presence of TH by measuring the steady-state levels of selected transcripts. *GH*, *DIOI*, and *PRL* are TH-responsive transcripts and we tested *HSP70* transcript to assess if there was a stress response associated with the exposure of nanosilver and QDs.

In total, the GH3 cells were exposed to 5 different chemical exposures (nanosilver, QDs, TCS, mTCS, and TCC) for this thesis. In these experiments, *GH* and *DIOI* transcript levels consistently increased in response to T_3 treatment. *PRL* transcript levels did not consistently increase in response to T_3 treatment and *HSP70* transcript levels were either decreased of unaffected by T_3 treatment.

GH3 cells were exposed to 0.1, 0.5, 1, 5, and 10 nM nanosilver in the absence and presence of 10 nM T_3 (Figure 4.3). There was significant cell death at 10 nM nanosilver and we were unable to obtain enough quality mRNA in order to measure the levels of selected transcripts (data not shown). Overall significant differences were observed for *DIOI* and *HSP70* (p=0.0001 for both, Kruskal-Wallis).

T_3 treatment alone resulted in 2.8 fold increase in *GH* (p=0.046, Mann-Whitney U), a 26-fold increase in *DIOI* transcript (p=0.002, Mann-Whitney U), and a 5-fold decrease in the *HSP70* transcript (p=0.005, Mann-Whitney U), relative to vehicle control; *PRL* transcript levels were not significantly changed with T_3 treatment alone.

Exposure to nanosilver alone did not cause any overall significant changes in the levels of *GH*, *PRL*, and *HSP70* transcripts (Figure 4.3). In contrast, *DIOI* transcript showed overall significance and was significantly decreased at 0.5, 1, and 5 nM nanosilver by 1.5-fold (p=0.025, Kruskal-Wallis; p=0.036, Mann-Whitney U), 1.6-fold (p=0.021, Mann-Whitney U), and 2.8-fold (p=0.014, Mann-Whitney U), respectively, relative to the vehicle control (Figure 4.3).

When co administered with T_3, nanosilver did not show any overall significance with all four transcripts. There was no significant change from the normal response to evident exposure to 5 nM nanosilver drastically reduced the variation in the responses

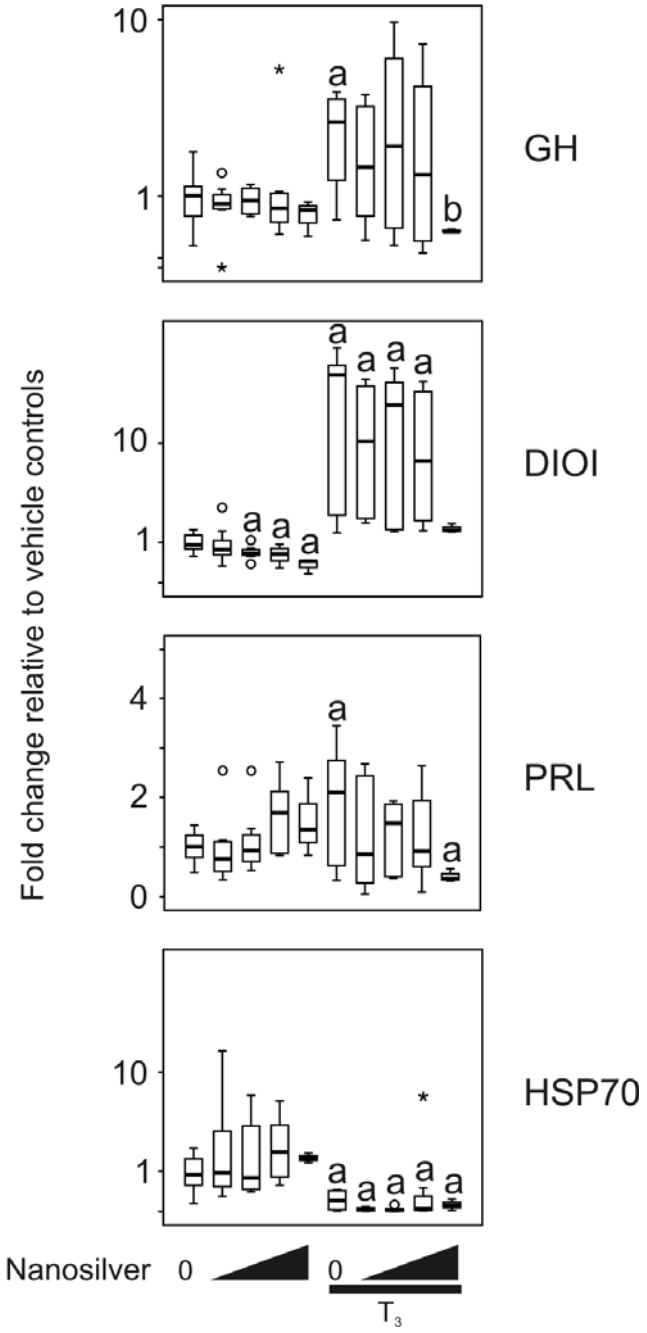


Figure 4.3. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to nanosilver in the absence and presence of 10 nM T_3. GH3 cells were exposed to vehicle control (0) and the indicated test chemical for 48 h. Test chemical concentrations were 0.1, 0.5, 1, and 5 nM for nanosilver. Exposure to 10 nM nanosilver resulted in extensive cell death (data not shown). Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, Mann-Whitney U) and relative to T_3 treatment by 'b' ($p \leq 0.05$, Mann-Whitney U). See Figure 1 legend for more graph details.

T_3 observed with *DIOI*, *PRL*, and *HSP70* transcripts (Figure 4.3). However, it was of all transcripts examined (Figure 4.3) and significantly reduced *GH* by 16-fold relative to T_3 treatment alone (p=0.014, Mann-Whitney U).

GH3 cells were exposed to 0.1, 0.5, 1, and 5 nM of QDs in the absence and presence of 10 nM T_3 (Figure 4.4). There was significant cell death at 5 nM and 10 nM QDs and we were unable to obtain enough quality mRNA in order to measure the levels of the selected transcripts (data not shown). Overall significance was observed with all 4 transcripts tested (p=0.0001, *GH*; p=0.0001, *DIOI*; p=0.034, *PRL*; p=0.0001, *HSP70*, Kruskal-Wallis).

T_3 treatment alone resulted in similar trends observed in Figure 4.3 with a 3-fold increase in *GH* transcript levels relative to vehicle control (p=0.004, Mann-Whitney U) and a 12-fold increase in *DIOI* transcript levels relative to vehicle control (p=0.004, Mann-Whitney U) although *PRL* transcripts were elevated, but not significantly compared to the vehicle control (Figure 4.4), a similar pattern observed in Figure 4.3. *HSP70* transcript levels were not changed in response to T_3 treatment in this experiment (Figure 4.4) as in contrast to the significant decrease in *HSP70* transcript levels in response to T_3 treatment.

Exposure to QD alone resulted in significant changes within the *DIOI* and *HSP70* transcripts (p=0.002 and p=0.0001, respectively, Kruskal-Wallis); there was no significant changes within the *GH* and *PRL* transcripts when cells were exposed to QD alone. Exposure to 0.5 and 1 nM QDs significantly decreased the transcript levels of respectively, relative to vehicle control (Figure 4.4). QD exposure significantly

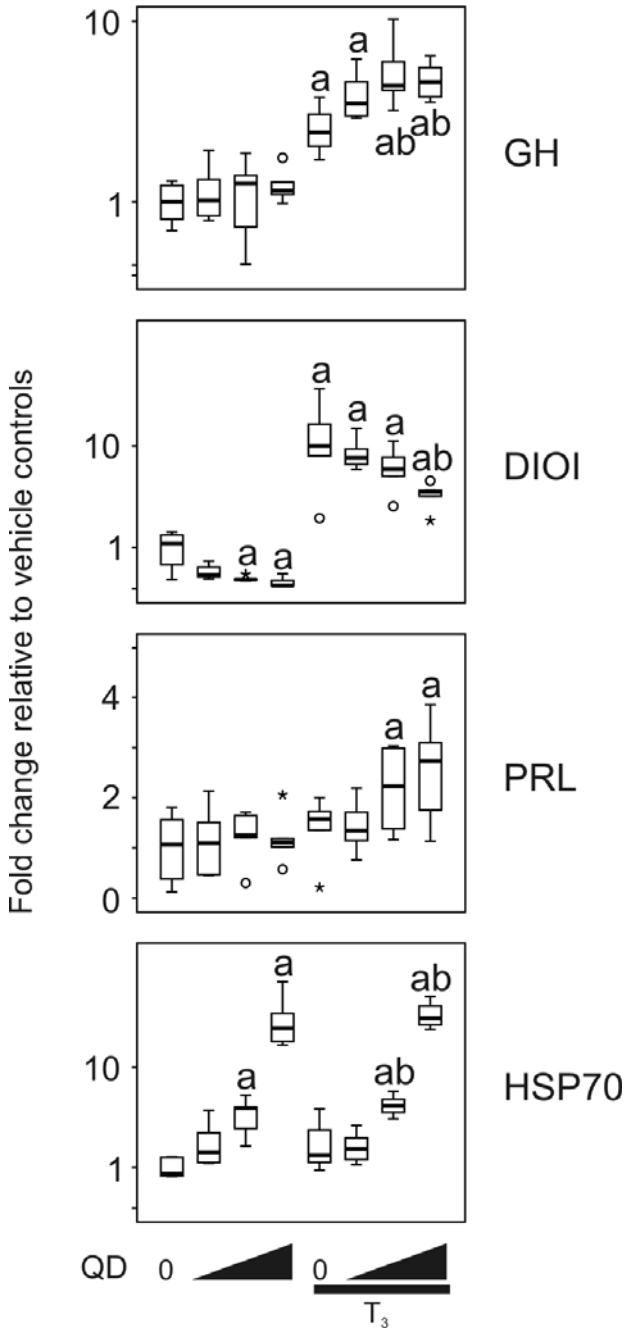


Figure 4.4. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to quantum dots (QD) in the absence and presence of 10 nM T_3. GH3 cells were exposed to vehicle control (0) and the indicated test chemical for 48 h. Test chemical concentrations were 0.1, 0.5, and 1 nM for QD. Exposure to 5 and 10 nM QDs resulted in substantial cell death and transcript determinations were not possible (data not shown). Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, Mann-Whitney U) and relative to T_3 treatment by 'b' ($p \leq 0.05$, Mann-Whitney U). See Figure 1 legend for more graph details.

DIOI by 5.5-fold (p=0.016, Mann-Whitney U) and 9.2-fold (p=0.006, Mann-Whitney U), increased the *HSP70* at 0.5 and 1 nM by 4-fold (p=0.006, Mann-Whitney U) and 24-fold (p=0.006, Mann-Whitney U), respectively, relative to vehicle control (Figure 4.4). No effects on *GH* or *PRL* transcripts were observed (Figure 4.4).

Coexposure of QDs with T_3, caused significant changes in the *GH* (p=0.009, Kruskal-Wallis), *DIOI* (p=0.014, Kruskal-Wallis), and *HSP70* (p=0.0001, Kruskal-Wallis) transcript levels. When the cells were exposed to both hormone and QD, there was an increase in *HSP70* transcript levels, as was observed without T_3 present, of 4-fold (0.5 nM QDs p=0.018, Mann-Whitney U) and 26-fold (1 nM QDs p=0.006, Mann-Whitney U), respectively, relative to T_3 treatment alone (Figure 4.4). Similarly, *GH* transcript levels were significantly increased upon coexposure of T_3 with 0.5 and 1 nM QDs relative to T_3 treatment alone by 5.9-fold (p=0.006, Mann-Whitney U) and 5.3-fold (p=0.006, Mann-Whitney U), respectively, (Figure 4.4). *PRL* transcripts did not show any significant effects when exposed to QDs in this context, but a trend reminiscent of the *GH* and *HSP70* transcript responses was evident (Figure 4.4). In contrast, the transcript levels of *DIOI* were significantly decreased (p=0.037, Mann-Whitney U) by 3-fold relative to T_3 treatment alone at the highest concentration of QDs tested in the presence of T_3 (Figure 4.4).

4.3.5 Context within Water Quality Guidelines

Given that the above C-fin experiments used the same number (molarity), functionalization (carboxyl) and sizes of NPs, the observed differential responses to these NPs were likely due to the metal composition. Safety guidelines for drinking water

and supporting aquatic life exist for silver, cadmium, and zinc. The United States drinking water guidelines generally concur with the Canadian guidelines and are less stringent for the support of aquatic life compared to the Canadian guidelines (EPA, 2006; EPA, 2009).

The current Canadian Council of Ministers of the Environment (CCME) guidelines for zinc are 30 µg/L for supporting aquatic life (CCME, 2007) and 5 mg/L for drinking water (Health Canada, 2008). In the C-fin above experiments, the concentrations of nanozinc oxide tested ranged from 0.1 nM – 10nM NP (equivalent to 0.19 mg/L -19 mg/L of NP) and were equal to a metal concentration range of 23 µg/L – 2.3 mg/L. Thus, nanozinc oxide exposure did not show any effects at concentrations bracketing current North American guidelines.

In contrast, the current CCME guideline for sustaining aquatic life for silver is 0.1 µg/L. There is no upper limit given for silver in drinking water as it is not considered a human health risk (Health Canada, 2008). The current CCME guideline for cadmium is 0.017 µg/L and the Health Canada drinking water guideline for cadmium is 5 µg/L. In the above experiments, the NP metal ranges tested were 0.055 - 5.5 mg/L silver and 0.22-22.0 mg/L cadmium for the C-fin assay experiments. For the GH3 cell experiments the NP metal range tested was 0.055 – 5.5 mg/L silver, where cell death occurred at 5.5 mg/L and transcript data could not be obtained. For the GH3 cell experiments with QDs, the NP metal range was 0.22 – 11 mg/L cadmium where cell death occurred at 11 mg/L cadmium and transcript data could not be obtained. Given there were sufficient indications that lower concentrations of these two NPs may affect

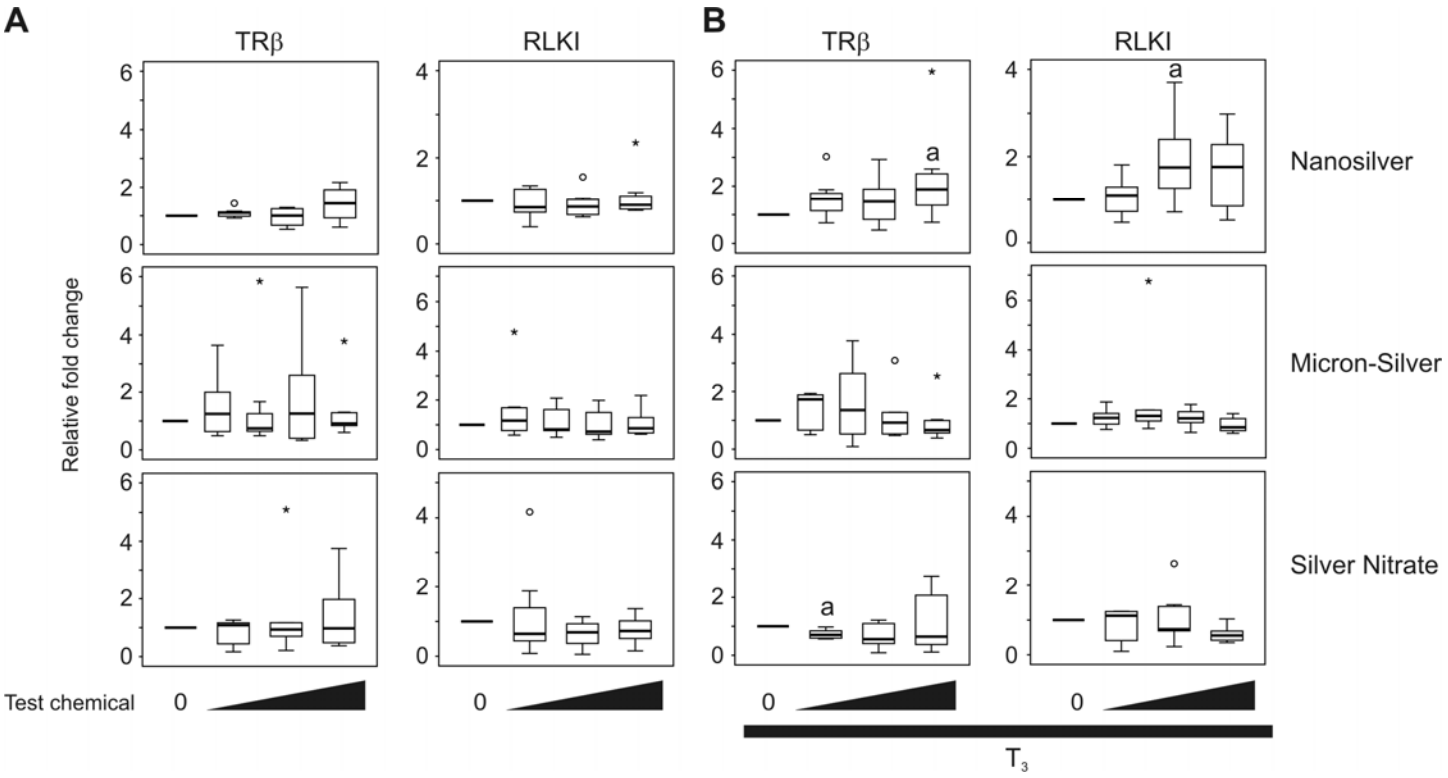


Figure 4.5. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to nanosilver, micron-silver, and silver nitrate in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=6-14) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were applied in 10-fold increments ranging from 0.06 µg/L - 6 µg/L nanosilver particles, micron-silver and silver nitrate. See Figure 1 legend for more graph details. Adapted from (Hinther *et al.*, 2010B).

target endpoints, we performed additional exposures using metal concentrations that bracketed the respective silver and cadmium limits for supporting aquatic life.

4.3.6 TH- and Stress Response Gene Transcript Levels upon Exposure to Low Concentrations of Nanosilver

In the second set of C-fin experiments, we used a concentration of nanosilver particles ranging from 0.06 µg/L – 6 µg/L (equivalent to 0.018 µg/L – 1.8 µg/L silver). While there was no effect of nanosilver on *TRβ* and *RLKI* transcript levels on its own (Figure 4.5A), the T_3-dependent increase in *TRβ* transcript levels was elevated an additional 2.2-fold in the presence of 6 µg/L nanosilver (p=0.036; Figure 4.5B). Moreover, the T_3-dependent repression of *RLKI* transcript levels was attenuated up to 1.7-fold (p=0.025, 0.6 µg/L; p=0.093, 6.0 µg/L; Figure 4.5B).

Micron-silver was tested at similar metal concentrations as nanosilver to determine if the effects observed with the nanometal were dependent on particle size. Micron-silver exerted no significant effects on *TRβ* and *RLKI* transcript levels (Figure 4.5A) even in the presence of T_3 (Figure 4.5B).

Given the possibility that the effects observed with nanosilver could be due to the presence of the silver cation, we exposed biopsies to equimolar amounts of ionic silver in the form of silver nitrate. In general, silver nitrate showed little effect on *TRβ* and *RLKI* transcript levels (Figure 4.5) except for a 1.7-fold attenuation in the T_3-dependent increase in *TRβ* transcript levels at the lowest silver nitrate concentration tested (p=0.028;Figure 4.5B).

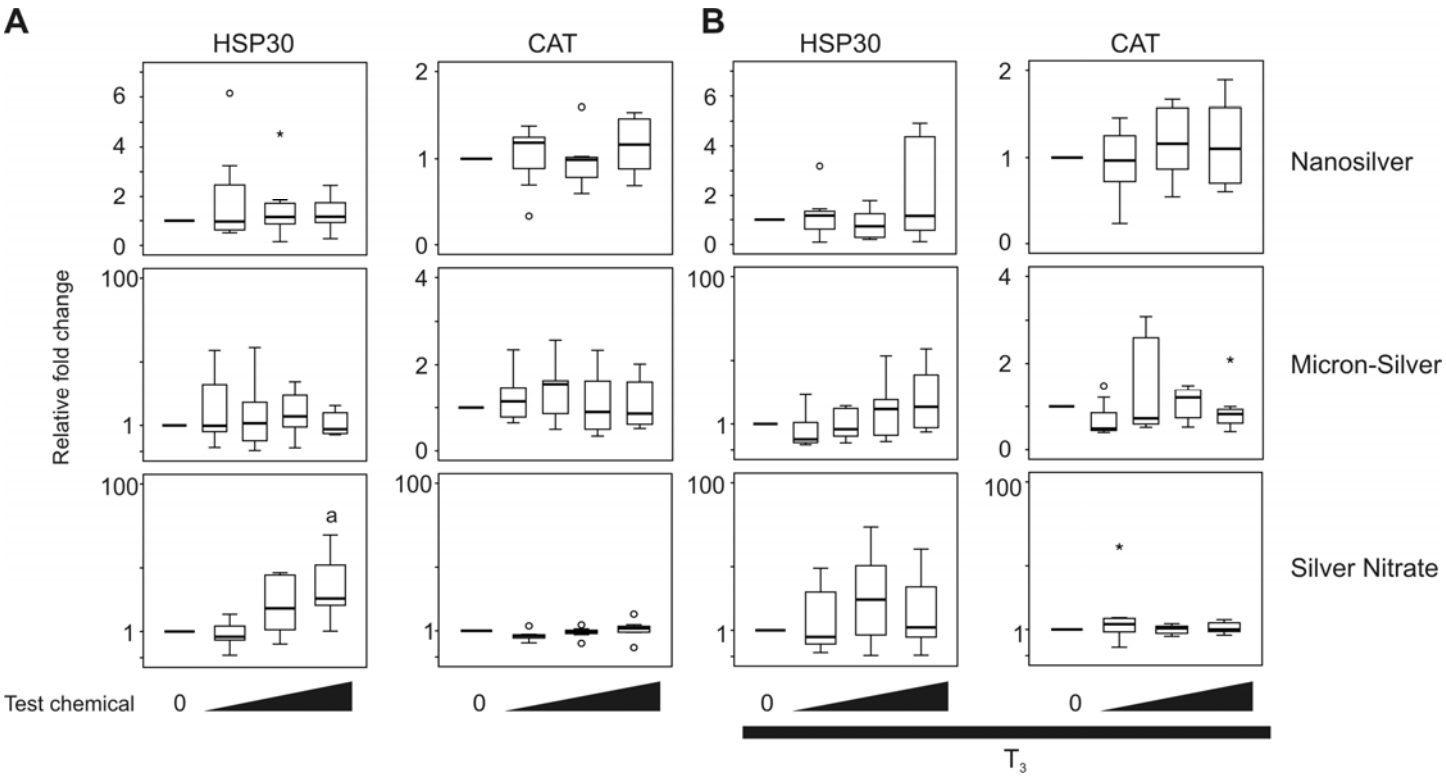


Figure 4.6. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nanosilver, micron-silver, and silver nitrate in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=6-14) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were applied in 10-fold increments ranging from 0.06 µg/L - 6 µg/L nanosilver particles, micron-silver and silver nitrate. See Figure 1 legend for more graph details. Adapted from (Hinther *et al.*, 2010B).

Nanosilver did not cause any significant changes to the *HSP30* and *CAT* transcript levels in both the absence and presence of T_3 (Figure 4.6). Micron-silver also showed no significant effects on either the *HSP30* and *CAT* transcripts although there was greater variation observed in the levels of *HSP30* transcripts in the micron-silver exposures (Figure 4.6). Silver nitrate exposure elicited a 4-fold (p=0.063) and 9-fold (p=0.018) increase in *HSP30* transcript levels at 0.6 and 6.0 µg/L, respectively (Figure 4.6A).

4.3.7 TH- and Stress Response Gene Transcript Levels upon Exposure to Low Concentrations of QDs

QDs were examined at a NP concentration range of 0.25 – 25 µg/L (equivalent to 0.022 µg/L – 2.2 µg/L cadmium). *TRβ* transcript levels increased by 1.4-fold in the presence of T_3 at 0.25 µg/L (p=0.028) compared to the control (Figure 4.7B). The QDs did not show any other significant changes in *TRβ* and *RLKI* transcript levels in the absence or presence of T_3 (Figure 4.7). We tested micron-sized cadmium telluride (micron-CdTe) to evaluate if the effect observed with QDs was due to chemical composition (both are made with CdTe) or associated with size. Tail fin biopsies exposed to micron-CdTe showed no significant changes in *TRβ* and *RLKI* transcript levels (Figure 4.7).

QDs did not elicit a stress response at the lower concentrations tested with *HSP30* and *CAT* transcript levels remaining unchanged regardless of T_3 status (Figure 4.8). Micron-CdTe did not cause significant changes in the *HSP30* and *CAT* transcript

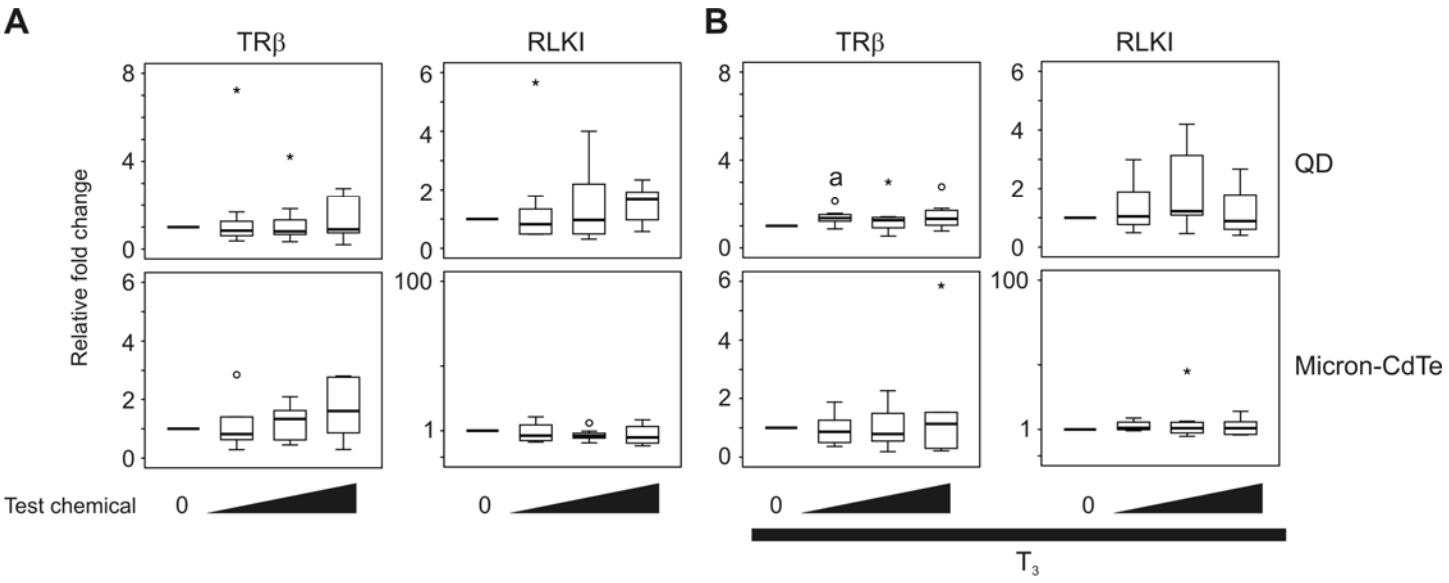


Figure 4.7. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to quantum dots (QDs) and micron-cadmium telluride (micron-CdTe) in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=6-14) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were applied in 10-fold increments ranging from 0.025 – 2.5 µg/L QDs or micron-CdTe. See Figure 1 legend for more graph details. Adapted from (Hinther *et al.*, 2010B).

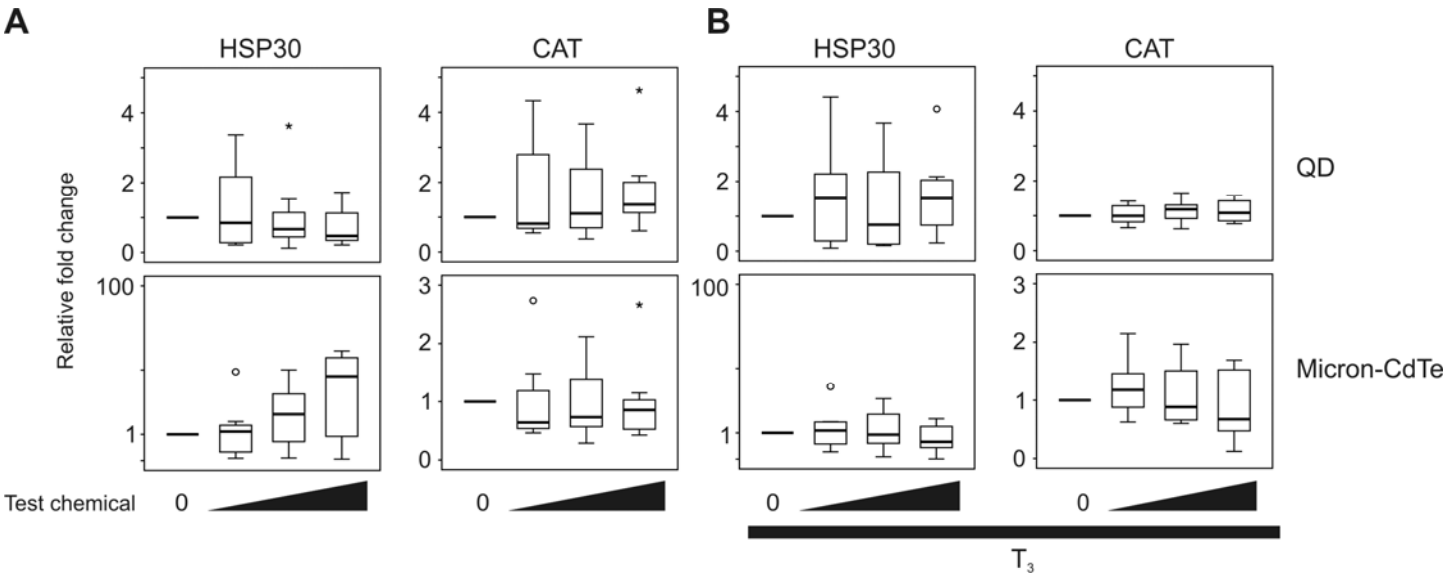


Figure 4.8. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to quantum dots (QDs) and micron-cadmium telluride (micron-CdTe) in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=6-14) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were applied in 10-fold increments ranging from 0.025 – 2.5 µg/L QDs or micron-CdTe. See Figure 1 legend for more graph details. Adapted from (Hinther *et al.*, 2010B).

levels in both the absence and presence of T_3 (Figure 4.8). However we did observe an increasing trend in *HSP30* transcript levels (2.5 µg/L, p=0.093).

4.4 Discussion

The present work shows frog tissue responds to nanosilver and QDs exposure by exhibiting signs of cellular stress through alterations of *HSP30* and *CAT* transcripts. We observed indications of cellular stress at concentrations of 0.22 mg/L cadmium and 2.75 mg/L silver and higher in the context of nanoparticles (Figure 4.2). This cellular stress response was independent of TH status (Figure 4.2) and appears to be related to the metal composition of the NP since nanozinc oxide had no effect at similar NP concentrations.

The *HSP70* transcript levels were measured in the GH3 cells as an indicator of cellular stress. HSP70 proteins act as a molecular chaperone and prevents proteins from misfolding and aggregating during *de novo* synthesis and under stressful conditions (Gray *et al.*, 1999; Papp *et al.*, 2003); however prolonged high levels of HSP70 proteins can be detrimental and has been shown to be cytotoxic in the absence of stress to rat pheochromocytoma cells (Arispe *et al.*, 2004; Li and Mak, 2009). HSP70 proteins levels will increase in response to a stress event but further stress events show little increase in HSP70 protein levels (Theodorakis *et al.*, 1999). *HSP70* mRNA half-life is 1 hour in cells after stress (Theodorakis and Morimoto, 1987; De Maio, 1999) and a parallel decrease in *HSP70* mRNA levels was shown with an accumulation of HSP70 protein levels. Balakrishnan and De Maio (2006) showed HSP70 protein can bind *HSP70* mRNA possibly as a mechanism to limit its protein expression and the decrease

in mRNA levels is thought to be dependent on the HSP70 protein levels (Balakrishnan and De Maio, 2006).

The *HSP70* transcript levels either decreased or were unaffected with T_3 treatment alone. SinceT_3 can increase the levels of heat shock proteins in amphibian tail fins and liver, there is reason to believe HSP70 protein levels did increase in the GH3 cells following T_3 treatment (Ghisari and Bonefeld-Jorgensen, 2005); however, the short half life of its mRNA and the 48 hours exposure period, could explain why we observed a inconsistent decrease in *HSP70* transcript levels relative to the control.

Nanosilver did not induce cellular stress as observed by the *HSP70* transcript levels (Figure 4.3). These observations indicate that mammalian cells are less sensitive to these nanoparticles than frog tissue. However, the mammalian cells showed marked cell death at 5 nM NP and above, which was not observed in the tail biopsies suggesting that the mammalian cells have a very narrow window of response before marked cytotoxicity is evident.

The rat pituitary cells showed signs of cellular stress when exposed to QDs at concentrations equivalent to 1.1 mg/L cadmium and higher through the perturbations in the *HSP70* transcript levels (Figure 4.4). Previous literature supports this by showing that there are both stimulatory and inhibitory T_3-responsive elements in regulation of the expression of the PRL gene in GH3 cells (Day and Maurer, 1989) and may explain the variable steady-state *PRL* transcript levels in response to T_3 treatment.

From the little toxicological information that exists, nanosilver appears to target the mitochondria through unknown mechanisms, increasing oxidative stress, and contributing to cell membrane damage in skin, respiratory system, liver, and the

gastrointestinal tract (Chen and Schluesener, 2008). Nanosilver may interact with protein and enzyme thiol groups and affect transcription in bacteria (Chen and Schluesener, 2008). However, these data are difficult to extrapolate to other systems and in many cases are not environmentally-relevant. Chen and Schluesener (2008) noted that nanosilver may affect gene and protein expression in human skin cells similar to QDs (Chen and Schluesener, 2008; Zhang *et al.*, 2008) though no studies have examined this to date. Cadmium is a highly toxic metal and this is reflected in the lower allowable concentrations in drinking water and in regulations for supporting aquatic life (EPA, 2006; EPA, 2009; Health Canada, 2008; CCME, 2007). It is a constituent of many QDs in current use with a variety of formulations available that could further influence their toxic effects (Hardman, 2006). In general, cytotoxicity in mammalian cells has been observed in the micromolar (mg/L) range (Lovric *et al.*, 2005; Lovric *et al.*, 2005; Hardman, 2006) which is consistent with our observations with the GH3 cells.

Not only did we see a cellular stress response, we also observed evidence that nanosilver and QDs may affect TH signaling pathways important in frog development at a wider range of concentrations than was observed for cell stress indicators. Alteration of the transcriptome is an essential component in TH-mediated tadpole metamorphosis (Shi, 2000) and part of the change in the tail transcriptome includes an increase in *TRβ* transcripts and a decrease in *RLKI* transcripts (Domanski and Helbing, 2007). There is considerable precedent linking *TRβ* transcript levels to progression through TH-dependent metamorphosis where perturbations from expected levels are indicative of altered postembryonic development (Crump *et al.*, 2002; Opitz *et al.*, 2006; Veldhoen *et al.*, 2006; Zhang *et al.*, 2006; Helbing *et al.*, 2007; Helbing *et al.*, 2007; Ji *et al.*, 2007;

Skirrow *et al.*, 2008). In the present study, significant changes in *TRβ* and *RLKI* transcripts were observed upon exposure to nanosilver and QDs over several orders of magnitude. The patterns observed could be categorized into two general groupings defined by the concentration range of the NP.

In the first experimental set, both *TRβ* and *RLKI* transcript levels were decreased in response to nanosilver and QD exposure at the higher concentrations tested (≥0.22 mg/L QDs and ≥2.75 mg/L nanosilver; Figure 4.1) which corresponds to the concentrations when cellular stress responses were also observed. The presence of T_3 may have afforded some protection to this decrease, but the pattern was similar regardless of the hormone status of the cultured tail fin biopsies (Figure 4.1). Both stress and hormone response pathways can influence each other (Helbing and Atkinson, 1994) and the C-fin data suggest exposure to NPs activates stress response pathways to different degrees, which, in the presence of TH, modulates the hormone-dependent regulation of gene expression. NP aggregation with proteins has been demonstrated to alter protein conformation (Klein, 2007) and the observation that *HSP30* transcript levels increase upon nanosilver and QD exposure may be an indication of increased protein aggregation that can occur during a stress event (Abdulle *et al.*, 2002). Some selectivity in biological response must be present due to the inherent properties of the NPs examined because not all NPs behaved in the same way.

It should be noted these stress responses are not due to a general toxic effect since none of the treatments or concentrations examined in the present study resulted in an alteration in the levels of the normalizer mRNA transcript encoding ribosomal protein L8 ($p=0.301$, Kruskal-Wallis), while we observed clear indications that other

transcripts encoding protein products involved in TH signalling and/or stress were uniquely affected. Indeed, the concentrations at which effects were observed in the C-fin experiments were orders of magnitude lower than the LC_{50} values determined in whole animals. The LC_{50} value of silver as silver nitrate was determined to be 0.25 mg/L, while that of nanosilver was 0.95 mg/L nanoparticle (equivalent to 0.28 mg/L silver) (Hinther *et al.*, 2010B). No mortality was observed in tadpoles exposed to 100 mg/L QDs (equivalent to 10.6 mg/L cadmium) (Hinther *et al.*, 2010B). Decreased TH-inducible transcript levels associated with stress events has previously been reported presumably as a part of the cellular stress response (Helbing and Atkinson, 1994).

Again, with the GH3 cells, we also observed perturbations in TH-signalling pathways as well as a cellular stress response. As mentioned above, there were no indications of cellular stress when the GH3 cells were exposed to nanosilver, since there were no perturbations seen in the *HSP70* transcript levels (Figure 4.3). In the presence of TH, nanosilver caused perturbations in the *GH* transcript levels at the highest concentration of nanosilver tested (5 nM nanosilver) but did not affect any of the other TH-responsive transcripts tested in the presence of TH. It is interesting that there was a drastic drop in the *GH* transcript levels at 5 nM nanosilver in the presence of TH (and indications of a similar trend with *DIOI* and *PRL* transcripts) but we did not detect an increase in *HSP70* transcript levels (Figure 4.3). It therefore appears that nanosilver is affecting the hormone-mediated pathway without inducing cellular stress. Additional evidence for perturbation of TH-signalling pathway was observed in the marked decrease in *DIOI* transcripts upon exposure to nanosilver alone (Figure 4.3).

Exposure of GH3 cells to QDs resulted in a similar induction of cellular stress pattern that we observed with the C-fin assays (compare Figures 4.1 and 4.4). Where there was a cellular stress response, we also saw perturbations in TH-signalling pathways. *DIOI* transcripts decreased in the presence or absence of TH while *GH* transcripts were increased to levels higher than those expected by T_3 induction alone (Figure 4.4). DIOI enzyme is responsible for the deiodination of the hormone's inner and outer ring. This enzyme activity results in the activation of thyroxine (T_4), but in these experiments, T_3 was used. Thus the increase in *DIOI* transcripts by T_3 could be part of a mechanism to inactivate the hormone to T_2 (Berry *et al.*, 1990; Berry *et al.*, 1991). With this in mind, a concurrent decrease in T_3-induced *DIOI* transcript levels by QDs could result in a delayed inactivation of T_3 manifested by the significant increase of *GH* and trends towards increased *PRL* transcripts (Figure 4.4).

As with the C-fin assay data, levels of the normalizer mRNA transcript encoding ribosomal protein L8 in GH3 cells were of normal quality and quantity where overt cell death was not noted, while we observed clear indications that other transcripts encoding protein products involved in TH signalling and/or stress were uniquely affected. It is clear that both nanosilver and QDs exerted some effects in terms of disrupting the actions of TH at the transcript level that is not due to overt toxicity. The mechanisms through which these NPs enact their TH disrupting effects is unclear but are at a cellular level and could perhaps occur upstream of TRs by NPs interacting with TH before it enters the cell or through affecting TH half-life. The effects NPs have on whole animals are yet to be determined.

Keeping the impact of NP exposure on aquatic organisms in mind, we performed a second set of C-fin exposures centred around metal levels currently set for water quality guidelines. In the range of 0.6 to 550 µg/L nanosilver (Figures 4.1 and 4.5), a different trend was observed compared to the higher concentrations tested in the present study that was dependent upon TH status reminiscent of a non-monotonic "inverted-U" type of response characteristic of endocrine disruption (Gore *et al.*, 2006). The expected T_3-dependent increase in *TRβ* transcript levels was significantly enhanced within this NP concentration range while the usual T_3-dependent reduction in *RLKI* transcript levels was attenuated (Figures 4.1 and 4.5). This pattern was only weakly observed at the lowest concentration of QDs tested (0.25 µg/L Figure 4.7) suggesting that the phenomenon observed may be more specific to nanosilver. These T_3-dependent effects were not observed upon exposure to silver nitrate, micron-silver, and micron-CdTe suggesting that metal composition as well as particle context (nano instead of micron or ionic) determines cellular response.

During frog postembryonic development, many gene transcripts are increased in response to elevated endogenous levels of TH of which *TRβ* represents a classic example. In these up-regulated genes, TR forms a heterodimer with the retinoid X receptor (RXR) and this complex binds to its TH response element (TRE) in the target gene. In the unliganded state, the TR-RXR complex binds to corepressors and functions as a negative regulator to repress the basal gene transcription. Upon ligand binding, the corepressors are released and coactivators are recruited that include histone acetyl-transferases (HATs), which facilitate gene transcription (Lee and Yen, 1999; Shi, 2009).

In contrast, there is little information known about genes that are down-regulated in response to TH exposure, such as *RLKI,* with thyroid stimulating hormone (*TSH*) being one of the few better known examples. Similar to TH-mediated up-regulated genes, corepressors are recruited by the unliganded TR-RXR heterodimer to the gene promoter and are associated with histone deacetylases (HDACs). However, in this context, the gene is active for mRNA transcription. When TH is present, corepressors are released and there is an apparent increase in histone H3 acetylation, which results in decreased transcription (Wang *et al.*, 2009). The underlying mechanisms of this TH-mediated dual control of gene regulation remain enigmatic. However, the nature of the impact of nanosilver exposure on transcript levels suggests a possible mechanism of action that involves affecting the recruitment of coactivators to TR-containing transcriptional complexes required for TH-mediated gene response (Lee and Yen, 1999). If true, this would represent a novel target and mechanism of action for NPs at lower concentrations.

From the classical toxicological perspective, the tested NPs are not particularly toxic relative to their bulk counterparts. However, in the case of nanosilver and QDs, these NPs are clearly able to alter the expression levels of key mRNA transcripts that suggest a possible deleterious effect on the organism. Adjusting the nanosilver and QD NPs for metal content, the effects observed on the TH signalling pathway fall at or below current North American water guidelines (EPA, 2006; EPA, 2009; Health Canada, 2008; CCME, 2007). Given that the water quality guidelines apply safety factors of 10 to 100 on the most sensitive toxicological endpoint, these data suggest that the values generated need to be reassessed on a broader range of species and endpoints. Thus

the present study points to another form of toxicity that must be addressed for nanomaterial safety. With QDs revolutionizing medical imaging and cell biology, and nanosilver appearing in a variety of medical and consumer products including hand sanitizers, textiles, and washing machines, the present study provides a note of caution in their use, particularly during sensitive life stages, as they may alter stress pathways and critical hormone-mediated cellular function.

Chapter 5: Nitrite and nitrate do not induce stress or disrupt thyroid hormone action in *Rana catesbeiana* cultured tadpole tail fin tissue

5.1 Introduction

Currently over 100,000 manufactured chemicals are produced in the marketplace (Commission, 2006). Many of these chemicals have endocrine disrupting abilities and more specifically, are disruptors of the thyroid hormone axis. Most endocrine disruptors can be classified as plasticizers, pesticides, industrial chemicals, heavy metals, or plant and fungal compounds; however, ions such as nitrate (NO_3) and nitrite (NO_2) have been shown to have endocrine disrupting abilities as well (Crain, 2000; Sampat, 2000; Gray *et al.*, 2001).

In the USA, the current public health maximal level for safe drinking water is 10 mg/L nitrate (measured as NO_3-N) and 1 mg/L nitrite (NO_2-N) (US, 2006; CCME, 2007; HealthCanada, 2008; US, 2009). In Canada, the Canadian Council of Ministers of the Environment (CCME) guideline for the protection of aquatic life has set the maximum level of nitrate at 13 mg/L (NO_3^-/L) in freshwater and 16 mg/L (NO_3^-/L) in marine water; the level for nitrite in freshwater is 60 µg/L (NO_2^-/L) and there is no level set for marine water (US, 2006; CCME, 2007; HealthCanada, 2008; US, 2009). Health Canada has set the maximal allowable concentration in drinking water at 10 mg/L nitrate (NO_3-N) and 3.2 mg/L nitrite (NO_2-N) (US, 2006; CCME, 2007; HealthCanada, 2008; US, 2009). Nitrate concentrations have been found as high as 25 mg/L NO_3-N in surface waters

and 100 mg/L NO_3-N in ground water, yet there is currently no guideline for the protection of wildlife (Rouse *et al.*, 1999; Camargo *et al.*, 2004).

Environmental nitrate can come from many sources and these include green leafy vegetables (physiological effects of dietary nitrate in vegetables are mitigated by co-occurring vitamin C), preservatives in meat and fish, agriculture fertilizer, waste from animal production and burning fossil fuels, industrial effluent, and wastewater treatment plant discharges (Rouse *et al.*, 1999; Camargo *et al.*, 2004; De Groef *et al.*, 2006). In aquatic environments, nitrogen exists in four forms: ammonium ion, ammonia, nitrite, and nitrate, listed from most to least toxic. Although nitrate is the least toxic form of the four, it is the most stable and therefore the most abundant. Under aerobic conditions, ammonia and ammonium can be oxidized to nitrite by *Nitrosomonas* bacteria, and then to nitrate by *Nitrobacter and Nitrospira bacteria (Sharma and Ahlert, 1977)*. When oxygen is low, denitrifying bacteria can use nitrate as a terminal electron acceptor and make nitrogen gas (N_2) (reviewed in (Camargo *et al.*, 2005)).

Aquatic animals are exposed to nitrate and nitrite through ingestion or epithelial absorption across skin or gills (Onken *et al.*, 2003). High levels of these contaminants cause methemoglobinemia, also called “brown blood” disease in fish and amphibians and “blue baby” syndrome in humans. Methemoglobin is formed from nitrate/nitrite-induced oxidation of hemoglobin, which prevents normal oxygen binding and leads to hypoxia (Porter *et al.*, 1999). Toxicity of nitrite and nitrate depends on body size and developmental stage, increases with increasing concentration and exposure time and decreases with water salinity and environmental adaptation (Rouse *et al.*, 1999; Camargo *et al.*, 2004).

In addition to the toxic effects of nitrate and nitrite, exposure to these chemicals has been shown to adversely affect the thyroid hormone (TH) axis in multiple vertebrate species, including rodents and anurans (reviewed in (Edwards *et al.*, 2006)). Nitrate can competitively depress iodine uptake in rats with alterations in thyroid gland morphology and function (Wyngaarden *et al.*, 1952; Wyngaarden *et al.*, 1953). High doses of nitrate for five months caused goitre and depressed serum T_4 and T_3 in exposed male rats (Zaki *et al.*, 2004). Likewise, tadpoles exposed to nitrate exhibit altered metamorphic development, suggesting that TH-signalling is affected by nitrate (Xu and Oldham, 1997; Edwards *et al.*, 2006; Ortiz-Santaliestra and Sparling, 2007). These observations may be explained, in part, because nitrate and nitrite compete with iodine uptake, transport, and retention in the thyroid gland, and can impair thyroid hormone synthesis, especially at high doses (Crow *et al.*, 2001; Hampel and Zollner, 2004).

Given that amphibian metamorphosis from a tadpole into a frog is completely dependent on TH, tadpoles provide an excellent model for studying chemicals which may disrupt the TH-signalling pathway. There are three major changes that take place during metamorphosis and these include: i) the death and resorption of larval organs and tissues used only by the tadpole (eg. tail), ii) remodelling of the larval organs to adult form and function (eg. intestine), which involves the coordinated apoptosis of larval cells with the proliferation and differentiation of adult precursor cells, and iii) *de novo* development of new organs and tissues for adult use (eg. hindlimbs) (Shi, 2000). The 3 stages in metamorphosis are: premetamorphosis, prometemorphosis, and metamorphic climax (Shi, 2000). During, premetamorphosis, tadpoles are not

producing any TH but can be precociously induced to undergo metamorphosis through exposure to TH (Shi, 2000). Not only can whole tadpoles respond to TH and undergo metamorphosis, but also their organs can be cultured and respond to TH. Cultured tail fins exposed to TH, have been shown to undergo apoptosis (Ji *et al.*, 2007; Skirrow *et al.*, 2008).

Most studies of the effects of nitrate and nitrite on amphibian metamorphosis have used whole-animal assays. The ability of these chemicals to affect signalling at the cellular level in TH-responsive tissues has not been investigated, although this is an additional mechanism by which nitrate and nitrite could disrupt metamorphosis. This study uses the recently developed "C-fin" assay to expose *Rana catesbeiana* premetamorphic tadpole tail fin biopsies to nitrate and nitrite with or without T_3 to determine if nitrate and nitrite are affecting TH-signalling within a TH-responsive tissue. We assessed TH-signalling by quantifying steady state levels of TH-responsive gene transcripts, thyroid hormone receptor β (*TRβ*) and Rana larval type I keratin (*RLKI*), as well as cellular stress markers, heat shock protein (*HSP30*) and catalase (*CAT*). Alteration of the transcriptome is an essential component in TH-mediated tadpole metamorphosis (Shi, 2000) and part of the change in the tail transcriptome includes an increase in *TRβ* transcripts and a decrease in *RLKI* transcripts (Domanski and Helbing, 2007). There is considerable precedent linking *TRβ* transcript levels to progression through TH-dependent metamorphosis where perturbations from expected levels are indicative of altered postembryonic development (Crump *et al.*, 2002; Opitz *et al.*, 2006; Veldhoen *et al.*, 2006; Zhang *et al.*, 2006; Helbing *et al.*, 2007; Helbing *et al.*, 2007; Ji *et al.*, 2007; Skirrow *et al.*, 2008).

5.2 Results

Using the C-fin assay, 6 mm tail biopsies were exposed to nitrate and nitrite in the absence and presence of T_3. T_3 treatment alone increased the *TRβ* transcript levels by 10-fold ± 1.5 (n=23; data not shown) and decreased *RLKI* transcript levels by 3-fold ± 0.5 (n=27; data not shown), results consistent with those previously reported (Hinther *et al.*, 2010A; Hinther *et al.*, 2010B). After exposure to 0.5 and 5 mg/L NO_2-N (in the form of $NaNO_2$) for 48 h did not have any effect on the TH-responsive gene transcripts, *TRβ* and *RLKI*, in the absence (p=0.683 and 0.257, respectively; Kendall's W) or presence of T_3 (p=0.282 and 0.751, respectively, Kendall's W; Figure 5.1). Exposure to the 5 mg/L sodium control (in the form of sodium gluconate (Na-G)) also did not result in a significant effect (p=0.300 – 0.875, Wilcoxon; Figure 5.1).

Exposure to 5 and 50 mg/L NO_3-N (in the form of $NaNO_3$) did not result in a change in TH-responsive transcript levels in the absence (p=0.565 and 0.913, respectively, Kendall's W) or presence of T_3 (p=0.066 and 0.529, respectively, Kendall's W). However, a greater degree of variation in the *RLKI* transcript was observed in the presence of 10 nM T_3 at the higher nitrate concentration. The 50 mg/L sodium control (in the form of sodium gluconate (Na-G)) in this experiment had no effect as well (p=0.480-1.000, Wilcoxon; Figure 5.1).

Exposure to 0.5 and 5 mg/L NO_2-N did not affect *HSP30* and *CAT* transcript levels in the absence (p=0.444 and 0.185, respectively, Kendall's W) or presence of T_3 (p=0.570 and 0.779, respectively, Kendall's W) (Figure 5.2). T3 treatment alone increased the *HSP30* transcript levels by 3.5-fold ± 1.5 (n=31; data not shown) and did not alter the *CAT* transcript levels (n=32; data not shown), results consistent with those

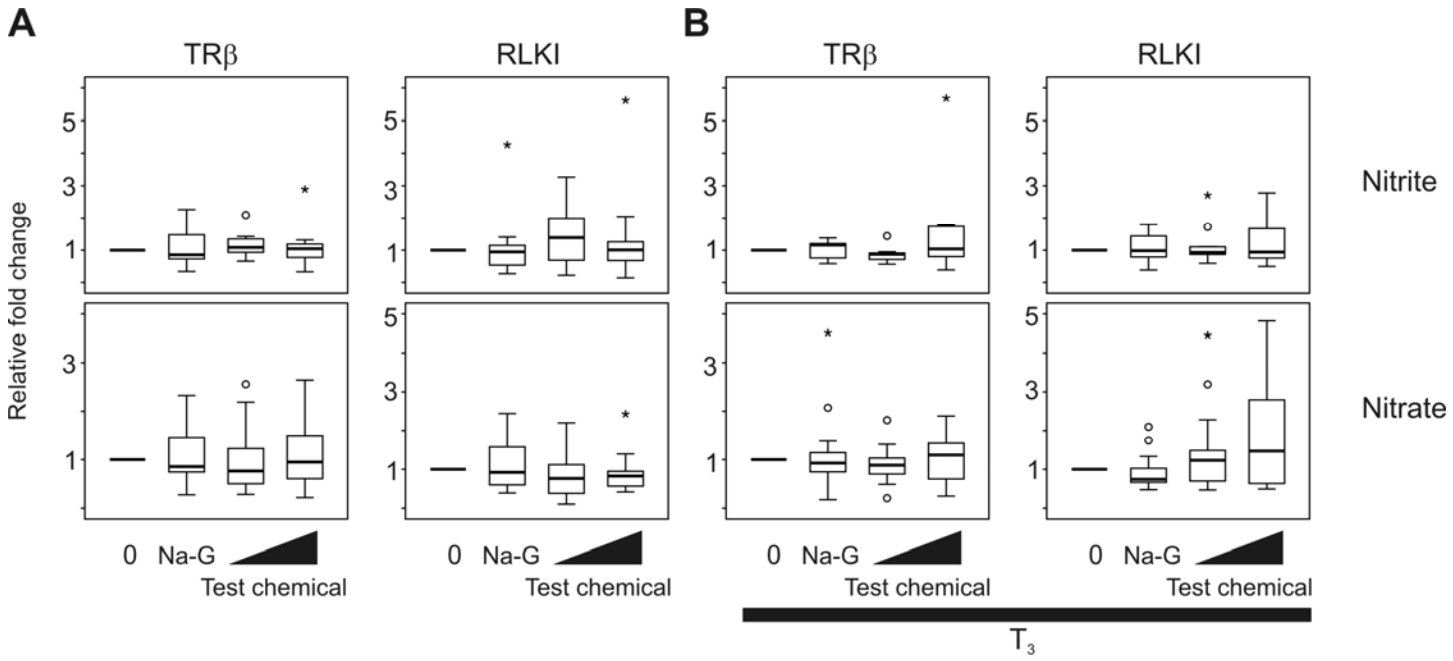


Figure 5.1. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to nitrite and nitrate in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=14-16) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: Nitrite: 5 mg/L sodium (in the form of sodium gluconate (Na-G)), 0.5 mg/L nitrite (in the form of $NaNO_2$), and 5 mg/L nitrite; nitrate: 50 mg/L sodium (in the form of sodium gluconate (Na-G)), 5 mg/L nitrate (in the form of $NaNO_3$), and 50 mg/L nitrate. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in Chapter 2 section. The medians are shown as solid black lines within the box, and the box indicates the first and third quartiles. The whiskers indicate minimum and maximum values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Increasing concentrations of test chemicals are represented by bevels. Significance is indicated by an 'a' ($p \leq 0.05$ relative to control; Wilcoxon).

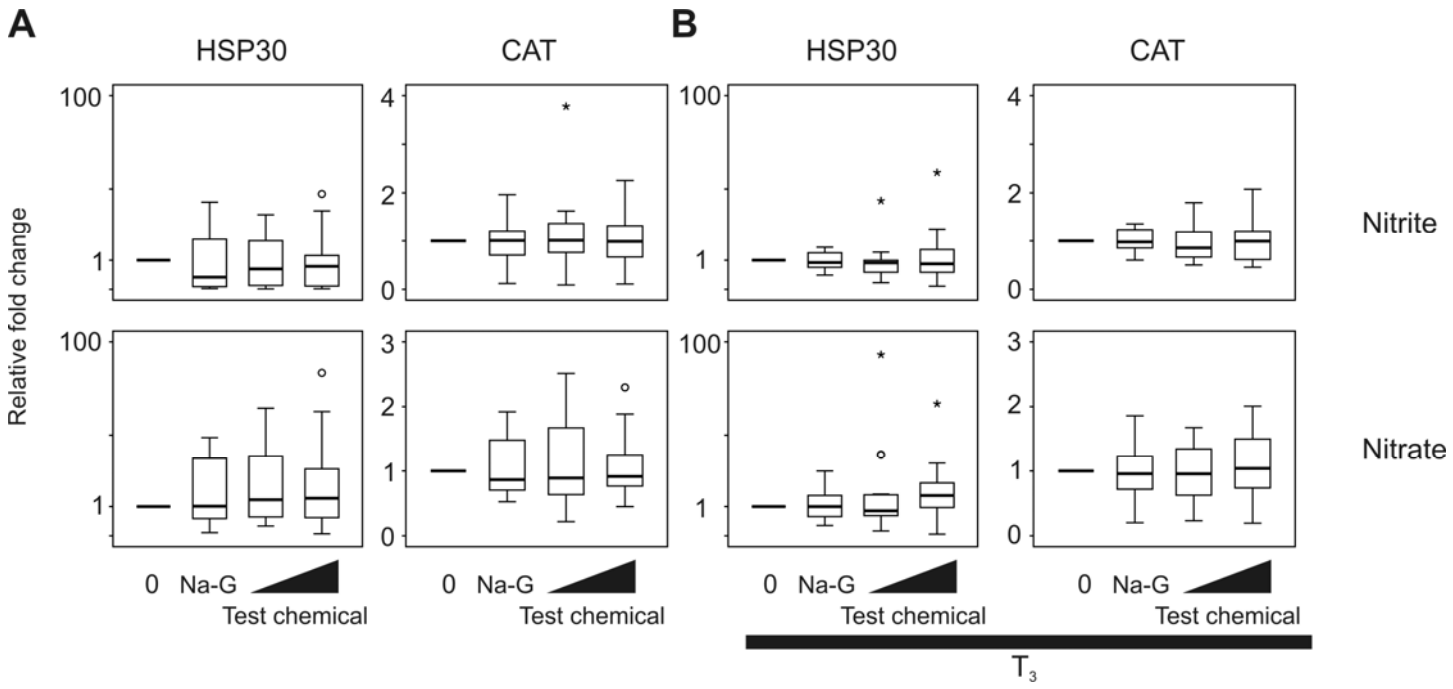


Figure 5.2. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nitrite and nitrate in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=15-16) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as in Figure 5.1. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Methods section. See Figure 5.1 legend for more detail.

previously reported (Hinther *et al.*, 2010A; Hinther *et al.*, 2010B). Exposure to 5 mg/L Na-G also did not result in a significant effect (p=0.438 – 0.717, Wilcoxon; Figure 5.2).

Exposure to 5 and 50 mg/L NO_3-N did not result in a change in stress-responsive transcript levels in the absence (p=0.282 and 0.819, *HSP30* and *CAT* transcripts respectively, Kendall's W) or presence of T_3 (p=0.074 and 0.819, respectively, Kendall's W; Figure 5.2). Exposure to 50 mg/L Na-G had no effect on the stress-responsive transcripts (p=0.796-1.000, Wilcoxon; Figure 5.2).

However, it should be noted that a high degree of variation was observed between individual animal *HSP30* transcript levels upon exposure to nitrite and this variation was even more apparent upon exposure to nitrate (note the $\log_{10}$ scale in Figure 5.2). This variation was not observed in the presence of T_3 (Figures 5.2).

5.3 Discussion

We exposed tail fin biopsies to nitrate and nitrite and measured the effects of these chemicals on TH-responsive and cellular stress-related gene transcript levels. The C-fin assay is useful in that it can give on an indication of the mechanism whereby a chemical may or may not be acting. Given that there were no marked effects with nitrate and nitrite as detected by the C-fin assay with respect to TH-responsive gene transcripts, this study indicates that nitrate and nitrite do not affect the TH axis through direct changes in *TRβ* and *RLKI* gene expression at the tissue level. It is interesting that nitrate and nitrite did not cause significant cellular stress due to their known toxic nature.

There have been multiple studies reported that suggest nitrate has an effect on the thyroid axis; however, the majority of these studies were whole animal exposures. Toad tadpoles (*Bufo bufo*) exposed to nitrate began and completed metamorphosis earlier and grew faster than controls (Xu and Oldham, 1997). *Rana cascadae* exposed to nitrate metamorphosed more slowly than controls but emerged from the water at the same time even though they were not developmentally ready (Marco and Blaustein, 1999). Edwards, McCoy et al. 2006 showed that *Bufo terrestris* tadpoles completed metamorphosis earlier in nitrate-contaminated laboratory water (0 – 30 mg/L NO_3-N in reverse-osmosis purified water with salts added back for osmotic balance), but were delayed when exposed to 30 mg/L NO_3-N in spring water. Altered iodine uptake following nitrate exposure was shown in fish and in bulls (Lahti *et al.*, 1985; Zraly *et al.*, 1997). Additionally, adult bulls orally exposed to nitrate had depressed thyroid activity and sheep exposed to nitrate developed goiters (Zraly *et al.*, 1997; Kursa *et al.*, 2000). Ortiz-Santaliestra and Sparling (2007) showed that nitrate at 100 mg/L (NO_3^-/L) increased larval mortality and reduced the growth of developing southern leopard frog tadpoles. *Nyctibatrachus major* and *Fejervarya limnocharis* tadpoles exposed to increasing concentrations of nitrate (0.1 - 5 mg/L NO_3-N) exhibited increasing rates of abnormalities, including a swollen body and head, paralysis, vertical swimming, and intestinal haemorrhaging (Krishnamurthy *et al.*, 2008). Given these studies it is clear that nitrate has detrimental effects through alterations in osmotic balance and/or TH signalling. In whole animals, nitrate can impair the TH-axis by competing with the transport of iodine into cells of the thyroid gland at the sodium-iodide symporter (NIS);

DeGroef *et al*. (2006) showed that the two ions generated similar steady state inward currents.

The C-fin assay results demonstrate that the negative impact of nitrate and nitrite on the thyroid axis is not mediated through changes in transcription of *TRβ* or *RLKI* in TH-responsive target tissue. *TRβ* and *RLKI* transcript levels have been shown to increase and decrease, respectively, in the tail fin during metamorphosis. TRβ is a known direct TH response gene and because nitrate and nitrite are not perturbing *TRβ* transcript levels, the present study suggests that nitrate and nitrite may not directly target TRs. It is, however, possible that nitrate acts directly on other tissues such as the thyroid gland. The recent description of a tadpole thyroid gland organ culture system (Opitz *et al.*, 2006) may assist in determining if this indeed is the case.

Additionally, nitrite and nitrate could act as nitric oxide donors (Guillette and Edwards, 2005; Hannas *et al.*, 2010). Nitric oxide donors have been shown to mimic the ability of T_4 to suppress catalase activity associated with tail shortening and apoptosis *in vitro* (Kashiwagi *et al.*, 1999). This suggests a non-genomic mechanism by which nitrate and nitrite could speed up metamorphosis by acting on T_4-responsive tissues in tadpole tails.

Chapter 6: Evaluation of triclocarban, triclosan, and its metabolite, methyl-triclosan for thyroid hormone disruptive effects in frog and mammalian systems

6.1 Introduction

Triclosan (TCS) and triclocarban (TCC) are broad-spectrum, synthetic, lipophilic, antimicrobial agents used in a variety of personal care products (PCPs). TCS was originally introduced in 1972 as an active ingredient in a surgical scrub for professional health care and is now used in antibacterial mouthwash and toothpaste, detergents, shampoos, deodorants, plastic cutting boards, sports equipment, textiles and furniture; TCC is mostly added to antibacterial soaps and body washes (Tierno, 1999; Bester, 2003; Sabaliunas *et al.*, 2003). TCS works through the inhibition of the enzyme enoyl-acyl carrier-protein reductase (ENR), which catalyzes an essential lipid biosynthesis step in membranes of Gram-positive and Gram-negative bacteria, as well as in many types of fungi (Heath *et al.*, 2000). TCC acts predominantly against Gram-positive bacteria (McDonnell and Pretzer, 1998; McMurry *et al.*, 1998; Heath *et al.*, 2000).

TCC typically accounts for 2% by weight of antimicrobial soaps, whereas TCS accounts for a lower amount in the range of 0.1-0.3% by weight although some formulations used in hospitals can be as high as 5% (Sabaliunas *et al.*, 2003). Approximately 45% of liquid and bar soaps contain these antimicrobials and it is estimated that approximately 1 million pounds of TCC are produced for the US market per year (Perencevich *et al.*, 2001; Sapkota *et al.*, 2007). Because of the large amount

of use of these antimicrobials, there is widespread environmental contamination. TCS and TCC have been detected in wastewater effluent in the US, UK, and Japan. They have been detected at microgram per litre levels in both the US and Switzerland (Koplin *et al.*, 2002; Lindstrom *et al.*, 2002; Halden and Paull, 2005). Not only are humans exposed to these antimicrobials through environmental contamination, but TCS and TCC are also readily absorbed through the gastrointestinal tract and oral mucosa. It has been reported that 0.39% (0.54mg) of an average 138 mg of TCC applied to the entire body was absorbed after a typical whole-body shower lather (Scharpf *et al.*, 1975). TCC is absorbed through the forearm with the abdomen, scalp, and scrotum allowing almost total absorption (Maibach *et al.*, 1971; Scharpf *et al.*, 1975; Sandborgh-Englund *et al.*, 2006). TCS has been found at levels as high as 2,000 μg/kg lipid in human breast milk and has also been found in urine samples from young girls (Adolfsson-Erici *et al.*, 2002; Dayan, 2007; Wolff *et al.*, 2007).

Not only can TCS bioaccumulate in the tissues of wildlife species, but its biological metabolite, methyl-TCS (mTCS), has also been detected in fish exposed to wastewater (Balmer *et al.*, 2004). Methyl-TCS is an environmental methylation product of TCS; however, the environmental fate of TCS and mTCS are quite different, with TCS being easily degraded by photolysis and mTCS being much more persistent (Lindstrom *et al.*, 2002).

TCS is structurally similar to thyroid hormone (TH) (Figure1.1 and Figure 1.6C) and there have been several studies looking at the effects TCS has on thyroid-mediated processes. Exposure of *Rana catesbeiana* tadpoles to TCS resulted in accelerated TH-dependent metamorphosis as well as decreased expression of TH receptor β (*TRβ*)

transcript (Veldhoen *et al.*, 2006). *Xenopus laevis* XTC-2 cells coexposed to TCS and TH showed an increase in the mRNA levels of *TR*β and TH receptor α (TRα) (Veldhoen *et al.*, 2006). Moreover, *Xenopus laevis* premetamorphic tadpoles exposed to TCS through prometamorphosis exhibited an increase in *TR*β transcript levels in stage-matched tadpoles at metamorphic climax (Veldhoen *et al.*, 2006; Fort *et al.*). This same study also found tadpole development was significantly accelerated upon exposure to TCS although thyroid morphology and thyroxine levels were unchanged (see (Helbing *et al.*, 2010) for further information).

In mammals, TCS decreased the levels of thyroxine (T_4), triiodothyronine (T_3), but did not affect the levels of thyroid stimulating hormone (TSH) when fed to rats (Crofton *et al.*, 2007; Zorrilla *et al.*, 2009; Paul *et al.*, 2010). Evidence suggests TCS may upregulate hepatic catabolism of THs, which may lead to the decreased levels of circulating T_4 and T_3 (Crofton and Zoeller, 2005; Crofton, 2008).

In addition, recent studies also found evidence TCC and TCS disrupt steroid hormone action. TCC was shown to enhance testosterone -induced androgen mediated transcription in the presence of testosterone. It also increased the size of male sex accessory organs in castrated male rats when they were fed both testosterone and TCC (Chen *et al.*, 2008). TCC also potentiated the estrogen receptor-α-mediated signal transcriptional activity induced by estradiol (Chen *et al.*, 2008). TCS was shown to be a weak androgen when tested in *Oryzias laptipes* (Foran *et al.*, 2000). Since TCC altered the transcriptional activity of the androgen and ERα receptor, there is potential that other nuclear receptor signal transduction systems could also be affected (Chen *et al.*, 2008). The TH receptors, TRβ and TRα, are nuclear receptors, and therefore, may

be affected by TCC. The mechanism of TCS is still unclear and further studies are still needed looking into the effects TCS has regarding the actions of TH. Since mTCS is an abundant biological metabolite of TCS, it is also important to study the effects mTCS has on the TH system.

Our laboratory recently developed a screening assay, which allows high throughput screening of potential endocrine disrupting chemicals. The *in vitro* cultured tail fin biopsy ("C-fin") assay uses cultured premetamorphic *Rana catesbeiana* tadpole tail fin biopsies that are responsive to TH exposure (Hinther *et al.*, 2010A). Using the C-fin assay, we studied the effects TCS, mTCS, and TCC have on TH-responsive gene transcripts in both the absence and presence of TH. This study also used rat pituitary (GH3) cells to study the effects these chemicals may have on TH-responsive gene transcripts in the absence and presence of TH.

In the present study, we used quantitative real time polymerase chain reaction (QPCR) to measure the effects of TCS, mTCS, and TCC on frog tail fin biopsies and mammalian cells. Disruption of TH action was assessed by measurement of the mRNA abundance of TH-induced TH receptor β (*TRβ*) and TH-repressed *Rana* larval keratin type I (*RLKI*) (Domanski and Helbing, 2007). We also determined the effects of these chemicals on cellular stress by measuring the transcript levels of the heat shock protein 30 (*HSP30*) and catalase (*CAT*). Using the GH3 cells, we measured disruption of TH-action through the measurement of mRNA levels of TH-induced growth hormone (*GH*), deiodinase I (*DIOI*), and prolactin (*PRL*). We determined the cellular stress effects of these chemicals by measuring the transcript levels of the heat shock protein 70 (*HSP70*). Alterations in the steady-state levels of these transcripts corresponded to the

specific type of chemical and are suggestive of the potential for perturbing hormone-dependent postembryonic development.

6.2 Results

6.2.1 Mammalian cell exposures

GH3 cells were exposed to each TCS, mTCS, and TCC in both the presence and absence of 10 nM T_3 to examine the effects that these chemicals have on the levels of *GH*, *DIOI*, *PRL*, and *HSP7*0 transcripts after 48 h of exposure. In the first experimental set, GH3 cells were exposed to 1-1,000 nM TCS (Figure 6.1). Overall, significant differences were observed for *GH, DIOI* and *HSP70* transcripts (p=0.0001 for all, Kruskal-Wallis). The ~3- and 6-fold increase in *GH* and *DIOI* mRNAs relative to the vehicle control, respectively, upon exposure to T_3 (leftmost bar, Figure 6.1; p=0.0001 for both; Mann-Whitney U) was the sole contributor to the observed significance. Therefore, TCS did not alter the hormone-dependent induction of either transcript nor did it have an effect on basal levels in the absence of T_3 (Figure 6.1).

PRL transcripts have previously been shown to be increased or decreased by TH treatment depending upon the cell type (Forman *et al.*, 1988). Overall, *PRL* transcripts did not show a significant difference (p=0.105, Kruskal-Wallis) (Figure 6.1). *PRL* transcript was a borderline responder to T_3 treatment, if responding at all. In most experiments, no significant alterations in *PRL* transcript levels were observed (compare Figures 4.3 and 4.4 and Figures 6.1-6.3). Where a significant increase was observed, it was very modest at 1.4-fold (p=0.011, Mann-Whitney U) (Figure 6.1) relative to vehicle

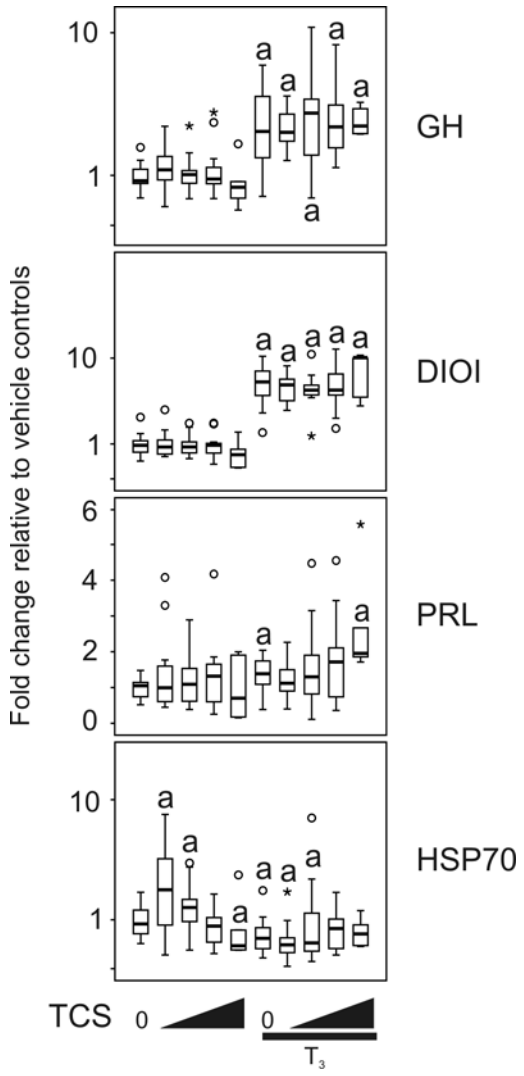


Figure 6.1. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to TCS. These cells (n=5-17) were exposed to vehicle control (0) or 1, 10, 100, and 1000 nM TCS in the absence and presence of 10 nM T_3 for 48 h. The results are expressed as fold change relative to the vehicle control. The medians are shown as solid black lines within the box, and the box indicates the first and third quartiles. The whiskers indicate minimum and maximum values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Increasing concentrations of test chemicals are represented by bevels. Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, Mann-Whitney U) and relative to T_3 treatment by 'b' ($p \leq 0.05$, Mann-Whitney U).

control. In the presence of T_3 treatment, 1,000 nM TCS significantly increased *PRL* transcript levels relative to T_3 treatment alone by 2-fold (p=0.006, Mann Whitney U) (Figure 6.1).

In contrast, exposure to TCS resulted in a significant alteration in *HSP70* transcript levels (p=0.0001, Kruskal-Wallis; Figure 6.1). This was due to multiple factors. First, TCS treatment alone increased *HSP70* transcript levels (p=0.007, Kruskal Wallis for control and TCS only concentrations) at 1 and 10 nM relative to the vehicle control by 2.6-fold (p=0.044, Mann-Whitney U) and 1.5-fold (p=0.034, Mann-Whitney U), respectively. Exposure to 1,000 nM TCS resulted in the slight reduction of *HSP70* transcript levels relative to the vehicle control by 1.2-fold (p=0.050, Mann-Whitney U) (Figure 6.1).

T_3 treatment alone reduced the *HSP70* transcript levels relative to vehicle control by 9.1-fold (p=0.009, Mann-Whitney U), but this response to hormone was largely unchanged by administration of TCS (p=0.393, Kruskal-Wallis for T_3 and T_3 plus TCS concentrations) (Figure 6.1).

In the second experimental set, GH3 cells were exposed to 1-1,000 nM mTCS (Figure 6.2). All four gene transcripts showed significant changes among all treatments (*GH* p=0.0001; *DIOI* p=0.0001; *PRL* p=0.013; *HSP70* p=0.001; Kruskal-Wallis) (Figure 6.2). Closer examination of the data revealed that 1,000 nM mTCS treatment significantly reduced *GH* transcript levels by 2.1-fold (p=0.025, Mann-Whitney U) (Figure 6.2) while no effect of mTCS was observed on *DIOI* transcripts (Figure 6.2). In contrast, mTCS affected *PRL* and *HSP70* mRNA abundance (p=0.02 and 0.004,

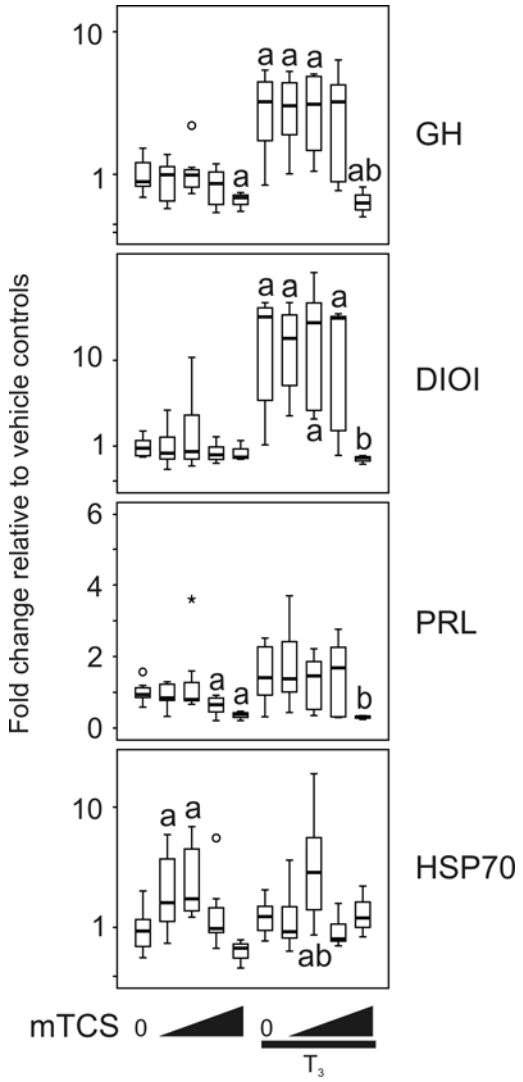


Figure 6.2. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to mTCS. These cells (n=3-8) were exposed to vehicle control (0) or 1, 10, 100, and 1000 nM mTCS in the absence and presence of 10 nM T_3 for 48 h. The results are expressed as fold change relative to the vehicle control. Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, Mann-Whitney U) and relative to T_3 treatment by 'b' ($p \leq 0.05$, Mann-Whitney U). Further graph details are presented in the Figure 6.1 legend.

respectively; Kruskal Wallis for control and TCS only concentrations). Specifically, 100 and 1,000 nM mTCS reduced *PRL* transcript levels significantly relative to vehicle control by 1.6-fold (p=0.016, Mann-Whitney U) and 2.8-fold (p=0.014, Mann-Whitney U) (Figure 6.2) and *HSP70* transcript levels were significantly increased by 1 and 10 nM mTCS by 2.8-fold (p=0.046, Mann-Whitney U) and 3.3-fold (p=0.005, Mann-Whitney U), respectively (Figure 6.2).

The response to T_3 alone was similar to experimental set 1 for *GH* and *DIOI* of transcripts with 3.6-fold (p=0.006, Mann-Whitney U) and 19-fold (p=0.004, Mann-Whitney U) increases, respectively (Figure 6.2). Analysis of the T_3 exposed set of cells showed both transcripts approaching significance (p=0.093 and 0.061 respectively, Kruskal-Wallis). The major contributing factor was a marked reduction in the T_3-induced response of both transcripts (7.9-fold, p=0.014; 35-fold, p=0.014, respectively; Mann-Whitney U) when the cells were coexposed to 1,000 nM mTCS (Figure 6.2).

The reduction in *PRL* transcript levels observed with mTCS exposure alone was also observed at the highest concentration tested in the presence of T_3 where 1,000 nM mTCS significantly reduced the *PRL* transcript levels relative to T_3 treatment alone by 18-fold (p=0.025, Mann-Whitney U) (Figure 6.2).

T_3 treatment alone did not significantly alter the *HSP70* transcript levels (Figure 6.2). However, there was a significant effect of TCS detected in the context of T_3 treatment (p=0.015, Kruskal Wallis) where exposure to 10 nM mTCS increased *HSP70* transcript levels by 3.6-fold relative to T_3 treatment alone (p=0.021, Mann-Whitney U) (Figure 6.2).

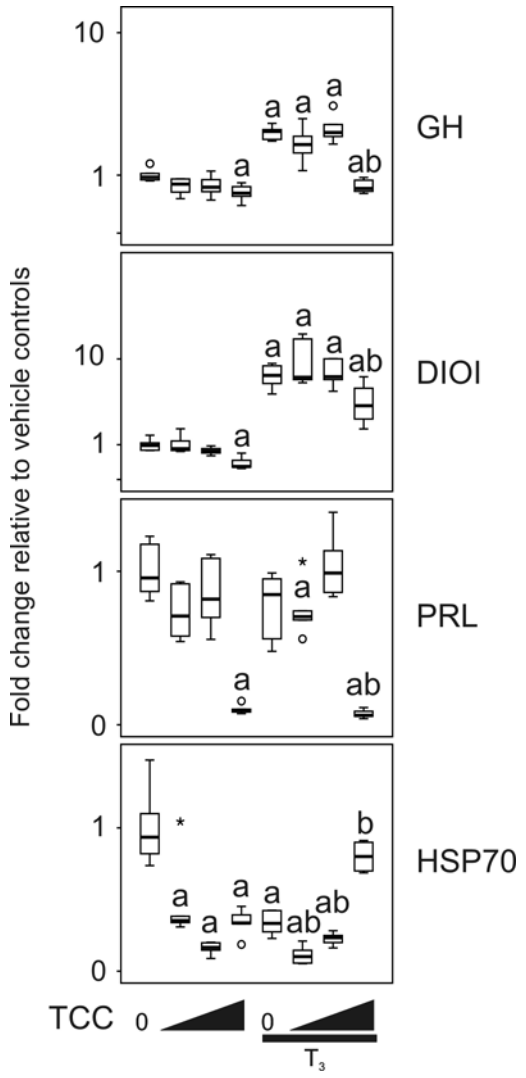


Figure 6.3. QPCR analysis of growth hormone (*GH*), deiodinase I (*DIOI*), prolactin (*PRL*), and heat shock protein 70 (*HSP70*) transcript levels in GH3 cells exposed to TCC. These cells (n=6) were exposed to vehicle control (0) or 10, 100, and 1000 nM TCC in the absence and presence of 10 nM T_3 for 48 h. The results are expressed as fold change relative to the vehicle control. Statistical significance of the mean relative fold change values compared to the vehicle control is denoted by 'a' ($p \leq 0.05$, Mann-Whitney U) and relative to T_3 treatment by 'b' ($p \leq 0.05$, Mann-Whitney U). Further graph details are presented in the Figure 6.1 legend.

In the third experimental set, GH3 cells were exposed to 10-1,000 nM TCC (Figure 6.3). TCC exposure resulted in significant changes in all four transcript abundances (p=0.0001 for all, Kruskal-Wallis) (Figure 6.3). TCC significantly reduced *GH* transcript levels relative to the vehicle control (p=0.021, Kruskal-Wallis) with 1,000 nM TCC resulting in a significant decrease relative to vehicle control by 1.6-fold (p=0.004, Mann-Whitney U) (Figure 6.3). TCC significantly reduced *DIOI* transcript levels relative to the vehicle control (p=0.002, Kruskal-Wallis) with 1,000 nM TCC resulting in a significant decrease relative to vehicle control by 2.6-fold (p=0.004, Mann-Whitney U) (Figure 6.3). Moreover, TCC significantly reduced *PRL* transcript levels relative to the vehicle control (p=0.001, Kruskal-Wallis) with 1,000 nM TCC resulting in a significant decrease relative to vehicle control by 2.2-fold (p=0.004, Mann-Whitney U) (Figure 6.3). Finally, the most marked response to TCC exposure was evident with the *HSP70* transcripts (p=0.0001, Kruskal-Wallis) where TCC reduced *HSP70* transcript levels relative to vehicle control at 10, 100, and 1,000 nM TCC by 2.2-fold (p=0.025, Mann-Whitney U), 6.3-fold (p=0.004, Mann-Whitney U), and 2.9-fold (p=0.004, Mann-Whitney U), respectively (Figure 6.3).

Again, the response to T_3 was similar to Figures 6.1 and 6.2 for the *GH* and *DIOI* transcripts (2.3-fold, p=0.004; 7-fold, p=0.006, Mann-Whitney U) (Figure 6.3). *PRL* transcript abundance was unchanged while T_3 treatment alone reduced the *HSP70* transcript levels relative to vehicle control by 2.9-fold (p=0.004, Mann-Whitney U) (Figure 6.3). Thus the response to T_3 varies between none to a decrease in mRNA

abundance in comparing the different experimental sets. Additional experiments (data not shown) also confirmed this observation. The reason for this is not clear.

Coadministration of T_3 and TCC resulted in significant changes in all four transcripts relative to T_3 alone (*GH* p=0.002; *DIOI* p=0.037; *PRL* p=0.001; *HSP70* p=0.0001; Kruskal-Wallis; Figure 6.3). *GH, DIOI* and *PRL* mRNAs showed lesser induction levels in the presence of 1,000 nM TCC (3.1-fold, p=0.004; 1.8-fold, p=0.028; 11-fold, p=0.004, respectively; Mann-Whitney U) (Figure 6.3).

TCC altered the *HSP70* transcript levels at all concentrations tested when coadministered with T_3. At 10 and 100 nM TCC, *HSP70* transcript levels were reduced relative to T_3 treatment alone by 3.9-fold (p=0.004, Mann-Whitney U) and 1.5-fold (p=0.037, Mann-Whitney U), respectively. At 1,000 nM TCC, *HSP70* levels were increased by 2.5-fold relative to T_3 treatment alone (p=0.004, Mann-Whitney U) (Figure 6.3).

6.2.2 Amphibian organ culture exposures

In the C-fin assay, four of the eight tail biopsies from each individual tadpole were exposed to solvent control and three concentrations of TCS (1, 10, and 100 nM), mTCS (1, 10, and 100 nM), or TCC (10, 100, and 1,000 nM). *TRβ* and *RLKI* transcript levels were not significantly affected by TCS treatment alone (Figure 6.4A). The *TRβ* transcript was significantly increased at 10 nM mTCS by 1.4-fold (p=0.028, Wilcoxon) relative to vehicle control with a tendency toward increased levels over the concentration range tested (p=0.178, Kendall's W) while *RLKI* transcripts were

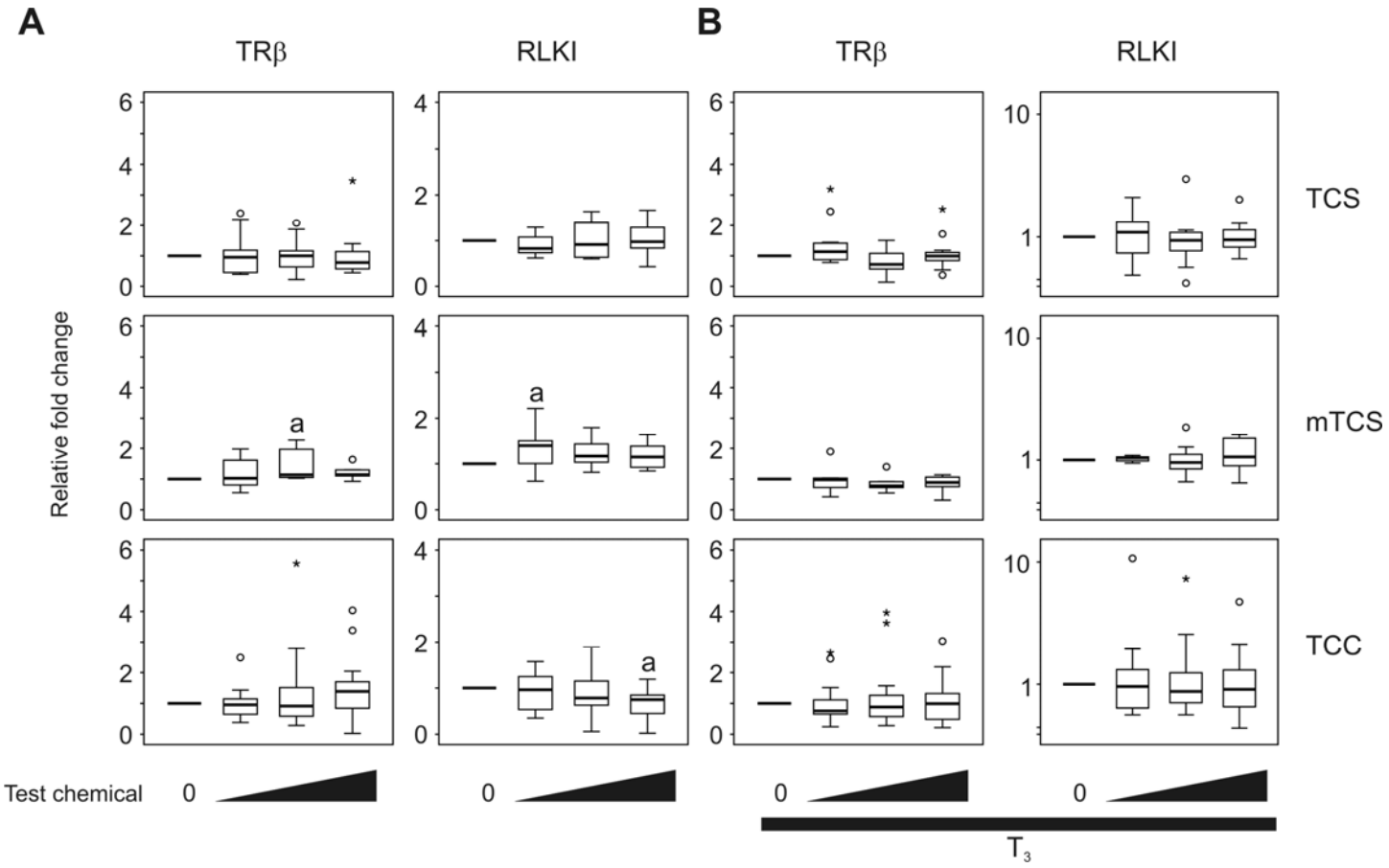


Figure 6.4. QPCR analysis of thyroid hormone receptor β (*TRβ*) and *Rana* larval keratin type I (*RLKI*) transcript levels in the C-fin assay after exposure to triclosan, methyl-triclosan, and triclocarban in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=8-24) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: 1, 10, and 100 nM triclosan or methyl-triclosan, and 10, 100, and 1,000 nM triclocarban. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Methods section. Significance is indicated by an 'a' ($p \leq 0.05$ relative to control; Wilcoxon). See Figure 6.1 legend for more graph details.

marginally increased upon exposure to 1 nM mTCS by 1.3-fold (p=0.050, Wilcoxon) relative to vehicle control (Figure 6.4A). TCC exposure did not affect *TRβ* transcript levels whereas *RLKI* transcript levels were significantly decreased (p=0.021, Kendall's W) with 1,000 nM TCC showing a 1.5-fold reduction (p=0.001, Wilcoxon) relative to vehicle control (Figure 6.4A).

The remaining four biopsies from each animal were exposed to 10 nM T_3 or 10 nM T_3 plus three concentrations of TCS (1, 10, and 100 nM), mTCS (1, 10, and 100 nM), or TCC (10, 100, and 1,000 nM). This level of hormone alone increased *TRβ* transcript levels by 15-fold ± 3-fold (n=41; data not shown) (mean=9.6±0.8; median=7.8; n=50) and reduced *RLKI* transcript levels by 3-fold ± 0.03-fold (n=38; data not shown); results consistent with those reported previously (Hinther *et al.*, 2010A; Hinther *et al.*, 2010B). Since we wished to determine whether TCS, mTCS, and TCC exposure altered TH-dependent responses of biopsies from each individual animal, we expressed the T_3/test chemical exposure data relative to the transcript levels obtained with T_3 alone (Figure 6.4B). In the presence of T_3, TCS, mTCS, and TCC did not significantly affect the steady-state levels of *TRβ* and *RLKI* transcript levels (Figure 6.4B).

HSP30 and *CAT* transcript levels were measured as indicators of cellular stress. Exposure to 1 and 10 nM TCS significantly increased the *HSP30* transcript levels relative to vehicle control by 4.5-fold (p=0.010, Wilcoxon) and 3.7-fold (p=0.050, Wilcoxon), respectively, with an overall tendency towards significance over the concentration range (p=0.074, Kendall's W) (Figure 6.5A). *CAT* transcript levels were increased upon exposure to TCS (p=0.050, Kendall's W) with the 10 nM TCS increasing *CAT* levels by 2.4-fold (p=0.026, Wilcoxon) (Figure 6.5A). *HSP30* transcript levels were

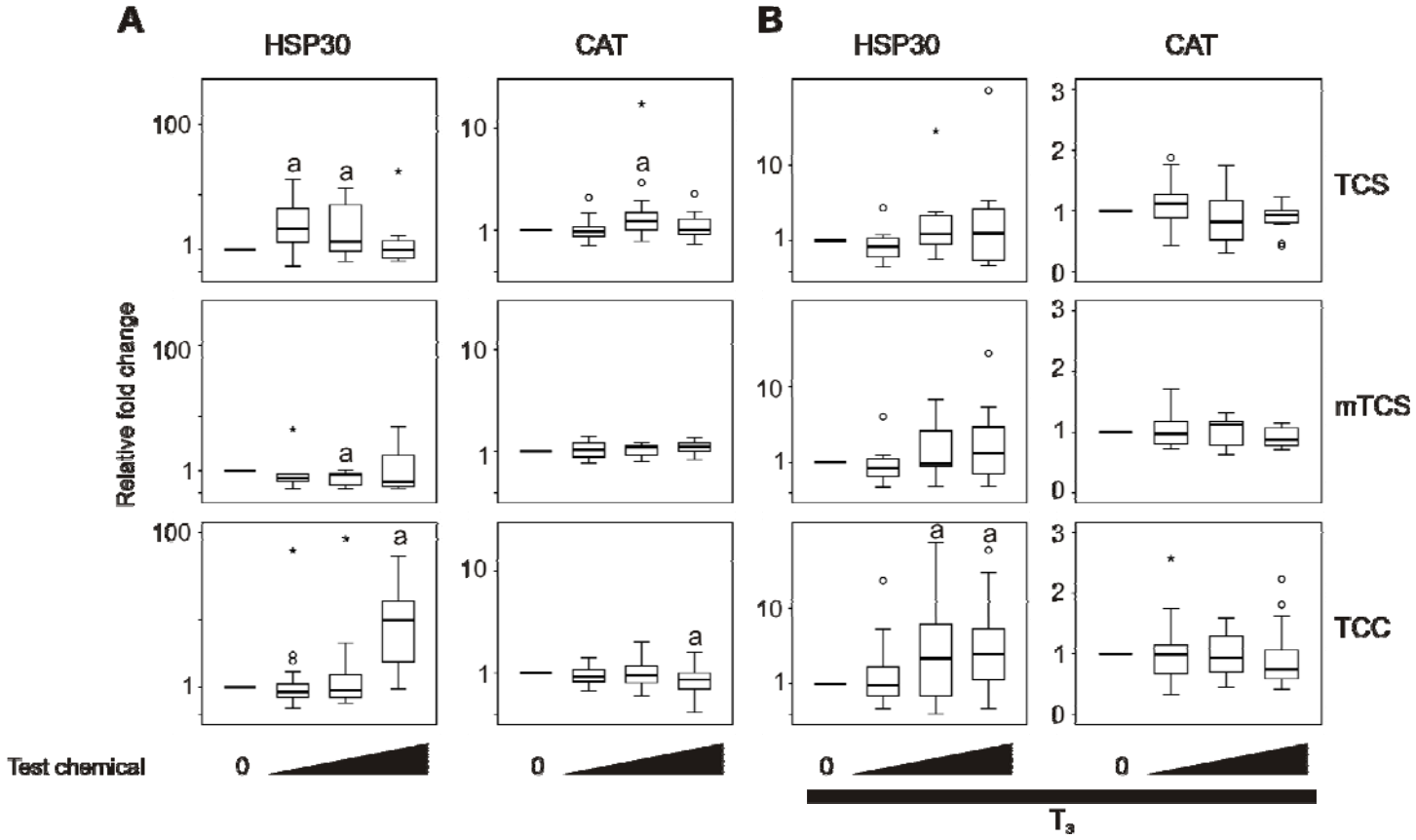


Figure 6.5. QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to triclosan, methyl-triclosan, and triclocarban in the absence (A) or presence (B) of 10 nM T_3. Tail fin biopsies (n=8-24) were exposed to vehicle control (0) and the indicated test chemicals for 48 h. Test chemical concentrations were as follows: 1, 10, and 100 nM triclosan or methyl-triclosan, and 10, 100, and 1,000 nM triclocarban. The results are expressed as fold change relative to the vehicle control (A) or to the T_3-induced levels (B) as described in the Methods section. Significance is indicated by an 'a' ($p \leq 0.05$ relative to control; Wilcoxon). See Figure 6.1 legend for more graph details.

significantly decreased with 10 nM mTCS treatment by 1.6-fold (p=0.028; Wilcoxon)

relative to vehicle control while *CAT* transcript levels were not significantly affected by

mTCS (Figure 6.5A). TCC significantly increased the *HSP30* transcript levels (p=0.0001, Kendell's W; p=0.0001 for 1,000 nM, Wilcoxon) whereas *CAT* transcript levels were increased at 1,000 nM TCC by 1.29-fold (p=0.042, Wilcoxon) (Figure 6.5A). *HSP30* transcript levels have been shown to increase when exposed to TH (Helbing *et al.*, 1996; Hinther *et al.*, 2010A). Treatment of biopsies with T_3 resulted in a 10 ± 4-fold (n=41; data not shown) induction of *HSP30* transcript levels relative to the vehicle control, consistent with previously reported data (Helbing *et al.*, 1996; Hinther *et al.*, 2010A). TH induces oxidative stress and previous results have shown *CAT* transcript levels to decrease in response to TH treatment (Kashiwagi *et al.*, 1999; Hinther *et al.*, 2010B). T_3 treatment alone resulted in a 1.4 ± 0.3-fold (n=45; data not shown) decrease in *CAT* transcript levels relative to the vehicle control, which is consistent with previous reported observations (Kashiwagi *et al.*, 1999; Hinther *et al.*, 2010B). TCS and mTCS did not have an effect on the T_3-induced levels of *HSP30* and *CAT* transcripts (Figure 6.5B). In contrast, *HSP30* transcripts levels were significantly increased upon exposure to TCC (p=0.004, Kendall's W) with the increase occurring at 100 and 1,000 nM TCC (7.5-fold, p=0.028; 6.2-fold, p=0.001, respectively; Wilcoxon) relative to T_3 treatment alone (Figure 6.5B). T_3-induced levels of *CAT* transcripts were not significantly changed by TCC treatment (Figure 6.5B).

6.3 Discussion

TCS is an abundant bactericide and has been shown to be present in urine and breast milk (Adolfsson-Erici *et al.*, 2002; Dayan, 2007; Wolff *et al.*, 2007). TCS has been detected in wastewater treatment plant effluents in North America, Europe and

Australia at concentrations ranging from 0.12 to 9.3 nM, with the highest mean concentration of 4.0 nM in the US (Ying *et al.*, 2007). Given the structural similarity of TCS to TH, there is concern for its effect on TH-mediated pathways. Recent studies have shown TCS to have an effect on TH-mediated anuran metamorphosis and TH-associated gene expression at environmentally-relevant concentrations. TCS has also been shown to decrease circulating T_4 levels, in a dose dependent manner, when fed to rats. Veldhoen *et al*. (2006) showed TCS to accelerate T_3-mediated metamorphosis, based on hindlimb morphology, when *Rana catesbeiana* tadpoles were exposed to TCS. Veldhoen *et al*. (2006) also observed a decrease in the *TR*β gene transcript levels in the tail fins of exposed tadpoles; XTC-2 cells were also exposed to TCS in the absence and presence of TH and TCS was shown to alter the gene transcript levels. The results of the XTC-2 cells suggest the whole animal does not have to be present to elicit the effects of TCS. This current study, however, did not show effects with respect to TH-responsive gene transcripts when the tail biopsies or cultured GH3 cells were exposed to TCS. The differences in results may be due to the nature of the cells and tissue types used in the respective studies.

It is interesting to note, although TCS did not alter the TH-responsive gene transcript levels, in either models tested, it did elicit a cellular stress response in the biopsies as indicated by the altered *CAT*, *HSP30*, and *HSP70* transcript levels. This suggests a possible mechanism for TCS accelerating metamorphosis in intact *Rana catesbeiana* tadpoles through a stress response.

It is known stress can accelerate metamorphosis. Environmental stress can lead to an increase in corticotropin releasing hormone (CRH), which then can further

stimulate the thyroid system (Denver, 1997). *Scaphiopus hammondii* tadpoles were exposed to water reduction, representing a desiccating environment, and showed an increase in T_3 and T_4, as well as corticosterone followed by external metamorphic change. This response was rapid and occurred within 48 hours after exposure to the reduction in water volume (Denver, 1998).

Heat shock proteins have been shown to protect thermally or chemically stressed cells (Schlesinger, 1990). Metamorphosis is a drastic process which involves a fine balance between cell proliferation, differentiation, and degradation. Heat shock proteins have been shown to increase in the liver of *R. catesbeiana* tadpoles exposed to T_3, which suggests there is crosstalk between the regulation of heat shock proteins and the metamorphic program (Helbing *et al.*, 1996). Previous studies have also shown catalase activity and transcript levels to decrease in *Rana japonica* tadpole tails when exposed to T_3. The decrease in catalase activity results in an increase in hydrogen peroxide, which causes oxidative stress. The oxidative stress might activate the cysteine-dependent aspartate-directed protease-3, leading to apoptosis (Kashiwagi *et al.*, 1999). Whether the induction of a stress response by TCS is the mechanism contributing to accelerated metamorphosis remains to be determined, however, another possibility for explaining the lack of TH-response effects of TCS in the models tested here compared to previous studies could be the bacterial methylation product, mTCS, may have conferred TH-disrupting properties rather than the parent compound.

The present study showed that mTCS increased the levels of both TH-responsive gene transcripts, *TR*β and *RLKI*, in the tail biopsies and disrupted *GH*, *DIOI*, and *PRL* transcript levels in the GH3 cells. Thus, mTCS was capable of acting at a

cellular level in an intact tissue and cell line. It would be worthwhile to study the whole animal effects of mTCS on frog tadpoles particularly since mTCS is more persistent in the environment (Lindstrom *et al.*, 2002).

We also found TCC disrupted the transcript levels of TH-responsive genes in the GH3 cells and in the tail fin biopsies. A modest effect was observed for *RLKI* transcript (Figure 6.5) but no effect on *TR*β transcript was observed in the frog tissue. The mammalian cells showed an effect in all three TH-responsive transcripts (*GH, DIOI*, and *PRL*) but only at the highest concentration (1,000 nM). Overall, our results are consistent with the speculation that TCC may act on nuclear hormone receptors given the previous effects TCC had on androgen, estrogen, and cortisone mediated pathways (Chen *et al.*, 2007; Chen *et al.*, 2008).

CAT, *HSP30*, and *HSP70* transcript levels were all affected by TCC exposure at concentrations as low as 1 nM (Figures 6.3 and 6.5). These results suggest that TCC is acting at a cellular level on the tail fin tissue and may be triggering a stress response. The clear reduction of *HSP70* transcripts in the GH3 cells in several of the treatment conditions suggests the cellular capacity to respond to stress events may also have been affected. Another possibility for the reduction of the *HSP70* transcript levels following TCC treatment is the reduction in *HSP70* transcript levels could be a result of a stress response caused by TCC. High levels of HSP70 proteins can be detrimental to the cell (Arispe *et al.*, 2004). *HSP70* mRNA half-life is 1 hour in cells after stress (Theodorakis and Morimoto, 1987; De Maio, 1999) and a parallel decrease in *HSP70* mRNA levels was shown with an accumulation of HSP70 protein levels. Balakrishnan and De Maio (2006) showed HSP70 protein can bind *HSP70* mRNA possibly as a

mechanism to limit its protein expression and the decrease in mRNA levels is thought to be dependent on the HSP70 protein levels. Since the chemical exposure was 48 hours in length, TCC could have caused a stress event, and increased *HSP70* transcript levels; however, at the time point the transcript levels were measured, the *HSP70* mRNA levels could have already decreased (Figure 6.4). It should be noted that the cellular stress as observed with TCC was not due to overt toxicity because the normalizer gene transcript, ribosomal protein *L8*, was of good quality and quantity as compared to other studies (Chapters 3-5; (Hinther *et al.*, 2010A; Hinther *et al.*, 2010B)). Given the observed effects in cell and organ culture, examination of the effects of TCC on postembryonic development of frog tadpoles is warranted.

Chapter 7: Discussion and Future Directions

Presented in this thesis is a novel assay to screen the growing number of anthropogenic chemicals released into the environment that may have disrupting effects on the thyroid hormone axis. There are many advantages to using the C-fin assay. For example, tail fin biopsies are taken from *R. catesbeiana* tadpoles at the premetamorphic stage, when these animals produce no confounding endogenous THs. Studies of TH action using mammalian model systems typically rely on physical or chemical ablation of the thyroid gland to abolish endogenous THs, however these methods lead to disease states in the animals. The use of premetamorphic tadpoles in the C-fin assay thus allows true determination of chemical effects in the absence of TH in perfectly healthy animals. Moreover, these tadpoles are at a very sensitive period in their lifetime and act as sentinel species in aquatic environments, where they are constantly exposed to xenobiotics absorbed through their skin. Furthermore, we can mimic a prometamorphic state by administering exogenous TH to determine any perturbations in the normal TH transcriptional response.

There are also benefits to using intact tail biopsies as opposed to a cell line because the tissue has not been transformed in any way and is a true representation of the complex tissue organization and extracellular interactions including inherent biological variation. The anuran metamorphosis model also has the advantage of independent tissue responsiveness to THs, allowing the *ex vivo* use of organs and tissues as screening tools for chemically-induced perturbations in TH signalling, both in the presence and absence of exogenous THs.

Through the use of intact tail biopsies from stage VI-VIII *R. catesbeiana* tadpoles, it is possible to expose one animal to multiple treatments. Not only does this reduce the number of animals used per experiment, but it also allows the normalization of each individual's response across treatments to minimize inter-individual variation and determine each individual's biological response. More specifically, one can determine if there is a bimodal response taking place, where some of the animals' transcript levels are increasing in response to a chemicals treatment, and other animals' transcript levels are decreasing in response to the same treatment. This would indicate differential responses to the test chemical within the group of animals used in the exposure.

The C-fin assay can also give us mechanistic insight into chemical action. If anomalies are observed for a given chemical of interest, then it can be concluded that the chemical is acting at a cellular level in a TH-responsive tissue, independent of TH synthesis and transport. However, an important caveat of this assay is that the converse does not necessarily hold true; lack of significant effects as assessed by C-fin does not imply that a chemical will not interfere with TH signalling in the context of an intact animal or a different tissue.

This thesis demonstrated the usefulness of the C-fin assay in the context of screening specific nanochemicals (nanosilver, QDs, and nanozinc oxide) for their potential TH-disrupting effects. The industrial production and use of nanoparticles has been drastically increasing, and due to their small size they have very different chemical and physical properties than their larger non-nano sized counterparts. In spite of this, little research has investigated potential deleterious effects of these particles. This thesis demonstrated nanosilver and QDs can both cause perturbations in the TH axis at

the transcriptional level in *R. catesbeiana*. Though a cellular stress response was induced, as measured via changes in *HSP30* and *CAT* transcript levels, within the C-fin assay, it is interesting to note overt toxicity did not occur, as the uniform quality and quantity of the mRNA steady-state levels of a housekeeping gene (ribosomal protein L8) were not affected by nanoparticle exposure. This further demonstrates the importance of having a complex tissue, as the NP-induced stress response was not detected within the GH3 cell line.

Chapter 3 of this thesis demonstrates the sensitivity of the C-fin assay in detecting perturbations in the TH axis at the transcriptional level by measuring the mRNA steady-state levels of two TH responsive genes, *TRβ* and *RLKI*. In addition, the usefulness of this assay could easily be extended to proteomic analyses of the changes occurring as a response to chemical exposures. For instance, it would be interesting to look at the RLKI protein fragment levels after exposure to NPs. Further studies will have to be conducted to fully understand the mechanism through which these NPs are causing the perturbations in the transcript levels tested. The future use of other experimental approaches, such as microarrays and chromatin immunoprecipitations (ChIPs), will be beneficial in supplementing the data obtained using the C-fin assay, as they will provide more comprehensive mechanistic information about specific endocrine disruptors. Microarrays, for example, can provide additional information as to other genes affected by these chemicals, thus providing insight into the pathways that are being disrupted. ChIPs can be used to detect EDC-dependent changes in transcription complex composition at specific thyroid-responsive promoters. Experiments in our lab are currently underway to determine whether nanosilver binds T_3 and/or affects cellular

T_3 uptake. Also, whole animal exposures are being performed with nanosilver, thus other affected tissues and pathways may soon be discovered.

Another group of chemicals examined in this thesis were the antimicrobials TCC and TCS, and the bacterial TCS metabolite methyl-TCS (m-TCS). These were assayed for their TH-disrupting potential both by the C-fin assay and through exposures in mammalian GH3 cells. Methyl-TCS appeared to be more potent as a thyroid disruptor than TCS, and TCC also showed evidence of possible endocrine disruption. Although the underlying mode of action for this perturbation remains unclear, manipulation of cellular stress pathways may be one component. In order to further elucidate the mechanistic details, microarray and ChIP analyses would also be beneficial in this context. Examination of the effects of these chemicals on other tissues and in the intact animal is also warranted.

An additional level of complexity in the field of EDC studies arises from the fact that several of these potential disruptors occur together in nature. Thus combinatorial effects should be taken into account, as these chemicals could be causing synergistic (more than additive) effects when simultaneously present in the environment. The opposite situation, attenuation of effects by chemical mixtures, could also hold true. To investigate these possibilities within the scope of the present work, the effects of TCS, TCC, and mTCS should be investigated in combination with the nanoparticles. This would represent a particularly salient chemical mixture since TCS/TCC is commonly found in toothpaste and nanosilver is now being manufactured into toothbrushes; this allows people to be exposed to both these chemicals simultaneously. The potential interactions of these chemicals are not known.

This thesis provides additional information to the endocrine disrupting field by investigating novel TH disrupting chemicals as well as further studying known TH disrupting chemicals. This thesis focused on the disruption of steady-state mRNA transcript levels; however, the mechanism of perturbation has yet to be determined. Due to these chemicals' large production volumes, further investigation is warranted in order to determine exactly how these chemicals exert their effects and whether it will adversely affect the health of humans and wildlife.

Bibliography

Abdulle, R., Mohindra, A., Fernando, P. and Heikkila, J. J. (2002). "Xenopus small heat shock proteins, Hsp30C and Hsp30D, maintain heat- and chemically denatured luciferase in a folding-competent state." Cell Stress & Chaperones **7**(1): 6-16.

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J. and Sturve, J. (2002). "Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden." Chemosphere **46**(9-10): 1485-1489.

Arispe, N. J., Simakova, O., Doh, M., Diaz, J. and De Maio, A. (2004). "Toxic extracellular interaction of heat shock proteins with cells is mediated by phosphatidylserine." Biophysical Journal **86**(1): 90a-90a.

Arrigo, A. P. and Tanguay, R. M. (1991). "Expression of heat shock proteins during development in Drosophila." Results Problems in Cell Differentiation **17**: 106-119.

Atkinson, B. G., Helbing, C. and Chen, Y. (1994). Reprogramming of gene expression in the liver of *Rana catesbeiana* tadpoles during spontaneous and thyroid hormone-induced metamorphosis. Perspectives in Comparative Endocrinology. K. G. Davey, S. S. Tobe and R. G. Peter. Ottawa, ON, National Research Council of Canada**:** 416-423.

Atkinson, B. G., Raizada, M., Bouchard, R. A., Frappier, R. H. and Walden, D. B. (1993). "The independent stage-specific expression of the 18-kDa heat shock protein genes during microsporogenesis in Zea mays L." Developmental Genetics **14**(1): 15-26.

Badger, T. M., Ronis, M. J. J., Hakkak, R., Rowlands, J. C. and Korourian, S. (2002). "The health consequences of early soy consumption." Journal of Nutrition **132**(3): 559s-565s.

Baker, B. S. and Tata, J. R. (1992). "Prolactin prevents the autoinduction of thyroid hormone receptor mRNAs during amphibian metamorphosis." Developmental Biology **149**: 463-467.

Balakrishnan, K. and De Maio, A. (2006). "Heat shock protein 70 binds its own messenger ribonucleic acid as part of a gene expression self-limiting mechanism." Cell Stress & Chaperones **11**(1): 44-50.

Balmer, M. E., Poiger, T., Droz, C., Romanin, K., Bergqvist, P. A., Muller, M. D. and Buser, H. R. (2004). "Occurrence of methyl triclosan, a transformation product of the bactericide triclosan, in fish from various lakes in Switzerland." Environmental Science & Technology **38**(2): 390-395.

Barbash, J., Thelin, G., Kolpin, D. and Gilliom, R. (2001). "Major herbicides in ground water: results from the National Water-Quality Assessment." Journal of Environmental Quality **30**: 831-845.

Bassett, J., Harvey, C. B. and Williams, G. R. (2003). "Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions." Molecular and Cellular Endocrinology **213**: 1-11.

Becker, K. B., Stephens, K. C., Davey, J. C., Schneider, M. J. and Galton, V. A. (1997). "The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in Rana catesbeiana tadpoles." Endocrinology **138**(7): 2989-2997.

Bernal, J., Guadano-Ferraz, A. and Morte, B. (2003). "Perspectives in the study of thyroid hormone action on brain development and function." Thyroid **13**(11): 1005-1012.

Berry, M. J., Banu, L. and Larsen, P. R. (1991). "Type I iodothyronine deiodinase is a selenocysteine-containing enzyme." Nature **349**(6308): 438-440.

Berry, M. J., Kates, A. L. and Larsen, P. R. (1990). "Thyroid hormone regulates type I deiodinase messenger RNA in rat liver." Molecular Endocrinology **4**(5): 743-748.

Bester, K. (2003). "Triclosan in a sewage treatment process - balances and monitoring data." Water Research **37**(16): 3891-3896.

Bianco, A. C. and Kim, B. W. (2006). "Deiodinases: implications of the local control of thyroid hormone action." Journal of Clinical Investigation **116**(10): 2571-2579.

Blaser, S. A., Scheringer, M., Macleod, M. and Hungerbuhler, K. (2008). "Estimation of cumulative aquatic exposure and risk due to silver: contribution of nano-functionalized plastics and textiles." Science of Total Environment **390**(2-3): 396-409.

Boas, M., Feldt-Rasmussen, U., Skakkebaek, N. E. and Main, K. M. (2006). "Environmental chemicals and thyroid function." European Journal of Endocrinology **154**(5): 599-611.

Bregman, D. B., Pestell, R. G. and Kidd, V. J. (2000). "Cell cycle regulation and RNA polymerase II." Frontiers in Bioscience **5**: D244-D257.

Brucker-Davis, F. (1998). "Effects of environmental synthetic chemicals on thyroid function." Thyroid **8**: 827-856.

Buchholz, D. R., Paul, B. D., Fu, L. and Shi, Y. B. (2006). "Molecular and developmental analyses of thyroid hormone receptor function in Xenopus laevis, the African clawed frog." General and Comparative Endocrinology **145**(1): 1-19.

Calamandrei, G., Maranghi, F., Venerosi, A., Alleva, E. and Mantovani, A. (2006). "Efficient testing strategies for evaluation of xenobiotics with neuroendocrine activity." Reproductive Toxicology **22**(2): 164-174.

Camargo, J. A., Alonso, A. and de la Puente, M. (2004). "Multimetric assessment of nutrient enrichment in impounded rivers based on benthic macroinvertebrates." Environment Monititoring and Assessment **96**(1-3): 233-249.

Camargo, J. A., Alonso, A. and Salamanca, A. (2005). "Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates." Chemosphere **58**(9): 1255-1267.

Campos-Barros, A., Hoell, T., Musa, A., Sampaolo, S., Stoltenburg, G., Pinna, G., Eravci, M., Meinhold, H. and Baumgartner, A. (1996). "Phenolic and tyrosyl ring iodothyronine deiodination and thyroid hormone concentrations in the human central nervous system." Journal of Clinical Endocrinology and Metabolism **81**(6): 2179-2185.

CCME (2007). Canadian water quality guidelines for the protection of aquatic life E. Canada. Ottawa, Canadian Council of Ministers of the Environment

Chae, Y. J., Pham, C. H., Lee, J., Bae, E., Yi, J. and Gu, M. B. (2009). "Evaluation of the toxic impact of silver nanoparticles on Japanese medaka (Oryzias latipes)." Aquatic Toxicology **94**(4): 320-327.

Chassande, O., Fraichard, A., Gauthier, K., Flamant, F., Legrand, C., Savatier, P., Laudet, V. and Samarut, J. (1997). "Identification of transcripts initiated from an internal promoter in the c-erbA alpha locus that encode inhibitors of retinoic acid receptor-alpha and triiodothyronine receptor activities." Molecular Endocrinology **11**(9): 1278-1290.

Cheek, A. O., Ide, C. F., Bollinger, J. E., Rider, C. V. and McLachlan, J. A. (1999). "Alteration of leopard frog (*Rana pipiens*) metamorphosis by the herbicide acetochlor." Archives of Environmental Contamination and Toxicology **37**: 70-77.

Cheek, A. O., Kow, K., Chen, J. and McLachlan, J. A. (1999). "Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin." Environmental Health Perspectives **107**: 273-278.

Chen, J., Ahn, K. C., Gee, N. A., Ahmed, M. I., Duleba, A. J., Zhao, L., Gee, S. J., Hammock, B. D. and Lasley, B. L. (2008). "Triclocarban enhances testosterone action: a new type of endocrine disruptor?" Endocrinology **149**(3): 1173-1179.

Chen, J., Ahn, K. C., Gee, N. A., Gee, S. J., Hammock, B. D. and Lasley, B. L. (2007). "Antiandrogenic properties of parabens and other phenolic containing small molecules in personal care products." Toxicological Applied Pharmacology **221**(3): 278-284.

Chen, J. G., Ahn, K. C., Gee, N. A., Ahmed, M. I., Duleba, A. J., Zhao, L., Gee, S. J., Hammock, B. D. and Lasley, B. L. (2008). "Triclocarban enhances testosterone action: A new type of endocrine disruptor?" Endocrinology **149**(3): 1173-1179.

Chen, S., Chang, Y., Wu, Y. and Lin, K. (2003). "Mitogen-activated protein kinases potentiate thyroid hormone receptro transcriptional activity by stabilizing its protein." Endocrinology **144**: 1407-1419.

Chen, X. and Schluesener, H. (2008). "Nanosilver: A nanoproduct in medical application." Toxicology Letters **176**: 1-12.

Commission, E. (2006). Environment fact sheet: REACH - a new chemicals policy for the EU.

Crain, E. F. (2000). "Environmental threats to children's health: a challenge for pediatrics: 2000 Ambulatory Pediatric Association (APA) Presidential Address." Pediatrics **106**(4 Suppl): 871-875.

Crofton, K. M. (2008). "Thyroid disrupting chemicals: mechanisms and mixtures." International Journal of Andrology **31**(2): 209-222.

Crofton, K. M., Craft, E. S., Hedge, J. M., Gennings, C., Simmons, J. E., Carchman, R. A., Carter, W. H., Jr. and DeVito, M. J. (2005). "Thyroid-hormone-disrupting chemicals: evidence for dose-dependent additivity or synergism." Environmental Health Perspectives **113**(11): 1549-1554.

Crofton, K. M., Paul, K. B., De Vito, M. J. and Hedge, J. M. (2007). "Short-term in vivo exposure to the water contaminant triclosan: Evidence for disruption of thyroxine." Environmental Toxicology and Pharmacology **24**(2): 194-197.

Crofton, K. M. and Zoeller, R. T. (2005). "Mode of action: neurotoxicity induced by thyroid hormone disruption during development--hearing loss resulting from exposure to PHAHs." Critical Review of Toxicology **35**(8-9): 757-769.

Crow, G. L., Luer, W. H. and Harshbarger, J. C. (2001). "Histological assessment of goiters in elasmobranch fishes." Journal of Aquatic Animal Health **13**(1): 1-7.

Crump, D., Werry, K., Veldhoen, N., Van Aggelen, G. and Helbing, C. C. (2002). "Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and metamorphosis in Xenopus Laevis." Environmental Health Perspectives **110**(12): 1199-1205.

Damstra, T., Page, S. W., Herrman, J. L. and Meredith, T. (2002). "Persistent organic pollutants: potential health effects?" Journal of Epidemiology and Community Health **56**(11): 824-825.

Das, B., Matsuda, H., Fujimoto, K., Sun, G., Matsuura, K. and Shi, Y. B. "Molecular and genetic studies suggest that thyroid hormone receptor is both necessary and sufficient to mediate the developmental effects of thyroid hormone." General and Comparative Endocrinology **168**(2): 174-180.

Das, B., Matsuda, H., Fujimoto, K., Sun, G. H., Matsuura, K. and Shi, Y. B. (2010). "Molecular and genetic studies suggest that thyroid hormone receptor is both necessary and sufficient to mediate the developmental effects of thyroid hormone." General and Comparative Endocrinology **168**(2): 174-180.

Davey, J., Schneider, M. and Galton, V. (1994). "Cloning of a thyroid hormone-responsive Rana catesbeiana c-erbA-beta gene." Developmental Genetics **15**(4): 339-346.

Davey, J. C., Becker, K. B., Schneider, M. J., St Germain, D. L. and Galton, V. A. (1995). "Cloning of a cDNA for the type II iodothyronine deiodinase." Journal of Biological Chemistry **270**: 26786-26789.

Davis, P. J., Davis, F. B. and Cody, V. (2005). "Membrane receptors mediateing thyroid hormone action." Trends in Endocrinology and Metabolism **16**: 429-435.

Davis, P. J., Davis, F. B. and Cody, V. (2005). "Membrane receptors mediating thyroid hormone action." Trends Endocrinology and Metabolism **16**(9): 429-435.

Davis, P. J., Leonard, J. L. and Davis, F. B. (2008). "Mechanisms of nongenomic actions of thyroid hormone." Frontiers in Neuroendocrinology **29**(2): 211-218.

Day, R. N. and Maurer, R. A. (1989). "Thyroid hormone-responsive elements of the prolactin gene: evidence for both positive and negative regulation." Molecular Endocrinology **3**(6): 931-938.

Dayan, A. D. (2007). "Risk assessment of triclosan [Irgasan (R)] in human breast milk." Food and Chemical Toxicology **45**(1): 125-129.

de Escobar, G. M., Obregon, M. J. and del Rey, F. E. (2007). "Iodine deficiency and brain development in the first half of pregnancy." Public Health Nutrition **10**(12A): 1554-1570.

De Groef, B., Decallonne, B. R., Van der Geyten, S., Darras, V. M. and Bouillon, R. (2006). "Perchlorate versus other environmental sodium/iodide symporter inhibitors: potential thyroid-related health effects." European Journal of Endocrinology **155**(1): 17-25.

De Maio, A. (1999). "Heat shock proteins. Facts, thoughts, and dreams. Reply." Shock **12**(4): 324-325.

DeAzevedo, W. F., Leclerc, S., Meijer, L., Havlicek, L., Strnad, M. and Kim, S. H. (1997). "Inhibition of cyclin-dependent kinases by purine analogues - Crystal structure of human cdk2 complexed with roscovitine." European Journal of Biochemistry **243**(1-2): 518-526.

Demonacos, C. V., Karayanni, N., Hatzoglou, E., Tsiriyiotis, C., Spandidos, D. A. and Sekeris, C. E. (1996). "Mitochondrial genes as sites of primary action of steroid hormones." Steroids **61**(4): 226-232.

Denver, R. J. (1997). "Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis." Hormonal Behaviour **31**(2): 169-179.

Denver, R. J. (1998). "Hormonal correlates of environmentally induced metamorphosis in the Western spadefoot toad, Scaphiopus hammondii." General and Comparative Endocrinology **110**(3): 326-336.

Denver, R. J. (1998). "The molecular basis of thyroid hormone-dependent central nervous system remodeling during amphibian metamorphosis." Comparative Biochemistry and Physiology Part C **119**: 219-228.

Denver, R. J., Hu, F., Scanlan, T. S. and Furlow, J. D. (2008). "Thyroid hormone receptor subtype specificity for hormone-dependent neurogenesis in Xenopus laevis." Developmental Biology.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A. O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McMaster, S., McClain, M., McConnell, E., Meier, C., Miller, R., Tietge, J. and Tyl, R. (1999). "Screening methods for thyroid hormone disruptors." Environmental Health Perspectives **107**(5): 407-415.

Di Cosmo, C., Liao, X., Dumitrescu, A.M., Nancy, P.L., Weiss, R.E., Refetoff, S. (2010). "Mice deficient in MCT8 reveal a mechanism regulating thyroid hormone secretion." The Journal of Clinical Investigation **120**(9): 3377-3388.

Dodd, M. and Dodd, J. (1976). The biology of metamorphosis. Physiology of the Amphibia. B. Lofts. New York, Academic Press**:** 467-599.

Doerge, D. R. and Sheehan, D. M. (2002). "Goitrogenic and estrogenic activity of soy isoflavones." Environmental Health Perspectives **110**: 349-353.

Domanski, D. and Helbing, C. C. (2007). "Analysis of the *Rana catesbeiana* tadpole tail fin proteome and phosphoproteome during T_3-induced apoptosis: identification of a novel type I keratin." BMC Developmental Biology **7**: 94.

Dorshkind, K. and Horseman, N. D. (2000). "The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency." Endocrinology Review **21**(3): 292-312.

Duarte-Guterman, P., Langlois, V. S., Pauli, B. D. and Trudeau, V. L. "Expression and T3 regulation of thyroid hormone- and sex steroid-related genes during Silurana (Xenopus) tropicalis early development." General and Comparative Endocrinology **166**(2): 428-435.

Duarte-Guterman, P. and Trudeau, V. L. "Regulation of thyroid hormone-, oestrogen- and androgen-related genes by triiodothyronine in the brain of Silurana tropicalis." Journal of Neuroendocrinology **22**(9): 1023-1031.

Duarte-Guterman, P. and Trudeau, V. L. "Transcript profiles and triiodothyronine regulation of sex steroid- and thyroid hormone-related genes in the gonad-mesonephros complex of Silurana tropicalis." Molecular Cell Endocrinology.

Eckey, M., Moehren, U. and Baniahmad, A. (2003). "Gene silencing by the thyroid hormone receptor." Molecular Cell Endocrinology **213**(1): 13-22.

Edwards, T. M., McCoy, K. A., Barbeau, T., McCoy, M. W., Thro, J. M. and Guillette, L. J., Jr. (2006). "Environmental context determines nitrate toxicity in Southern toad (Bufo terrestris) tadpoles." Aquatic Toxicology **78**(1): 50-58.

Eliceiri, B. and Brown, D. (1994). "Quantitation of endogenous thyroid hormone receptors α and β during embryogenesis and metamorphosis in *Xenopus laevis*." Journal of Biological Chemistry **269**: 24459-24465.

Eliceiri, B. P. and Brown, D. D. (1994). "Quantitation of endogenous thyroid hormone receptors alpha and beta during embryogenesis and metamorphosis in Xenopus laevis." Journal of Biological Chemistry **269**(39): 24459-24465.

Fekkes, D., Hennemann, G. and Visser, T. J. (1982). "Evidence for a single enzyme in rat liver catalysing the deiodination of the tyrosyl and the phenolic ring of iodothyronines." Biochemistry Journal **201**(3): 673-676.

Fernando, P. and Heikkila, J. J. (2000). "Functional characterization of Xenopus small heat shock protein, Hsp30C: the carboxyl end is required for stability and chaperone activity." Cell Stress Chaperones **5**(2): 148-159.

Fini, J. B., Le Mevel, S., Turque, N., Palmier, K., Zalko, D., Cravedi, J. P. and Demeneix, B. A. (2007). "An in vivo multiwell-based fluorescent screen for monitoring vertebrate thyroid hormone disruption." Environmental Science and Technology **41**(16): 5908-5914.

Flamant, F. and Samarut, J. (2003). "Thyroid hormone receptors: lessons from knockout and knock-in mutant mice." Trends in Endocrinology and Metabolism **14**(2): 85-90.

Foran, C. M., Bennett, E. R. and Benson, W. H. (2000). "Developmental evaluation of a potential non-steroidal estrogen: triclosan." Marine Environmental Research **50**(1-5): 153-156.

Forman, B. M., Yang, C. R., Stanley, F., Casanova, J. and Samuels, H. H. (1988). "c-erbA protooncogenes mediate thyroid hormone-dependent and independent regulation of the rat growth hormone and prolactin genes." Molecular Endocrinology **2**(10): 902-911.

Fort, D., Degitz, S. J., Tietge, J. and Touart, L. (2007). "The hypothalamic-pituitary-thyroid (HPT) axis in frogs and its role in frog development and reproduction." Critical Review of Toxicology **37**: 117-161.

Fort, D. J., Rogers, R. L., Gorsuch, J. W., Navarro, L. T., Peter, R. and Plautz, J. R. "Triclosan and anuran metamorphosis: no effect on thyroid-mediated metamorphosis in Xenopus laevis." Toxicological Science **113**(2): 392-400.

Fort, D. J., Rogers, R. L., Gorsuch, J. W., Navarro, L. T., Peter, R. and Plautz, J. R. (2010). "Triclosan and anuran metamorphosis: no effect on thyroid-mediated metamorphosis in Xenopus laevis." Toxicological Science **113**(2): 392-400.

Frieden, E. and Naile, B. (1955). "Biochemistry of amphibian metamorphosis: I. Enhancement of induced metamorphosis by glucocorticoids." Science **121**(3132): 37-39.

Gao, X., Cui, Y., Levenson, R. M., Chung, L. W. and Nie, S. (2004). "In vivo cancer targeting and imaging with semiconductor quantum dots." Natural Biotechnology **22**(8): 969-976.

Gernold, M., Knauf, U., Gaestel, M., Stahl, J. and Kloetzel, P. M. (1993). "Development and tissue-specific distribution of mouse small heat shock protein hsp25." Develompmental Genetics **14**(2): 103-111.

Ghisari, M. and Bonefeld-Jorgensen, E. C. (2005). "Impact of environmental chemicals on the thyroid hormone function in pituitary rat GH3 cells." Molecular Cell Endocrinology **244**(1-2): 31-41.

Goleman, W. L., Urquidi, L. J., Anderson, T. A., Smith, E. E., Kendall, R. J. and Carr, J. A. (2002). "Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in Xenopus laevis." Environmental Toxicology and Chemistry **21**(2): 424-430.

Gore, A., Heindel, J. and Zoeller, R. (2006). "Endocrine disruption for endocrinologists." Endocrinology **147**: S1-S3.

Gray, B. R., Hill, W. R. and Stewart, A. J. (2001). "Effects of development time, biomass and ferromanganese oxides on nickel sorption by stream periphyton." Environmental Pollution **112**(1): 61-71.

Gray, K. M. and Janssens, P. A. (1990). "Gonadal hormones inhibit the induction of metamorphosis by thyroid hormones in Xenopus laevis tadpoles in vivo, but not in vitro." General and Comparative Endocrinology **77**: 202-211.

Gray, T. A., Saitoh, S. and Nicholls, R. D. (1999). "An imprinted, mammalian bicistronic transcript encodes two independent proteins." Proceedings of the National Academy of Science **96**(10): 5616-5621.

Griffitt, R. J., Hyndman, K., Denslow, N. D. and Barber, D. S. (2009). "Comparison of Molecular and Histological Changes in Zebrafish Gills Exposed to Metallic Nanoparticles." Toxicological Sciences **107**(2): 404-415.

Griffitt, R. J., Weil, R., Hyndman, K. A., Denslow, N. D., Powers, K., Taylor, D. and Barber, D. S. (2007). "Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio)." Environmental Science and Technology **41**(23): 8178-8186.

Guigon, C. J. and Cheng, S. Y. (2009). "Novel non-genomic signaling of thyroid hormone receptors in thyroid carcinogenesis." Molecular Cell Endocrinology **308**(1-2): 63-69.

Guillette, L. J. and Edwards, T. M. (2005). "Is nitrate an ecologically relevant endocrine disruptor in vertebrates?" Integrative and Comparative Biology **45**(1): 19-27.

Halden, R. U. and Paull, D. H. (2005). "Co-occurrence of triclocarban and triclosan in US water resources." Environmental Science & Technology **39**(6): 1420-1426.

Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., Kester, M. H., Andersson, P. L., Legler, J. and Brouwer, A. (2006). "In vitro profiling of the endocrine-disrupting potency of brominated flame retardants." Toxicological Science **92**(1): 157-173.

Hampel, R. and Zollner, H. (2004). "Iodine supply and incidence of goitrogenics in Germany." Ernahrungs-Umschau **51**(4): 132-+.

Handy, R. D., Owen, R. and Valsami-Jones, E. (2008). "The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs." Ecotoxicology **17**(5): 315-325.

Handy, R. D., von der Kammer, F., Lead, J. R., Hassellov, M., Owen, R. and Crane, M. (2008). "The ecotoxicology and chemistry of manufactured nanoparticles." Ecotoxicology **17**(4): 287-314.

Hannas, B. R., Das, P. C., Li, H. and LeBlanc, G. A. (2010). "Intracellular Conversion of Environmental Nitrate and Nitrite to Nitric Oxide with Resulting Developmental Toxicity to the Crustacean Daphnia magna." Public Library of Science **5**(8): -.

Hardman, R. (2006). "A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors." Environmental Health Perspectives **114**(2): 165-172.

Hasunuma, I., Yamamoto, K. and Kikuyama, S. (2004). "Molecular cloning of bullfrog prolactin receptor cDNA: changes in prolactin receptor mRNA level during metamorphosis." General and Comparative Endocrinology **138**(3): 200-210.

Hayes, T., Chan, R. and Licht, P. (1993). "Interactions of temperature and steroids on larval growth, development and metamorphosis in a toad (*Bufo boreas*)." Journal of Experimental Zoology **266**: 206-215.

Hayes, T. B. (1997). "Steroids as potential modulators of thyroid hormone activity in anuran metamorphosis." American Zoologist **37**: 185-194.

HealthCanada (2008). Guidelines for Canadian Drinking Water Quality Summary Table. Environment. Ottawa, Government of Canada**:** 1-14.

Heath, R. J., Li, J., Roland, G. E. and Rock, C. O. (2000). "Inhibition of the Staphylococcus aureus NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene." Journal of Biological Chemistry **275**(7): 4654-4659.

Heath, R. J., Li, J., Roland, G. E. and Rock, C. O. (2000). "Inhibition of the Staphylococcus aureus NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene." Journal of Biological Chemistry **275**(7): 4654-4659.

Heath, R. J. and Rock, C. O. (2000). "A triclosan-resistant bacterial enzyme." Nature **406**(6792): 145-146.

Helbing, C., Bailey, C., Ji, L., Gunderson, M., Zhang, F., Veldhoen, N., Mu, R., Lesperance, M., Holcombe, G., Kosian, P., Tietge, J., Korte, J. J. and Degitz, S. (2007). "Identification of gene expression indicators for thyroid axis disruption in a

Xenopus laevis metamorphosis screening assay Part 1: Effects on the brain." Aquatic Toxicology **82**: 227-241.

Helbing, C., Gallimore, C. and Atkinson, B. G. (1996). "Characterization of a *Rana catesbeiana* hsp30 gene and its expression in the liver of this amphibian during both spontaneous and thyroid hormone-induced metamorphosis." Developmental Genetics **18**(3): 223-233.

Helbing, C., van Aggelen, G. and Veldhoen, N. (2010). "Triclosan affects thyroid hormone-dependent metamorphosis in anurans." Toxicological Sciences: 2.

Helbing, C. C. and Atkinson, B. G. (1994). "3,5,3'-Triiodothyronine-induced carbamyl phosphate synthetase gene expression is stabilized in the liver of Rana catesbeiana tadpoles during heat shock." Journal of Biological Chemistry **269**: 11743-11750.

Helbing, C. C., Gergely, G. and Atkinson, B. G. (1992). "Sequential up-regulation of thyroid hormone β receptor, ornithine transcarbamylase and carbamyl phosphate synthetase mRNAs in the liver of *Rana catesbeiana* tadpoles during spontaneous and thyroid hormone-induced metamorphosis." Developmental Genetics **13**: 289-301.

Helbing, C. C., Ji, L., Bailey, C. M., Veldhoen, N., Zhang, F., Holcombe, G. W., Kosian, P. A., Tietge, J., Korte, J. J. and Degitz, S. J. (2007). "Identification of gene expression indicators for thyroid axis disruption in a Xenopus laevis metamorphosis screening assay Part 2. Effects on the tail and hindlimb." Aquatic Toxicology **82**(4): 215-226.

Helbing, C. C., Maher, S. K., Han, J., Gunderson, M. P. and Borchers, C. (2010). "Peering into molecular mechanisms of action with frogSCOPE." General and Comparative Endocrinology **168**(2): 190-198.

Helbing, C. C., Ovaska, K. and Ji, L. (2006). "Evaluation of the effect of acetochlor on thyroid hormone receptor gene expression in the brain and behavior of *Rana catesbeiana* tadpoles." Aquatic Toxicology **80**: 42-51.

Hetzel, B. S. and Dunn, J. T. (1989). "The iodine deficiency disorders: their nature and prevention." Annual Review in Nutrition **9**: 21-38.

Hetzel, B. S. and Mano, M. T. (1989). "A review of experimental studies of iodine deficiency during fetal development." Journal of Nutrition **119**(2): 145-151.

Hinther, A., Domanski, D., Vawda, S. and Helbing, C. C. (2010). "C-fin: a cultured frog tadpole tail fin biopsy approach for detection of thyroid hormone-disrupting chemicals." Environmental Toxicology and Chemistry **29**(2): 380-388.

Hinther, A., Vawda, S., Skirrow, R. C., Veldhoen, N., Collins, P., Cullen, J. T., van Aggelen, G. and Helbing, C. C. (2010). "Nanometals Induce Stress and Alter Thyroid Hormone Action in Amphibia at or below North American Water Quality Guidelines." Environmental Science and Technology **44**: 8314-8321.

Hohenwarter, O., Waltenberger, A. and Katinger, H. (1996). "An in vitro test system for thyroid hormone action." Analytical Biochemistry **234**(1): 56-59.

Houlahan, J., Findlay, C., Schmidt, B., Meyer, A. and Kuzmin, S. (2000). "Quantitative evidence for global amphibian population declines." Nature **404**: 752-755.

Hsu, J. H. and Brent, G. A. (1998). "Thyroid hormone receptor gene knockouts." Trends in Endocrinology and Metabolism **9**(3): 103-112.

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y. and Taketani, Y. (2002). "Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure." Human Reproduction **17**(11): 2839-2841.

Jaffe, R. C. (1981). "Plasma concentration of corticosterone during Rana catesbeiana tadpole metamorphosis." General and Comparative Endocrinology **44**(3): 314-318.

Jakobs, T. C., Koehler, M. R., Schmutzler, C., Glaser, F., Schmid, M. and Kohrle, J. (1997). "Structure of the human type I iodothyronine 5'-deiodinase gene and localization to chromosome 1p32-p33." Genomics **42**(2): 361-363.

Jakobs, T. C., Schmutzler, C., Meissner, J. and Kohrle, J. (1997). "The promoter of the human type I 5'-deiodinase gene--mapping of the transcription start site and identification of a DR+4 thyroid-hormone-responsive element." European Journal in Biochemistry **247**(1): 288-297.

Ji, L., Domanski, D., Skirrow, R. and Helbing, C. (2007). "Genistein prevents thyroid hormone-dependent tail regression of *Rana catesbeiana* tadpoles by targetting protein kinase C and thyroid hormone receptor alpha." Developmental Dynamics **236**: 777-790.

Ji, L., Domanski, D., Skirrow, R. C. and Helbing, C. C. (2007). "Genistein prevents thyroid hormone-dependent tail regression of Rana catesbeiana tadpoles by targetting protein kinase C and thyroid hormone receptor alpha." Developmental Dynamics **236**(3): 777-790.

Jingxia, Z., Lanju, X., Tao, Z., Guogang, R. and Zhuo, Y. (2009). "Influences of nanoparticle zinc oxide on acutely isolated rat hippocampal CA3 pyramidal neurons." Neurotoxicology **30**: 220-230.

Jolivet Jaudet, G. and Leloup Hatey, J. (1984). "Variations in aldosterone and corticosterone plasma levels during metamorphosis in Xenopus laevis tadpoles." General and Comparative Endocrinology **56**(1): 59-65.

Jugan, M. L., Levy-Bimbot, M., Pomerance, M., Tamisier-Karolak, S., Blondeau, J. P. and Levi, Y. (2007). "A new bioluminescent cellular assay to measure the transcriptional effects of chemicals that modulate the alpha-1 thyroid hormone receptor." Toxicology in Vitro **21**(6): 1197-1205.

Kaltenbach, J. C. (1996). Endocrinology of amphibian metamorphosis. Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells. L. I. Gilbert, J. R. Tata and B. G. Atkinson. San Diego, Academic Press**:** 403-431.

Kashiwagi, A., Hanada, H., Yabuki, M., Kanno, T., Ishisaka, R., Sasaki, J., Inoue, M. and Utsumi, K. (1999). "Thyroxine enhancement and the role of reactive oxygen species in tadpole tail apoptosis." Free Radical Biology and Medicine **26**(7-8): 1001-1009.

Kikuyama, S., Kawamura, K., Tanaka, S. and Yamamoto, K. (1993). "Aspects of amphibian metamorphosis. Hormonal control." International Reviews in Cytology **145**: 105-148.

Kikuyama, S., Kawamura, K., Tanaka, S. and Yamamoto, K. (1993). "Aspects of amphibian metamorphosis: hormonal control." International Reviews in Cytology **145**: 105-148.

Kikuyama, S., Suzuki, M. R. and Iwamuro, S. (1986). "Elevation of plasma aldosterone levels of tadpoles at metamorphic climax." General and Comparative Endocrinology **63**(2): 186-190.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H. and Fujimoto, N. (2002). "Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A." Biochemistal and Biophysical Research Communications **293**(1): 554-559.

Kitamura, S., Jinno, N., Suzuki, T., Sugihara, K., Ohta, S., Kuroki, H. and Fujimoto, N. (2005). "Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture." Toxicology **208**: 377-387.

Kitamura, S., Kato, T., Iida, M., Jinno, N., Suzuki, T., Ohta, S., Fujimoto, N., Hanada, H., Kashiwagi, K. and Kashiwagi, A. (2005). "Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: Affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis." Life Science **76**(14): 1589-1601.

Klein, J. (2007). "Probing the interactions of proteins and nanoparticles." Proceedings of the National Academy of Science **104**(7): 2029-2030.

Kloas, W. (2002). "Amphibians as a model for the study of endocrine disruptors." International Reviews in Cytology **216**: 1-57.

Kloas, W., Lutz, I. and Einspanier, R. (1999). "Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo." The Science of the Total Environment **225**: 59-68.

Koenig, R. J., Lazar, M. A., Hodin, R. A., Brent, G. A., Larsen, P. R., Chin, W. W. and Moore, D. D. (1989). "Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing." Nature **337**(6208): 659-661.

Koplin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B. and Buxton, H. T. (2002). "Response to comment on "Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance"." Environmental Science & Technology **36**(18): 4004-4004.

Krain, L. and Denver, R. (2004). "Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*." Journal of Endocrinology **181**: 91-104.

Kress, E., Samarut, J. and Plateroti, M. (2009). "Thyroid hormones and the control of cell proliferation or cell differentiation: paradox or duality?" Molecular Cell Endocrinology **313**(1-2): 36-49.

Krishnamurthy, S. V., Meenakumari, D., Gurushankara, H. P. and Vasudev, V. (2008). "Nitrate-induced morphological anomalies in the tadpoles of Nyctibatrachus major and Fejervarya limnocharis (Anura : Ranidae)." Turkish Journal of Zoology **32**(3): 239-244.

Krug, E. C., Honn, K. V., Battista, J. and Nicoll, C. S. (1983). "Corticosteroids in serum of Rana catesbeiana during development and metamorphosis." General and Comparative Endocrinology **52**(2): 232-241.

Kursa, J., Travnicek, J., Rambeck, W. A., Kroupova, V. and Vitovec, J. (2000). "Goitrogenic effects of extracted rapeseed meal and nitrates in sheep and their progeny." Veterinarni Medicina **45**(5): 129-140.

Lahti, E., Harri, M. and Lindqvist, O. V. (1985). "Uptake and Distribution of Radioiodine, and the Effect of Ambient Nitrate, in Some Fish Species." Comparative Biochemistry and Physiology a-Physiology **80**(3): 337-342.

Langlois, V. S., Duarte-Guterman, P., Ing, S., Pauli, B. D., Cooke, G. M. and Trudeau, V. L. "Fadrozole and finasteride exposures modulate sex steroid- and thyroid hormone-related gene expression in Silurana (Xenopus) tropicalis early larval development." General and Comparative Endocrinology **166**(2): 417-427.

Law, R. J., Bersuder, P., Allchin, C. R. and Barry, J. (2006). "Levels of the flame retardants hexabromocyclododecane and tetrabromobisphenol A in the blubber of harbor porpoises (Phocoena phocoena) stranded or bycaught in the U.K., with evidence for an increase in HBCD concentrations in recent years." Environmental Science and Technology **40**(7): 2177-2183.

Leatherland, J. (2000). Contaminant-altered thyroid function in wildlife. Environmental Endocrine Disrupters: An Evolutionary Perspective. L. Guillette, Jr and D. Crain. New York, Taylor & Francis**:** 155-181.

Lee, H. and Yen, P. M. (1999). "Recent advances in understanding thyroid hormone receptor coregulators." Journal of Biomedical Science **6**(2): 71-78.

Legler, J. and Brouwer, A. (2003). "Are brominated flame retardants endocrine disruptors?" Environment International **29**(6): 879-885.

Leloup, J. and Buscaglia, M. (1977). "La triiodothyronine, hormone de la metamorphose des amphibiens." Comptes Rendues de l'Academie de les Sciences Paris Serie D **284**: 2261-2263.

Levy, C. W., Roujeinikova, A., Sedelnikova, S., Baker, P. J., Stuitje, A. R., Slabas, A. R., Rice, D. W. and Rafferty, J. B. (1999). "Molecular basis of triclosan activity." Nature **398**(6726): 383-384.

Levy, O., Dai, G., Riedel, C., Ginter, C. S., Paul, E. M., Lebowitz, A. N. and Carrasco, N. (1997). "Characterization of the thyroid Na+/I- symporter with an anti-COOH terminus antibody." Proceedings of the National Academy of Science **94**(11): 5568-5573.

Li, G. C. and Mak, J. Y. (2009). "Re-induction of hsp70 synthesis: An assay for thermotolerance." International Journal of Hyperthermia **25**(4): 249-257.

Lindquist, S. and Craig, E. A. (1988). "The heat-shock proteins." Annual Review in Genetics **22**: 631-677.

Lindstrom, A., Buerge, I. J., Poiger, T., Bergqvist, P. A., Muller, M. D. and Buser, H. R. (2002). "Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater." Environmental Science and Technology **36**(11): 2322-2329.

Lindstrom, A., Buerge, I. J., Poiger, T., Bergqvist, P. A., Muller, M. D. and Buser, H. R. (2002). "Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater." Environmental Science & Technology **36**(11): 2322-2329.

Livak, K. J. and Schmittgen, T. D. (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Lovric, J., Bazzi, H. S., Cuie, Y., Fortin, G. R., Winnik, F. M. and Maysinger, D. (2005). "Differences in subcellular distribution and toxicity of green and red emitting CdTe quantum dots." Journal of Molecular Medicine **83**(5): 377-385.

Lovric, J., Cho, S. J., Winnik, F. M. and Maysinger, D. (2005). "Unmodified cadmium telluride quantum dots induce reactive oxygen species formation leading to multiple organelle damage and cell death." Chemical Biology **12**(11): 1227-1234.

Maibach, H. I., Feldman, R. J., Milby, T. H. and Serat, W. F. (1971). "Regional variation in percutaneous penetration in man. Pesticides." Archives in Environmental Health **23**(3): 208-211.

Marco, A. and Blaustein, A. R. (1999). "The effects of nitrite on behavior and metamorphosis in cascades frogs (Rana cascadae)." Environmental Toxicology and Chemistry **18**(5): 946-949.

Mariash, C. N. (2003). "Thyroid hormone and the adipocyte." Journal of Clinical Endocrinology and Metabolism **88**(12): 5603-5604.

McConahey, W. M. (1972). "Hashimoto's thyroiditis." Medical Clinics of North America **56**(4): 885-896.

McDonnell, G. and Pretzer, D. (1998). "Action and targets of triclosan." American Society ofr Microbiology News **64**(12): 670-671.

McMurry, L. M., Oethinger, M. and Levy, S. B. (1998). "Triclosan targets lipid synthesis." Nature **394**(6693): 531-532.

McMurry, L. M., Oethinger, M. and Levy, S. B. (1998). "Triclosan targets lipid synthesis." Nature **394**(6693): 531-532.

Meerts, I. A., van Zanden, J. J., Luijks, E. A., van Leeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A. and Brouwer, A. (2000). "Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin in vitro." Toxicological Science **56**(1): 95-104.

Meijer, L., Borgne, A., Mulner, O., Chong, J., Blow, J., Inagki, N., Inagaki, M., Delcros, J. and Moulinoux, J. (1997). "Biochemical and cellular effects of roscovitine, a

potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5." European Journal of Biochemistry **243**: 527-536.

Messier, N., Laflamme, L., Hamann, G. and Langlois, M. F. (2001). "In vitro effect of Triac on resistance to thyroid hormone receptor mutants: potential basis for therapy." Molecular and Cellular Endocrinology **174**(1-2): 59-69.

Messier, N. and Langlois, M. F. (2000). "Triac regulation of transcription is T(3) receptor isoform- and response element-specific." Molecular Cell Endocrinology **165**(1-2): 57-66.

Miller, M. D., Crofton, K. M., Rice, D. C. and Zoeller, R. T. (2009). "Thyroid-Disrupting Chemicals: Interpreting Upstream Biomarkers of Adverse Outcomes." Environmental Health Perspectives **117**(7): 1033-1041.

Morreale de Escobar, G., Obregon, M. and Escobar del Rey, F. (2004). "Role of thyroid hormone during early brain development." European Journal of Endocrinology **151**: U25-U37.

Nieuwkoop, P. D., and Faber, F. (1994). Normal table of *Xenopus laevis*. New York, NY., Garland Publishing,.

Nishikawa, M., Toyoda, N., Yonemoto, T., Ogawa, Y., Tabata, S., Sakaguchi, N., Tokoro, T., Gondou, A., Yoshimura, M., Yoshikawa, N. and Inada, M. (1998). "Quantitative measurements for type 1 deiodinase messenger ribonucleic acid in human peripheral blood mononuclear cells: mechanism of the preferential increase of T3 in hyperthyroid Graves' disease." Biochemical and Biophysical Research Communications **250**(3): 642-646.

Nishimura, N., Fukazawa, Y., Uchiyama, H. and Iguchi, T. (1997). "Effects of estrogenic hormones on early development of Xenopus laevis." Journal of Experimental Zoology **278**(4): 221-233.

Oberdorster, G., Oberdorster, E. and Oberdorster, J. (2005). "Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles." Environmental Health Perspectives **113**(7): 823-839.

Okada, R., Iwata, T., Kato, T., Kikuchi, M., Yamamoto, K. and Kikuyama, S. (2000). "Cloning of bullfrog thyroid-stimulating hormone (TSH) beta subunit cDNA: expression of TSHbeta mRNA during metamorphosis." General and Comparative Endocrinology **119**(2): 224-231.

Onken, H., Tresguerres, M. and Luquet, C. M. (2003). "Active NaCl absorption across posterior gills of hyperosmoregulating Chasmagnathus granulatus." Journal of Experimental Biology **206**(6): 1017-1023.

Opitz, R., Hartmann, S., Blank, T., Braunbeck, T., Lutz, I. and Kloas, W. (2006). "Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in Xenopus laevis tadpoles." Toxicological Science **90**(2): 337-348.

Opitz, R., Lutz, I., Nguyen, N. H., Scanlan, T. S. and Kloas, W. (2006). "Analysis of thyroid hormone receptor beta A mRNA expression in Xenopus laevis tadpoles as a means to detect agonism and antagonism of thyroid hormone action." Toxicology and Applied Pharmacology **212**(1): 1-13.

Opitz, R., Trubiroha, A., Lorenz, C., Lutz, I., Hartmann, S., Blank, T., Braunbeck, T. and Kloas, W. (2006). "Expression of sodium-iodide symporter mRNA in the thyroid gland of Xenopus laevis tadpoles: developmental expression, effects of antithyroidal compounds, and regulation by TSH." Journal of Endocrinology **190**(1): 157-170.

Oppenheimer, J. H., Dillmann, W. H., Schwartz, H. L. and Towle, H. C. (1979). "Nuclear receptors and thyroid hormone action: a progress report." Fed Proc **38**(8): 2154-2161.

Oppenheimer, J. H. and Schwartz, H. L. (1997). "Molecular basis of thyroid hormone-dependent brain development." Endocrinology Review **18**(4): 462-475.

Ortiz-Santaliestra, M. E. and Sparling, D. W. (2007). "Alteration of larval development and metamorphosis by nitrate and perchlorate in southern leopard frogs (Rana sphenocephala)." Archives in Environmental Contaminant Toxicology **53**(4): 639-646.

Papp, E., Nardai, G., Soti, C. and Csermely, P. (2003). "Molecular chaperones, stress proteins and redox homeostasis." Biofactors **17**(1-4): 249-257.

Parkison, C., Ashizawa, K., McPhie, P., Lin, K. H. and Cheng, S. Y. (1991). "The monomer of pyruvate kinase, subtype M1, is both a kinase and a cytosolic thyroid hormone binding protein." Biochemical and Biophysical Res Communications **179**(1): 668-674.

Paul, B. D., Buchholz, D. R., Fu, L. and Shi, Y. B. (2005). "Tissue- and gene-specific recruitment of steroid receptor coactivator-3 by thyroid hormone receptor during development." Journal of Biology and Chemistry **280**(29): 27165-27172.

Paul, K. B., Hedge, J. M., DeVito, M. J. and Crofton, K. M. "Short-term exposure to triclosan decreases thyroxine in vivo via upregulation of hepatic catabolism in Young Long-Evans rats." Toxicological Science **113**(2): 367-379.

Paul, K. B., Hedge, J. M., DeVito, M. J. and Crofton, K. M. (2010). "Short-term Exposure to Triclosan Decreases Thyroxine In Vivo via Upregulation of Hepatic Catabolism in Young Long-Evans Rats." Toxicological Sciences **113**(2): 367-379.

Perencevich, E. N., Wong, M. T. and Harris, A. D. (2001). "National and regional assessment of the antibacterial soap market: A step toward determining the impact of prevalent antibacterial soaps." American Journal of Infection Control **29**(5): 281-283.

Planchais, S., Glab, N., Trehin, C., Perennes, C., Bureau, J. M., Meijer, L. and Bergounioux, C. (1997). "Roscovitine, a novel cyclin-dependent kinase inhibitor, characterizes restriction point and G2/M transition in tobacco BY-2 cell suspension." Plant Journal **12**(1): 191-202.

Plateroti, M., Gauthier, K., Domon-Dell, C., Freund, J. N., Samarut, J. and Chassande, O. (2001). "Functional interference between thyroid hormone receptor alpha (TRalpha) and natural truncated TRDeltaalpha isoforms in the control of intestine development." Molecular Cell Biology **21**(14): 4761-4772.

Porter, W. P., Jaeger, J. W. and Carlson, I. H. (1999). "Endocrine, immune, and behavioral effects of aldicarb (carbamate), atrazine (triazine) and nitrate (fertilizer) mixtures at groundwater concentrations." Toxicology and Industrial Health **15**(1-2): 133-150.

Porterfield, S. P. and Hendrich, C. E. (1993). "The role of thyroid hormones in prenatal and neonatal neurological development--current perspectives." Endocrinology Review **14**(1): 94-106.

Puzianowska-Kuznicka, M., Damjanovski, S. and Shi, Y. B. (1997). "Both thyroid hormone and 9-cis retinoic acid receptors are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in *Xenopus laevis*." Molecular and Cellular Biology **17**: 4738-4749.

Regard, E., Taurog, A. and Nakashima, T. (1978). "Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing Rana catesbeiana tadpoles and in adult anuran amphibia." Endocrinology **102**: 674-684.

Ritchie, J. W., Shi, Y. B., Hayashi, Y., Baird, F. E., Muchekehu, R. W., Christie, G. R. and Taylor, P. M. (2003). "A role for thyroid hormone transporters in transcriptional regulation by thyroid hormone receptors." Molecular Endocrinology **17**(4): 653-661.

Rollerova, E., Gasparova, Z., Wsolova, L. and Urbancikova, M. (2000). "Interaction of acetochlor with estrogen receptor in the rat uterus. Acetochlor-possible endocrine modulator?" General Physiology and Biophysics **19**: 73-94.

Rouse, J. D., Bishop, C. A. and Struger, J. (1999). "Nitrogen pollution: an assessment of its threat to amphibian survival." Environmental Health Perspectives **107**(10): 799-803.

Sabaliunas, D., Webb, S. F., Hauk, A., Jacob, M. and Eckhoff, W. S. (2003). "Environmental fate of Triclosan in the River Aire Basin, UK." Water Research **37**(13): 3145-3154.

Sampat, P. (2000). "Groundwater shock: the polluting of the world's major freshwater stores." World Watch: 10-22.

Samuels, H. H., Aranda, A., Casanova, J., Copp, R. P., Flug, F., Forman, B. M., Horowitz, Z. D., Janocko, L., Park, H. Y., Pascual, A. and et al. (1988). "Identification of the cis-acting elements and trans-acting factors that mediate cell-specific and thyroid hormone stimulation of growth hormone gene expression." Recent Progress in Hormone Research **44**: 53-114.

Sandborgh-Englund, G., Adolfsson-Erici, M., Odham, G. and Ekstrand, J. (2006). "Pharmacokinetics of triclosan following oral ingestion in humans." Journal of Toxicology and Environmental Health A **69**(20): 1861-1873.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Bhysdael, J., Leutz, A., Beug, H. and Vennstrom, B. (1986). "The c-erbA protein is a high affinity receptor for thyroid hormone." Nature **324**: 635-640.

Sapkota, A., Heidler, J. and Halden, R. U. (2007). "Detection of triclocarban and two co-contaminating chlorocarbanilides in US aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry." Environmental Research **103**(1): 21-29.

Sapkota, A., Heldler, J. and Halden, R. U. (2007). "Detection of triclocarban and two co-contaminating chlorocarbanilides in US aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry." Environmental Research **103**(1): 21-29.

Sato, Y., Buchholz, D. R., Paul, B. D. and Shi, Y. B. (2007). "A role of unliganded thyroid hormone receptor in postembryonic development in Xenopus laevis." Mechanisms of Development **124**(6): 476-488.

Scharpf, L. G., Jr., Hill, I. D. and Maibach, H. I. (1975). "Percutaneous penetration and disposition of triclocarban in man: body showering." Archives in Environmental Health **30**(1): 7-14.

Schlesinger, M. J. (1990). "Heat shock proteins." Journal of Biology and Chemistry **265**(21): 12111-12114.

Schneider, M. J., Davey, J. C. and Galton, V. A. (1993). "Rana catesbeiana tadpole red blood cells express an alpha, but not a beta, c-erbA gene." Endocrinology **133**(6): 2488-2495.

Schonfelder, G., Wittfoht, W., Hopp, H., Talsness, C. E., Paul, M. and Chahoud, I. (2002). "Parent bisphenol A accumulation in the human maternal-fetal-placental unit." Environmental Health Perspectives **110**(11): A703-A707.

Schriks, M., Zvinavashe, E., Furlow, J. D. and Murk, A. J. (2006). "Disruption of thyroid hormone-mediated Xenopus laevis tadpole tail tip regression by hexabromocyclododecane (HBCD) and 2,2',3,3',4,4',5,5',6-nona brominated diphenyl ether (BDE206)." Chemosphere **65**(10): 1904-1908.

Scribner, E. A., Thurman, E. M. and Zimmerman, L. R. (2000). "Analysis of selected herbicide metabolites in surface and ground water of the United States." Science of the Total Environment **248**(2-3): 157-167.

Setchell, K. D. R., Zimmer-Nechemias, L., Cai, J. N. and Heubi, J. E. (1998). "Isoflavone content of infant formulas and the metabolic fate of these phytoestrogens in early life." American Journal of Clinical Nutrition **68**(6): 1453s-1461s.

Sharma, B. and Ahlert, R. C. (1977). "Nitrification and Nitrogen Removal." Water Research **11**(10): 897-925.

Shi, Y.-B. (1996). Thyroid hormone-regulated early and late genes during amphibian metamorphosis. Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells. L. I. Gilbert, J. R. Tata and B. G. Atkinson. San Diego, Academic Press**:** 505-538.

Shi, Y.-B. (2000). Amphibian Metamorphosis: From morphology to molecular biology. New York, Wiley-Liss.

Shi, Y.-B., Liang, V. C., Parkison, C. and Cheng, S. Y. (1994). "Tissue-dependent developmental expression of a cytosolic thyroid hormone protein gene in *Xenopus*: Its role in the regulation of amphibian metamorphosis." FEBS Letters **355**(1): 61-64.

Shi, Y. (2009). "Dual function of thyroid hormone receptors in vertebrate development: the roles of histone-modifying cofactor complexes." Thyroid **19**: 987-999.

Shi, Y. B., Wong, J., Puzianowska-Kuznicka, M. and Stolow, M. A. (1996). "Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors." BioEssays **18**(5): 391-399.

Shibusawa, N., Hollenberg, A. N. and Wondisford, F. E. (2003). "Thyroid hormone receptor DNA binding is required for both positive and negative gene regulation." Journal of Biology and Chemistry **278**(2): 732-738.

Silva, J. E. (2005). "Thyroid hormone and the energetic cost of keeping body temperature." Biosci Rep **25**(3-4): 129-148.

Skirrow, R. C., Veldhoen, N., Domanski, D. and Helbing, C. C. (2008). "Roscovitine inhibits thyroid hormone-induced tail regression of the frog tadpole and reveals a role for cyclin C/Cdk8 in the establishment of the metamorphic gene expression program." Developmental Dynamics **237**(12): 3787-3797.

St Germain, D. L. and Galton, V. A. (1997). "The deiodinase family of selenoproteins." Thyroid **7**(4): 655-668.

St Germain, D. L., Schwartzman, R. A., Croteau, W., Kanamori, A., Wang, Z., Brown, D. D. and Galton, V. A. (1994). "A thyroid hormone-regulated gene in Xenopus laevis encodes a type III iodothyronine 5-deiodinase." Proceedings of the National Academy of Science **91**(16): 7767-7771.

Storey, N. M., Gentile, S., Ullah, H., Russo, A., Muessel, M., Erxleben, C. and Armstrong, D. L. (2006). "Rapid signaling at the plasma membrane by a nuclear receptor for thyroid hormone." Proceedings of the National Academy of Science **103**(13): 5197-5201.

Takase, M. and Iguchi, T. (2007). "Molecular cloning of two isoforms of Xenopus (Silurana) tropicalis estrogen receptor mRNA and their expression during development." Biochim et Biophys Acta **1769**(3): 172-181.

Tanguay, R. M., Wu, Y. and Khandjian, E. W. (1993). "Tissue-specific expression of heat shock proteins of the mouse in the absence of stress." Developmental Genetics **14**(2): 112-118.

Tashjian, A. H., Jr., Bancroft, F. C. and Levine, L. (1970). "Production of both prolactin and growth hormone by clonal strains of rat pituitary tumor cells. Differential effects of hydrocortisone and tissue extracts." Journal of Cell Biology **47**(1): 61-70.

Tata, J. R. (1967). "The formation, distribution and function of ribosomes and microsomal membranes during induced amphibian metamorphosis." Biochemistry Journal **105**(2): 783-801.

Tata, J. R. (1968). "Early metamorphic competence of Xenopus larvae." Developmental Biology **18**(5): 415-440.

Tata, J. R. (1993). "Gene expression during metamorphosis: an ideal model for post-embryonic development." BioEssays **15**(4): 239-248.

Tata, J. R. (2006). "Amphibian metamorphosis as a model for the developmental actions of thyroid hormone." Molecular & Cellular Endocrinology **246**: 10-20.

Tata, J. R. (2007). "A hormone for all seasons." Perspectoves in Biological Medicine **50**(1): 89-103.

Tata, J. R., Kawahara, A. and Baker, B. S. (1991). "Prolactin inhibits both thyroid hormone-induced morphogenesis and cell death in cultured amphibian larval tissues." Developmental Biology **146**(1): 72-80.

Tata, J. R. and Widnell, C. C. (1966). "Ribonucleic acid synthesis during the early action of thyroid hormones." Biochemistry Journal **98**(2): 604-620.

Taylor, A. C. and Kollros, J. J. (1946). "Stages in the normal development of Rana pipiens larvae." Anatomical Record **94**: 7-24.

Theodorakis, N. G., Drujan, D. and De Maio, A. (1999). "Thermotolerant cells show an attenuated expression of Hsp70 after heat shock." Journal of Biological Chemistry **274**(17): 12081-12086.

Theodorakis, N. G. and Morimoto, R. I. (1987). "Posttranscriptional Regulation of Hsp70 Expression in Human-Cells - Effects of Heat-Shock, Inhibition of Protein-Synthesis, and Adenovirus Infection on Translation and Messenger-Rna Stability." Molecular and Cellular Biology **7**(12): 4357-4368.

Thomsen, C., Lundanes, E. and Becher, G. (2001). "A simplified method for determination of tetrabromobisphenol A and polybrominated diphenyl ethers in human plasma and serum." Journal of Separation Science **24**(4): 282-290.

Tierno, P. M. (1999). "Efficacy of triclosan." American Journal of Infection Control **27**(1): 71-72.

Tonon, M. C., Cuet, P., Lamacz, M., Jegou, S., Cote, J., Gouteaux, L., Ling, N., Pelletier, G. and Vaudry, H. (1986). "Comparative effects of corticotropin-releasing factor, arginine vasopressin, and related neuropeptides on the secretion of ACTH and alpha-MSH by frog anterior pituitary cells and neurointermediate lobes in vitro." General and Comparative Endocrinology **61**(3): 438-445.

Toyoda, N., Zavacki, A. M., Maia, A. L., Harney, J. W. and Larsen, P. R. (1995). "A novel retinoid X receptor-independent thyroid hormone response element is present in the human type 1 deiodinase gene." Molecula and Cell Biology **15**(9): 5100-5112.

Turque, N., Palmier, K., Le Mevel, S., Alliot, C. and Demeneix, B. (2005). "A rapid, physiologic protocol for testing transcriptional effects of thyroid-disrupting agents in permetamorphic *Xenopus* tadpoles." Environmental Health Perspectives **113**: 1588-1593.

US, E. P. A. (2006). National recommended water quality criteria. O. o. W. a. S. Technology. Washington, D.C., US Environment Protection Agency.

US, E. P. A. (2009). National drinking water regulations. E. P. A. US.

Utiger, R. D. (1995). "Altered thyroid function in nonthyroidal illness and surgery. To treat or not to treat?" The New England Journal of Medicine **333**(23): 1562-1563.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. and Mazur, M. (2006). "Free radicals, metals and antioxidants in oxidative stress-induced cancer." Chemistry and Biology Interactions **160**(1): 1-40.

Veldhoen, N., Boggs, A., Walzak, K. and Helbing, C. C. (2006). "Exposure to tetrabromobisphenol-A alters TH-associated gene expression and tadpole metamorphosis in the Pacific Tree Frog *Pseudacris regilla*." Aquatic Toxicology **78**: 292-302.

Veldhoen, N., Crump, D., Werry, K. and Helbing, C. (2002). "Distinctive gene profiles occur at key points during natural metamorphosis in the *Xenopus laevis* tadpole tail." Developmental Dynamics **225**: 457-468.

Veldhoen, N. and Helbing, C. C. (2001). "Detection of environmental endocrine-disruptor effects on gene expression in live *Rana catesbeiana* tadpoles using a tail fin biopsy technique." Environmental Toxicology and Chemistry **20**(12): 2704-2708.

Veldhoen, N. and Helbing, C. C. (2005). Monitoring gene expression in *Rana catesbeiana* tadpoles using a tail fin biopsy technique and its application to the detection of environmental endocrine disruptor effects in wildlife species. Techniques in Aquatic Toxicology. Volume 2. G. K. Ostrander. Boca Raton, CRC Press**:** 315-327.

Veldhoen, N., Skirrow, R., Osachoff, H., Wigmore, H., Clapson, D., Gunderson, M., van Aggelen, G. and Helbing, C. (2006). "The bactericidal agent Triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development." Aquatic Toxicology **80**: 217-227.

Veldhoen, N., Skirrow, R. C., Ji, L., Domanski, D., Bonfield, E. R., Bailey, C. M. and Helbing, C. C. (2006). "Use of heterologous cDNA arrays and organ culture in the detection of thyroid hormone-dependent responses in a sentinel frog, *Rana catesbeiana*." Comparative Biochemistry and Physiology D **1**: 187-199.

Veldhoen, N., van Aggelen, G., Vijayan, M. and Helbing, C. (2002). "Acetochlor alters thyroid hormone receptor β gene expression during thyroid hormone-induced metamorphosis of *Rana catesbeiana* without modulating the thyroid axis." Comparative Biochemistry and Physiology D **1**: 187-199.

Wang, D. Q., Xia, X. M., Liu, Y., Oetting, A., Walker, R. L., Zhu, Y. L., Meltzer, P., Cole, P. A., Shi, Y. B. and Yen, P. M. (2009). "Negative Regulation of TSH alpha Target Gene by Thyroid Hormone Involves Histone Acetylation and Corepressor Complex Dissociation." Molecular Endocrinology **23**(5): 600-609.

Waring, R. H. and Harris, R. M. (2005). "Endocrine disrupters: a human risk?" Molecular Cell Endocrinology **244**(1-2): 2-9.

Weinberger, C., Thompson, C., Ong, E., Lebo, R., Gruol, D. and Evans, R. (1986). "The c-erbA gene encodes a thyroid hormone receptor." Nature **324**: 641-646.

White, B. A. and Nicoll, C. S. (1981). Hormonal control of amphibian metamorphosis. Metamorphosis: a problem in developmental biology. L. I. Gilbert and E. Frieden. New York, Plenum Publishing.

Williams, G. R. (2000). "Cloning and characterization of two novel thyroid hormone receptor beta isoforms." Molecular and Cellular Biology **20**(22): 8329-8342.

Williams, G. R., Franklyn, J. A. and Sheppard, M. C. (1991). "Thyroid hormone and glucocorticoid regulation of receptor and target gene mRNAs in pituitary GH3 cells." Molecular Cell Endocrinology **80**(1-3): 127-138.

Wolff, M. S., Teitelbaum, S. L., Windham, G., Pinney, S. M., Britton, J. A., Chelimo, C., Godbold, J., Biro, F., Kushi, L. H., Pfeiffer, C. M. and Calafat, A. M. (2007). "Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls." Environmental Health Perspectives **115**(1): 116-121.

Wong, J. and Shi, Y.-B. (1995). "Coordinated regulation of and transcriptional activation by Xenopus thyroid hormone and retinoid X receptors." Journal of Biological Chemistry **270**: 18479-18483.

Wong, J. and Shi, Y. B. (1995). "Coordinated regulation of and transcriptional activation by Xenopus thyroid hormone and retinoid X receptors." Journal Biological Chemistry **270**(31): 18479-18483.

Wong, J., Shi, Y. B. and Wolffe, A. P. (1995). "A role for nucleosome assembly in both silencing and activation of the Xenopus TR beta A gene by the thyroid hormone receptor." Genes and Development **9**(21): 2696-2711.

Woolfson, J. P. and Heikkila, J. J. (2009). "Examination of cadmium-induced expression of the small heat shock protein gene, hsp30, in Xenopus laevis A6 kidney epithelial cells." Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology **152**(1): 91-99.

Wyngaarden, J. B., Stanbury, J. B. and Rapp, B. (1953). "The effects of iodine, perchlorate, thiocyanate, and nitrate administration upon the iodide concentrating mechanism of the rat thyroid." Endocrinology **52**(5): 568-574.

Wyngaarden, J. B., Wright, B. M. and Ways, P. (1952). "The effect of certain anions upon the accumulation and retention of iodide by the thyroid gland." Endocrinology **50**(5): 537-549.

Xu, Q. and Oldham, R. S. (1997). "Lethal and Sublethal Effects of Nitrogen Fertilizer Ammonium Nitrate onCommon Toad (Bufo bufo) Tadpoles." Archives of Environmental Contamination and Toxicology **32**(3): 298-303.

Yaoita, Y., Shi, Y. B. and Brown, D. D. (1990). "Xenopus laevis alpha and beta thyroid hormone receptors." Proceedings of the National Academy of Science **87**(18): 7090-7094.

Yen, C., Huang, Y., Liao, C., Liao, C., Cheng, W., Chen, W. and Lin, K. (2006). "Mediation of the inhibitory effect of thyroid hormone on proliferation of hepatoma cells by transforming growth factor-beta." Journal of Molecular Endocrinology **36**: 9-21.

Yen, P. M. (2001). "Physiological and molecular basis of thyroid hormone action." Physiological Reviews **81**(3): 1097-1142.

Yen, P. M., Ando, S., Feng, X., Liu, Y., Maruvada, P. and Xia, X. (2006). "Thyroid hormone action at the cellular, genomic and target gene levels." Molecular and Cellular Endocrinology **246**: 121-127.

Ying, G. G., Yu, X. Y. and Kookana, R. S. (2007). "Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modelling." Environmental Pollution **150**(3): 300-305.

Zaki, A., Chaoui, A. A., Talibi, A., Derouiche, A. F., Aboussaouira, T., Zarrouck, K., Chait, A. and Himmi, T. (2004). "Impact of nitrate intake in drinking water on the thyroid gland activity in male rat." Toxicology Letters **147**(1): 27-33.

Zhang, C. Y., Kim, S., Harney, J. W. and Larsen, P. R. (1998). "Further characterization of thyroid hormone response elements in the human type 1 iodothyronine deiodinase gene." Endocrinology **139**(3): 1156-1163.

Zhang, F., Degitz, S. J., Holcombe, G. W., Kosian, P. A., Tietge, J., Veldhoen, N. and Helbing, C. C. (2006). "Evaluation of gene expression endpoints in the context of a *Xenopus laevis* metamorphosis-based bioassay to detect thyroid hormone disruptors." Aquatic Toxicology **76**: 24-36.

Zhang, J. and Lazar, M. A. (2000). "The mechanism of action of thyroid hormones." Annual Review in Physiology **62**: 439-466.

Zhang, L. W., Yu, W. W., Colvin, V. L. and Monteiro-Riviere, N. A. (2008). "Biological interactions of quantum dot nanoparticles in skin and in human epidermal keratinocytes." Toxicology and Applied Pharmacology **228**(2): 200-211.

Zhang, X. W., Li, Y., Wang, Z. L. and Li, P. (2006). "Glucocorticoid receptor subunit gene expression in thyroid gland and adenomas." Acta Oncologica **45**(8): 1073-1078.

Zoeller, R. (2005). "Environmental chemicals as thyroid hormone analogues: New studies indicate that thyroid hormone receptors are targets of industrial chemicals." Molecular and Cellular Endocrinology **242**: 10-15.

Zoeller, R. and Tan, S. (2007). "Implications of research on assays to characterize thyroid toxicants." Critical Review in Toxicol **37**: 195-210.

Zorrilla, L. M., Gibson, E. K., Jeffay, S. C., Croftonogy, K. M., Setzer, W. R., Cooper, R. L. and Stoker, T. E. (2009). "The Effects of Triclosan on Puberty and Thyroid Hormones in Male Wistar Rats." Toxicological Sciences **107**(1): 56-64.

Zraly, Z., Bendova, J., Svecova, D., Faldikova, L., Veznik, Z. and Zajicova, A. (1997). "Effects of oral intake of nitrates on reproductive functions of bulls." Veterinarni Medicina **42**(12): 345-354.

Appendix 1: Abbreviations

°C	Degree Celsius
µ	micro
µl	microlitre
aa	Amino acid
Ace	Acetochlor
ACTH	Adrenocorticotropin releasing hormone
AF-1	Activation function 1 domain
$AgNO_3$	Silver Nitrate
AR	Androgen Receptor
BFR	Brominated flame retardants
bp	Base pair
BTEB	Basic transcription element binding protein
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CBP	CREB-binding protein
CCME	Canadian Council of Ministers of the Environment
Cdk	Cyclin-dependent kinase
cDNA	Complementary deoxyribonucleic acid
C-fin Assay	Cultured Tail Fin Biopsy Assay
CREB	cAMP-response element-binding protein
CRF	Corticotropin releasing factor
CTD	C-terminal domain
CTHBP	Cytoplasmic TH binding proteins
Da	Dalton
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
DIO	Deiodinase
DIOI	Deiodinase I
DIOII	Type II 5‟-deiodinase
DIOIII	Type III 5-deiodinase
DIT	3-5-diiodinated tyrosine
DLS	Dynamic Light Scattering
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
DRIPs	Vitamin D receptor interacting proteins
EDC	Endocrine-disrupting compound

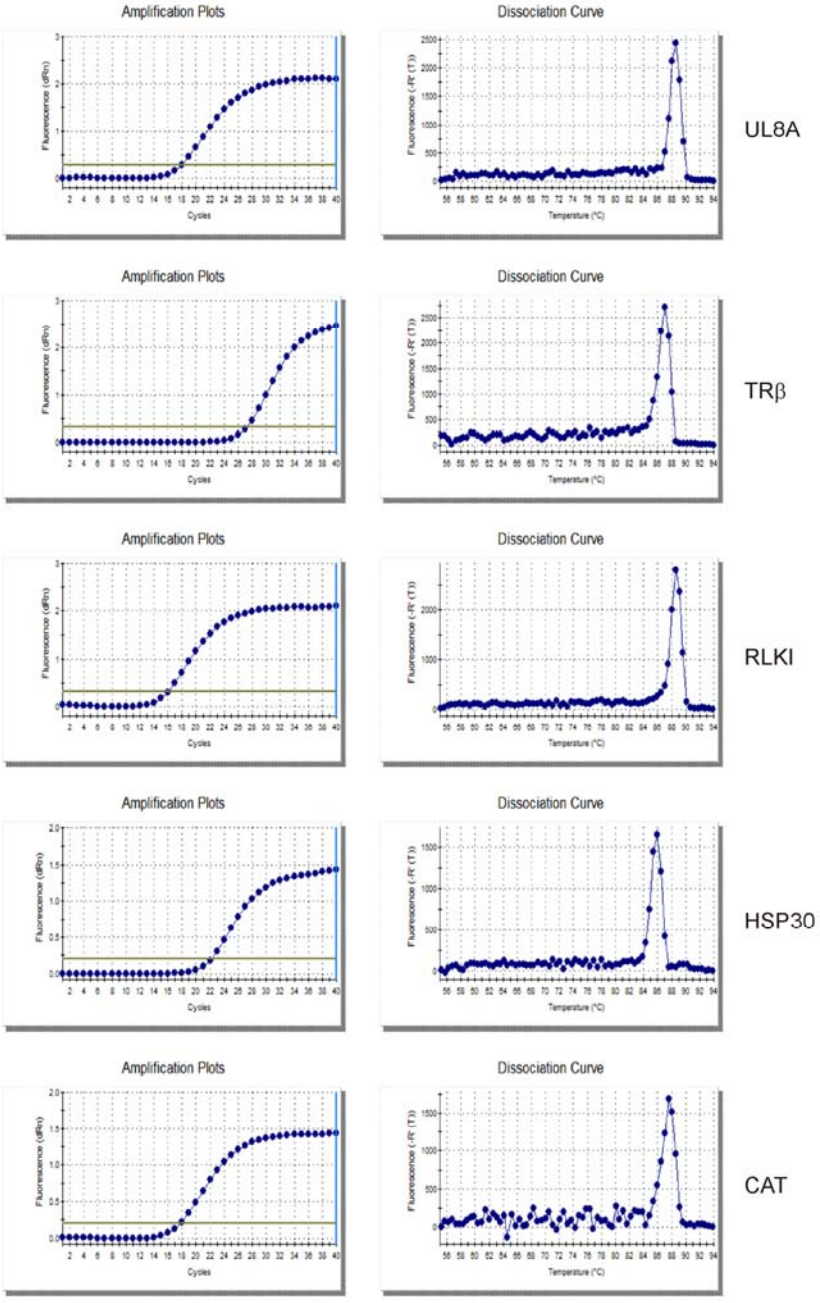
EDTA	Ethylenedinitrilo-tetraacetic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPA	U.S. Environmental Protection Agency
ERE	Estrogen response elements
ER	Endoplasmic reticulum
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
EtOH	Ethanol
GH	Growth hormone
GH3	Growth Hormone Cells
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
h	hour
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
H_2O_2	Hydrogen Peroxide
HPT	Hypothalamus-pituitary-thyroid axis
HRE	Hormone response element
HSP30	Heat Shock Protein 30
HSP70	Heat Shock Protein 70
I^-	Inorganic iodide
ICP	inductively coupled plasma
k	kilo
KCl	Potassium chloride
Kd	Dissociation constant
kDa	kiloDalton
LBD	Ligand binding domain
LB medium	Luria-Bertani medium
LC_{50}	Lethal Concentration 50 (having 50% chance causing death)
m	milli
M	molarity
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
mg	milligram
$MgCl_2$	Magnesium chloride
micron-CdTe	Micron-Cadmium Telluride
micron-Ag	Micron-Silver
min	minute

MIT	3-monoiodinated tyrosine
ml	millitre
mM	millimolar
mRNA	Messenger RNA
mTCS	methyl-Triclosan
mV	millivolts
NaCl	Sodium chloride
Na-Glu	Sodium Glucose
$NaH_2PO_4H_2O$	Sodium phosphate
$NaHCO_3$	Sodium bicarbonate
$Na\text{-}NO_2$	Sodium nitrite
$Na\text{-}NO_3$	Sodium nitrate
NaOH	Sodium hydroxide
N-CoR	Nuclear receptor co-repressor
NF	Niewkoop and Faber
NIS	sodium-iodine symporter pump
nm	nanometre
NP	Nanoparticle
NR	Nuclear receptor
nTRE	Negative TRE
OECD	Organisation for Economic Co-operation and Development
ORF	Open reading frame
P/CAF	p300/CBP-associated factor
p160/SRCs	Steroid receptor co-activators
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PESC	Environment Canada's Pacific Environmental Science Centre
PI3K	Phosphatidylinositol 3-kinase
PHAH	Polyhalogenated aromatic hydrocarbon
PKC	Protein kinase C
ppb	parts per billion
ppm	parts per million
PRL	Prolactin
pTRE	Positive TRE
QD	Quantum Dot
QPCR	Quantitative real-time polymerase chain reaction
RAR	Retinoic acid receptor
RBCs	Red blood cells
RLKI	*Rana catesbeiana* larval type I keratin

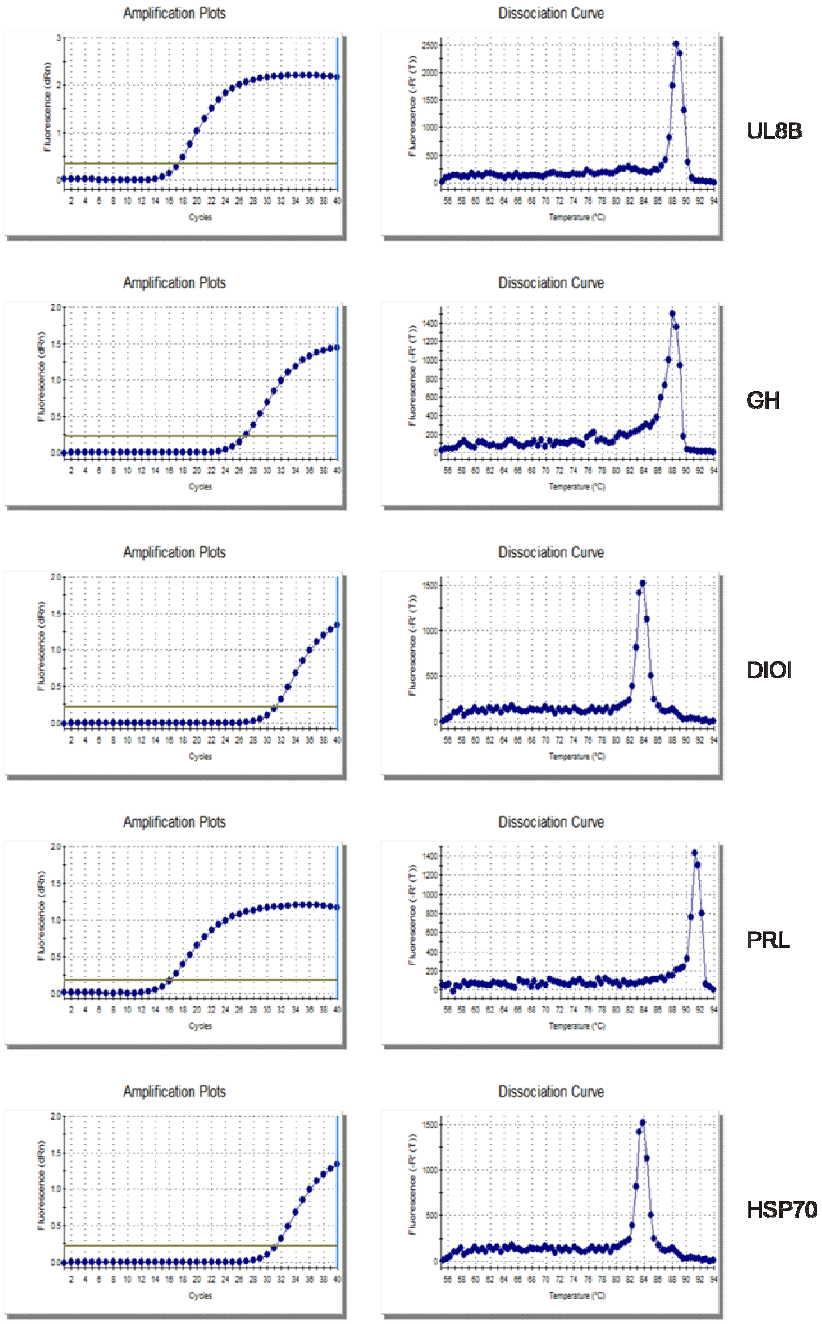
RNA	Ribonucleic acid
RNA Pol II	RNA Polymerase II
rT$_3$	Reverse T_3
RT-PCR	Reverse transcription-PCR
RXR	9-*cis*-retinoic acid receptor
s	second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH3	Src homology 3 domain
SMRT	Silencing mediators of retinoid and thyroid hormone receptors
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
T_2	Diiodothyronine
T_3	3,5,3‟-Triiodothyronine
T_4	3,5,3‟,5‟-tetraiodo-L-thyronine; Thyroxine
Taq	*Thermus aquaticus*
TBBPA	Tetrabromobisphenol A
TBG	Thyronine binding globulin
TBP	TATA box binding protein
TCC	Triclocarban
TCS	Triclosan
TEM	transmission electron microscopy
Tg	Thyroglobulin
TH	Thyroid hormone
TH/bZIP	TH-Responsive basic leucine zipper transcription factor
TK	Taylor and Kollros
TPO	thyroid peroxidase
TRAP	Thyroid hormone receptor associated protein
TRE	Thryoid hormone response element
TRH	Thyrotropin releasing hormone
TR	Thyroid hormone receptor
TRβ	Thyroid hormone receptor beta
TRα	Thyroid hormone receptor alpha
TSH	Thyroid stimulating hormone
TTR	Transthyretin

Appendix 2: Primer Information

Rana catesbeiana Primer Amplification Plots and Dissociation Curves



Rattus norvegicus Primer Amplification Plots and Dissociation Curves



Species	Primer	dCt Slope
Rana catesbeiana	TRβ	0.01*
Rana catesbeiana	RLKI	0.08*
Rana catesbeiana	HSP30	0.06*
Rana catesbeiana	CAT	0.04*
Rattus norvegicus	GH	0.001**
Rattus norvegicus	DIOI	0.03**
Rattus norvegicus	PRL	0.04**
Rattus norvegicus	HSP70	0.09**

*As compared to UL8A with a primer efficiency of 113%
**As compared to UL8B with a primer efficiency of 96%