Thyroid Hormone Disrupting Effects of Municipal Wastewater

by

Pola Wojnarowicz
B.Sc., University of Saskatchewan, 2009

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in the Department of Biochemistry and Microbiology

Supervisory Committee

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Abstract

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Current municipal wastewater treatment plants (MWWTP) technologies are insufficiently removing emerging contaminants of concern. These emerging contaminants are an issue as many are known endocrine disrupting compounds (EDCs). EDCs are contaminants that can have severe and irreversible impacts on highly conserved endocrine systems that are critical during developmental periods in vertebrates as well as during adult life. Many EDCs have non-monotonic dose-response curves yet they are not often tested at low, environmentally relevant concentrations. EDC research to date has focused heavily on xenoestrogenic compounds whereas thyroid hormone (TH) disruption has been largely overlooked.

TH is conserved in all vertebrates and plays crucial roles in neural development, basal metabolism, and thermoregulation. TH is comprised of thyroxine (T_4), often known as the transport form of TH, and triiodothyronine (T_3), the more bioactive form of TH. A TH spike occurs in the perinatal period of humans, and when disrupted, this spike can cause severe developmental defects. An analogous, but perhaps more overt, TH spike occurs in amphibians. TH is the sole hormone that drives amphibian metamorphosis, thus providing an excellent model for TH action. Our lab has previously developed the cultured tailfin (C-fin) assay, which uses biopsies from premetamorphic *Rana catesbeiana* tadpole tailfins cultured in the presence of an exogenous chemical of concern to assess perturbations to TH- and stress-responsive gene transcript levels by QPCR.

This thesis uses the C-fin assay to assess the efficacy of removal of biological TH- and stress-altering activity in conventional municipal wastewater treatment systems. We first assess the successive levels of a full-scale conventional activated sludge (CAS) MWWTP in its ability to reduce perturbations of mRNA transcript levels of the critical TH

receptors alpha (*thra*) and beta (*thrb*), and stress responsive gene transcripts superoxide dismutase (*sod*), catalase (*cat*) and heat shock protein 30 (*hsp30*). Secondary treatment of wastewater effluents removes cellular stress perturbations when compared to influents, but *thr* disruptions remain after conventional secondary wastewater treatment. We then assess three pilot-sized conventional secondary MWWTP configurations run at two operational conditions. The C-fin assay results suggest that the current understanding of operational conditions and the efficiency of complex MWWTP configurations is not clear-cut when assessed by biological endpoints such as the transcript abundance perturbations in the C-fin assay.

Finally, the C-fin assay is used to investigate transcript profiles of genes of interest when the tissues are treated with the endogenous hormones T_3, T_4, and estradiol (E_2). Our results indicate that T_4 acts as more than solely a T_3-prohormone and that gene expression levels in response to the two different forms of TH can be T_3 or T_4 specific. E_2 effects, although implicated in altering TH-mediated responses in other contexts, do not affect TH-responsive gene transcripts in the C-fin. The data presented use the novel C-fin assay to challenge and advance the currently accepted views of TH-action, as well as develop necessary yet practical biological knowledge for management of emerging contaminant release from MWWTPs.

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Abbreviations

Use of capitalization and italics for gene transcripts/proteins follows the example below as derived from http://www.xenbase.org/gene/static/geneNomenclature.jsp and http://www.informatics.jax.org/mgihome/nomen/gene.shtml.

Species	Gene Transcript	Protein
Mammal	*Thra*	Thra
Amphibian	*thra*	Thra

Acth	Corticotropin
AMA	Amphibian metamorphosis assay
BNR	Biological nutrient removal
CAS	Conventional activated sludge
Cat	Catalase
$cBOD_5$	Carbonaceous biochemical oxygen demand
CCME	Canadian Council of Ministers of the Environment
Cdk8	Cyclin dependent kinase 8
cDNA	Reverse transcribed RNA, complementary DNA
C-fin	Cultured tailfin assay
CNS	Central nervous system
Co-A	Coactivator complex
COD	Chemical oxygen demand
Co-R	Corepressor complex
Crf	Corticotropin releasing factor
Cs	Corticosteroids
CTHBP	Cytoplasmic thyroid hormone binding protein
DES	Diethylstilbestrol
Dio1	Deiodinase 1
Dio2	Deiodinase 2
Dio3	Deiodinase 3
DO	Dissolved oxygen
DR4	Direct repeat of a consensus sequence 4 nucleotides apart
E	Estrogen
E_2	17β-estradiol
EC	Emerging contaminant
EDC	Endocrine disrupting compounds
EE2	17α-ethynylestradiol
Eef1a	Eukaryotic translation elongation factor 1-alpha
ESI	Electrospray ionization
Esr	Estrogen receptor, either isoform

Esra	Estrogen receptor alpha
GMF	Gemfibrozil
Gn	Gonadotropin
Gnrh	Gonadotropin releasing hormone
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-thyroid
HRT	Hydraulic retention time
HSP	Heat shock protein
Hsp30	Heat shock protein 30
IBP	Ibuprofen
Klf9	krüppel-like factor 9
LAT	L-amino acid permease
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MMP2	Matrix metalloproteinase 2
MRM	Multiple reactions monitoring
mRNA	messenger RNA
MS	Mass spectrometry
MWWTP	Municipal wastewater treatment plant
NAS	Nitrifying activated sludge
NCoR	Nuclear receptor corepressor
NIS	Sodium/iodide symporter
NOAEL	No observed adverse effect level
Nr3c1	Glucocorticoid receptor
OATC	Organic anion transporter
OECD	Organization for Economic Cooperation and Economic Development
PKC	Protein kinase C
PLC	Phospholipase C
PPCP	Pharmaceuticals and personal care products
QPCR	Quantitative polymerase chain reaction
Rlk1	*Rana* larval keratin I

ROS	Reactive oxygen species
Rpl8	Ribosomal protein L8
Rps10	Ribosomal protein S10
R^2	Coefficient of determination
rT_3	Reverse triiodothyronine
Rxr	Retinoid X receptor
S/N	Signal-to-noise ratio
SMRT	Silencing mediator for RAR and TR
Sod	Superoxide dismutase
SPE	Solid phase extraction
SRC	Steroid receptor coactivator
SRT	Sludge retention time
T	Testosterone
T_2	3,5-Diiodothyronine
T_3	3,5,3'-Triiodothyronine
T_4	L-Thyroxine
TAN	Total ammonia nitrogen
TBG	Thyroxine binding globulin
TCC	Triclocarban
TCS	Triclosan
Tg	Thyroglobulin
TH	Thyroid hormone
THBP	Thyroid hormone binding protein
TH-EDC	Thyroid hormone endocrine disrupting compound
Thibz	Thyroid hormone basic leucine zipper transcription factor
Thr	Thyroid hormone receptor, either isoform
Thra	Thyroid hormone receptor alpha
Thrb	Thyroid hormone receptor beta
Thrb1	Thyroid hormone receptor beta – 1
TK	Taylor and Kollros developmental stage
TKN	Total kjeldahl nitrogen
TP	Total phosphorous
TPO	Thyroperoxidase
TRE	Thyroid hormone response element
Trh	Thyrotropin releasing hormone
Tsh	Thyroid stimulating hormone/Thyrotropin
TSHα	Thyroid stimulating hormone alpha
TSS	Total suspended solids
TTR	Transthyretin
US EPA	United States Environmental Protection Agency

VSS	Volatile suspended solids
WTC	Wastewater Technology Centre
Y2H	Yeast two-hybrid assay

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1 Introduction

1.1 Municipal Wastewater Treatment Overview

Municipal wastewater treatment plants (MWWTPs) are a major point source of contaminants to aquatic ecosystems. Although current MWWTP technologies effectively remove conventional contaminants, such as pathogens and high nutrient loads (Ternes et al., 2004), a group of chemicals known as emerging contaminants (ECs) or microcontaminants (found at μg/L concentrations in effluents) are recalcitrant to traditional MWWTP systems (Oulton et al., 2010). ECs are made up of a wide variety of chemical compounds, which most often include pharmaceuticals and personal care products (PPCPs), plasticizers, flame-retardants, and surfactants (Ratola et al., 2012).

Effective removal of ECs from MWWTPs largely depends on three factors: 1) the type and concentration of target EC for removal, 2) the MWWTP process, and 3) the operational conditions of the MWWTP. As an overabundance of ECs exists in consumer goods and novel, as-of-yet unregulated ECs are regularly being added to new products, this thesis focuses on MMWTP processes and operational conditions as potential practical points of EC removal management.

MWWTP processes remove ECs in three ways: biodegradation of ECs, removal *via* physical means, and removal *via* chemical oxidation. Although physical removal and chemical oxidation of ECs have their benefits (i.e. physical removal does not produce metabolites and chemical oxidation has high efficiency rates), these two MWWTP processes are considered advanced treatment systems and engineered advanced MWWTP systems can be cost prohibitive (Oulton et al., 2010). Biodegradation is a conventional MWWTP process and is the most cost effective method of EC treatment in MWWTPs

(Liu et al., 2009b; Oulton et al., 2010). Activated sludge bioreactors are the most widely used method of biodegradation in MWWTPs worldwide (Liu et al., 2009b), and are the major focus of my research.

Most full-scale, conventional MWWTP processes are comprised of multiple levels of treatment (or multiple steps along a treatment train). Raw wastewater from household drains such as sinks, showers, and toilets typically flows first through preliminary treatment such as raw wastewater screening and grit removal. A primary clarifier (or primary settling phase) then follows, which separates out heavy solids and oils. Some physical EC removal occurs during settling processes such as primary treatment as many ECs are lipophilic and adsorb to sludge particles ("sludge" being the concentrated settled portions of bioreactors including microorganisms) or are removed during fat separation (Carballa et al., 2004). Secondary treatment however is the major unit responsible for EC removal along conventional MWWTP treatment trains (Carballa et al., 2004; Oulton et al., 2010; Ternes et al., 2004). Conventional secondary treatment is typically composed of biological degradation of wastewater contaminants by microorganisms in a bioreactor and a subsequent secondary clarifier to separate sludge from the effluent water stream. In some higher-level treatment systems, where fresh water supplies are low or effluent receiving environments are particularly sensitive, tertiary treatment is also employed. Tertiary treatment often employs chemical oxidation or passive forms of wastewater treatment such as lagoons and wetlands which remove ECs with high efficiency, but the high costs and physical space constraints for such tertiary treatments limit most MWWTPs to secondary treatment prior to effluent release (Oulton et al., 2010).

As mentioned previously, operational conditions of treatment methods also play a major role in determining EC removal efficiency during MWWTP processes. Temperature, hydraulic retention time (HRT), and sludge retention time (SRT) of MWWTPs are important operational conditions implicated in altering EC removal efficiencies in conventional MWWTPs.

Temperature can alter biodegradation of ECs as it can directly affect bacterial growth and enzymatic reaction rates. Studies of seasonal variations of removal efficiencies of ECs in full-scale MWWTPs, when examined with conventional chemical endpoints, often result in lower concentrations of wastewater ECs in summer as compared to winter. Vieno *et al.* (2005) found a 25% reduction of removal efficiency of 5 ECs in winter as compared to summer in a MWWTP in Finland. Sui *et al*. (2011) studied 12 ECs regularly consumed in two MWWTPs in China and found that summer EC effluent concentrations were lower than winter concentrations likely due to higher biodegradation efficiencies in warmer months. Similar chemical fates have been reported in Germany (Zuehlke et al., 2006) and California (Yu et al., 2013) suggesting higher temperatures of bioreactors result in higher EC removal efficiencies.

HRT is the length of time that a compound in wastewater remains in the bioreactor (Kim et al., 2005) and may also play a role in the variability of MWWTP efficacy. HRT is a factor of the volume of the bioreactor tank and the flow rate of wastewater (Miège et al., 2009). As a larger bioreactor necessitates a higher biomass, it is difficult to distinguish between effects of HRT on EC removal and differences in biomass in the bioreactor (Kim et al., 2005; Vieno et al., 2007). Studies of variation in HRT, although

linked to EC removal efficiency, have not been investigated as extensively as the relationship between EC removal and SRT.

In order to maintain microbial populations in bioreactors, part of the sludge is regularly disposed of while part is recycled to inoculate the bioreactor for continued biodegradation. SRT is the mean residence time of bacteria in the bioreactor of MWWTPs. SRT is related to the growth rate of bacteria since only microorganisms which can reproduce within the SRT will enrich the bioreactor (Clara et al., 2005a). Mechanisms of enhanced biodegradation of ECs at higher SRTs therefore may be attributed to the development of slow growing bacteria and resultant diversification of bacterial populations or possibly to enzyme diversification of bacteria (Ternes et al., 2004). In a landmark study, Clara *et al.* (2005a) found strong correlations between SRT and removal efficiency of bisphenol A, three natural estrogens, ibuprofen, and a popular cholesterol medication (bezafibrate), concluding that a SRT of at least 10 days resulted in ~90% removal efficiencies. However, removal efficiencies of the artificial estrogen 17α-ethynylestradiol (EE2), the non-steroidal anti-inflammatory diclofenac, and the anti-epileptic carbamazepine were not significantly affected by longer SRTs. Many studies of individual ECs in lab-scale bioreactors have reflected the SRT/removal efficiency correlation seen by Clara *et al.* (2005a) (Clara et al., 2005b; Kim et al., 2005; Oppenheimer et al., 2007). However, in a survey of full-scale Ontario MWWTPs, Servos *et al.* (2005) found no effect of SRT on removal of two natural estrogens although the authors attributed the lack of correlation to a limited sample number of plants with a wide variety of treatment processes.

These studies have brought to light the complexity of EC removal in MWWTPs, highlighting that not all ECs are comparably biodegradable and that studies of individual contaminant removal fates, although informative, are not the complete picture for improved MWWTP efficiency. The inherent variability in municipal wastewater influents in addition to the perpetual novel production of chemicals in consumer products remain as major hurdles for municipalities aiming to control EC release. In Canada, upgrades to MWWTPs in recent years have improved effluent qualities (Holeton et al., 2011) but a substantial challenge lies ahead as, under the *Canada-wide Strategy for the Management of Municipal Wastewater Effluent* (CCME, 2009) all MWWTPs must meet effluent standards at a secondary treated level or equivalent by 2030. In order to meet these effluent standards, significant infrastructure and financial investment are necessary for some municipalities.

1.2 Endocrine Disrupting Compounds

The major concern about ECs in MWWTP effluents and receiving environments is that many ECs are known endocrine disrupting compounds (EDCs). EDCs, as defined by the Canadian Environmental Protection Act (1999), are exogenous compounds that interfere with "synthesis, secretion, transport, binding, action or elimination of endogenous hormones". Contaminants from extremely diverse classifications such as industrial surfactants, pesticides, pharmaceuticals, and plastics can act as EDCs and prediction of EDC activity in novel compounds based on structure has proven challenging, as EDCs do not necessarily share structural similarities (Diamanti-Kandarakis et al., 2009).

There are several critical characteristics of EDCs, however, that accentuate the unique gravity of endocrine disruption: firstly, EDCs often produce non-traditional dose-

response curves. Classic toxicological models are based on the premise of a monotonic dose-response curve: along a range of doses, the slope of the dose-response curve does not change between a positive and negative value (Vandenberg et al., 2012). That is, for example, a higher dose of a compound will always cause a stronger response in a given endpoint than a lower dose would, or *vice versa*. Risk management of environmental toxicants by governing bodies such as the United States Environmental Protection Agency (US EPA) and Environment Canada uses this basic premise to set guidelines of exposure. In defining reference doses, doses which can be considered safe, regulators empirically test for the no observed adverse effect level (NOAEL) of exposure and use safety factors (usually 10 or 100) to extrapolate down to a safe reference dose.

Numerous EDCs however, do not exhibit monotonic dose-response curves (Vandenberg et al., 2012). At low, environmentally relevant concentrations, EDCs can cause effects that are not predicted by higher dose exposures. The assumption that chemical exposure can be considered safe below a certain threshold has also been challenged in circumstances where exogenous chemicals share common mechanisms with endogenous compounds and the xenobiotic threshold is already exceeded by the endogenous chemical (Vandenberg et al., 2012). This was demonstrated by Sheehan *et al*. (1999) with estradiol-induced sex reversal in turtles with temperature-dependent sex determination. Turtle eggs were incubated at a temperature to produce a majority of males and treated with a range of exogenous 17β-estradiol (E_2) concentrations. The lowest E_2 treatment (0.4 ng E_2/egg), although significantly lower than endogenous E_2 concentrations (1.7 ng E_2/egg), caused a 14% sex reversal in the population suggesting that effects thresholds, if already exceeded by an endogenous hormone, can be easily

surpassed by the presence of additional very low concentrations of exogenous chemicals to cause lasting effects.

The second critical aspect of EDC toxicology is that the timing of EDC exposure during specific developmental windows can cause serious irreversible effects. Although the endocrine system is important in homeostasis and response to changing environmental factors in fully developed organisms, exposure to concentrations of EDCs that would not cause effects in adults can have serious effects on the embryo, fetus, or juvenile if a hormone signaling pathway is important in establishment and/or execution of critical developmental programs during early life stages. Disruption by exogenous chemicals, such as xenoestrogens, in humans has defined classical cases of developmental exposure to EDCs, which have caused both immediate and latent effects. The synthetic estrogen agonist diethylstilbestrol (DES) left pregnant mothers relatively unharmed while *in utero* exposure caused increased sexual organ malformations such as cryptorchidism in males and incidences of vaginal adenocarcinomas in so called "DES-sons" and "DES-daughters" (Herbst et al., 1971; Vandenberg et al., 2008; Virtanen and Adamsson, 2012). Exposure to synthetic estrogens such as the commonly used oral contraceptive EE2 at environmentally-relevant concentrations have also caused feminization of wildlife during critical sexual developmental periods (Hogan et al., 2008; Tompsett et al., 2012; Tompsett et al., 2013). EE2 was ultimately linked to not only intersex males and altered oogenesis in female fathead minnow (*Pimephales promelas*), but also to the near population extinction of the species due to feminization during critical mating periods in a landmark study conducted in the Experimental Lakes Area of north-western Ontario (Kidd et al., 2007).

The final crucial feature of EDC toxicological research is that currently, a disconnect exists between single-contaminant EDC research and the reality of exposure scenarios as mixtures of EDCs are now essentially ubiquitous. Whether or not contaminant mixtures can be treated as having additive, synergistic, or less than additive effects is a major concern that has seemingly endless permutations based on the amount of compounds suspected to be EDCs, the potential variations of EDC combinations, and the ratios at which the compound mixtures could be characterized. Although in compounds with similar modes of action, the United States Environmental Protection Agency (US EPA) has adopted an additive toxic equivalency method of risk assessment, additive effects are not always the case (Kortenkamp, 2007). Synergistic and less-than additive effects have been described for both estrogenic compounds (Rajapakse et al., 2004) and mixtures of other EDCs such as thyroid hormone (TH) active compounds (Crofton et al., 2005). Additionally, there is a lack of understanding of how compounds of different EDC classes, affecting different hormonal systems, may function together (Kortenkamp, 2007). Much of the current knowledge of EDCs is heavily weighted toward xenoestrogens but other essential hormones, such as TH, have in recent years gained much needed attention. Although many studies examining EDCs and ECs in wastewater effluents regularly characterize estrogenic compound concentrations, only one such study thus far has examined TH in effluents and that study has found significant TH levels in wastewaters (Svanfelt et al., 2010). TH disruption has similarly vital implications for vertebrates as xenoestrogens, only TH-EDCs have thus far been severely understudied.

1.3 Thyroid Hormone

TH acts on nearly every cell in the body and controls the bulk of physiological processes. TH is able to induce both anabolic and catabolic pathways to maintain homeostasis as it plays a critical role in regulating basal metabolism, energy balance, and thermogenesis (Cordeiro et al., 2013). At the cellular level, TH also plays a critical role in cell proliferation, differentiation, and apoptosis (Sirakov et al., 2013). TH causes crucial pleiotropic actions in programming differentiation throughout tissue homeostasis as well as during fetal development (Pascual and Aranda, 2013). During gestation in mammals, TH contributes to the regulation of genes involved in neuronal cell differentiation and myelination. TH action during the first weeks of gestation is solely dependent on maternal TH, as the fetal thyroid does not begin to secrete TH until about 16 weeks of development. TH levels then reach a peak around birth, and remain elevated for several months after birth (Patel et al., 2011) before decreasing to a basal level. Disruption in TH action during the perinatal period of humans results in severe irreversible developmental effects such as cretinism (Xue-Yi et al., 1994).

TH is conserved across all vertebrates and several TH-mediated mechanisms of development in humans are paralleled in other species. During fetal development, TH induces skin keratinization, urea cycle enzymes, and the switching from fetal to adult type hemoglobin (Grimaldi et al., 2013). All of these TH-mediated changes are paralleled in the process of amphibian metamorphosis.

1.4 TH-mediated Amphibian Metamorphosis

Investigations into the TH-mediated mechanisms of development in mammals are hampered by the difficulty of studying a uterus-enclosed system, which partially depends on maternal TH. Amphibian metamorphosis presents an excellent model of TH action, as

TH is the sole hormone that is necessary to drive metamorphosis (Figure 1.1). As opposed to mammalian systems, the premetamorphic tadpole is an independent living organism that is functionally athyroid (has no endogenous circulating TH). As amphibian TH-plasma levels increase in the tadpole, metamorphic processes begin to occur; this time period is known as the prometamorphic phase of development. At the peak of TH levels reached during metamorphic climax, most amphibians undergo significant biochemical and physiological change in preparation for the move from an aquatic to terrestrial habitat (Shi, 2000). This is paralleled in mammalian development as a perinatal TH-peak coincides with similar biochemical and physiological changes in preparation for the transition from an amniotic to terrestrial habitat. Although TH-mediated metamorphosis occurs naturally in tadpole development, the process can also be manipulated to study TH mechanisms of action: premetamorphic tadpoles, although functionally athyroid, are competent to respond to exogenous TH, which induces a precocious metamorphosis. In addition to this, all TH-responsive organs respond to TH stimulation independently; that is, *ex vivo* TH-treatment of tadpole organs in culture causes organ autonomous metamorphic responses.

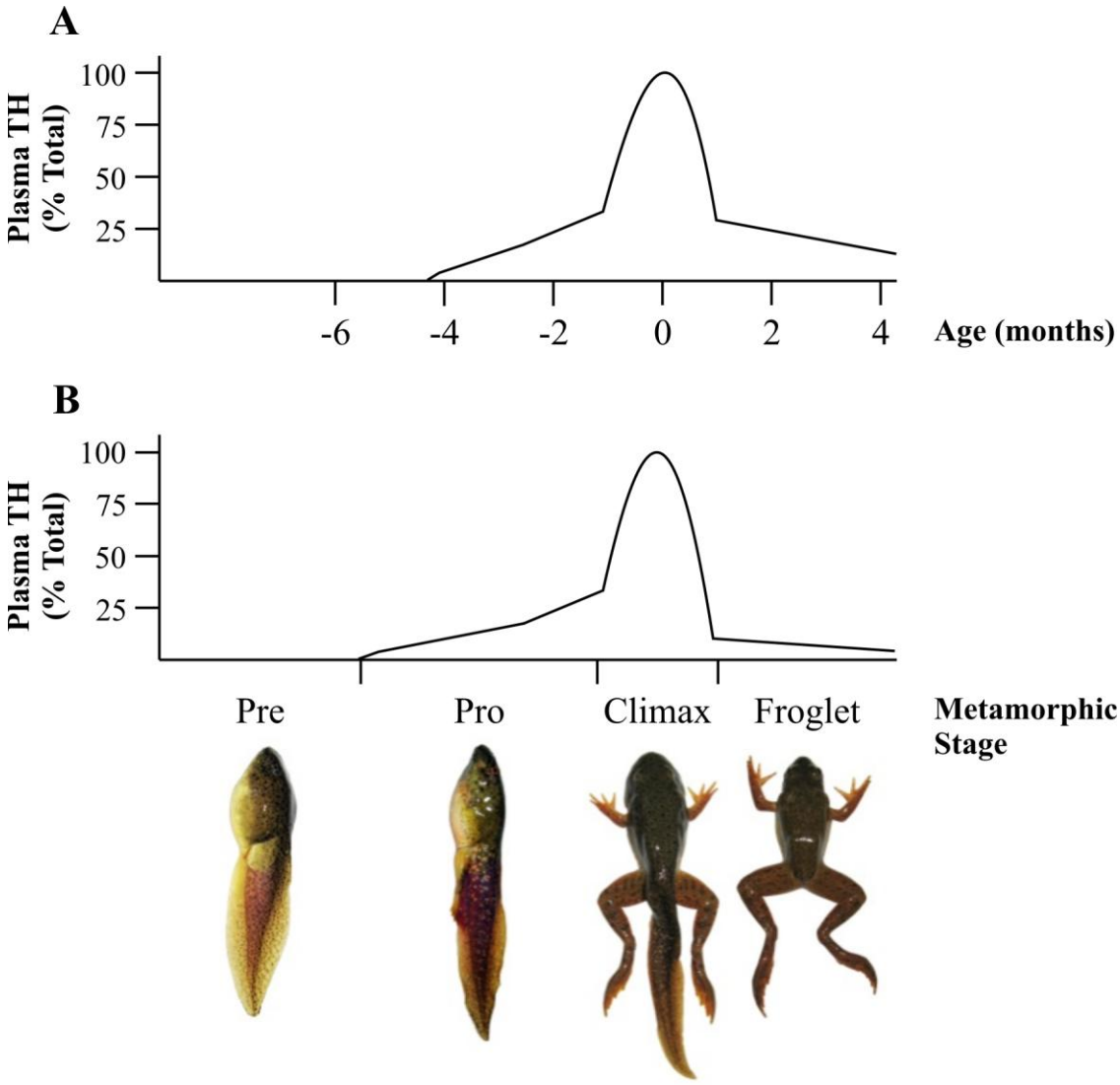

Figure 1.1 Plasma TH levels during A) the perinatal period of humans and B) metamorphosis of amphibians. *Rana catesbeiana* tadpoles are shown during premetamorphosis, prometamorphosis, metamorphic climax, and froglet stages. Adapted from (Leloup and Buscaglia, 1977; Tata, 1993).

TH causes pleiotropic effects that act on practically every tissue in the body during the process of amphibian metamorphosis. TH causes resorption and apoptosis of the tail and gills, *de novo* organogenesis of front and hind limbs, and reprogramming of organs such as the brain and liver. The metamorphic process involves seemingly disparate

mechanisms such as differentiation and apoptosis of TH-responsive tissues yet the primary regulator of these contrasting cellular fates is TH. Although the predominant mechanism of TH action is *via* nuclear TH receptors (Thrs), which act to alter TH-responsive gene transcription, the nuances of disparate TH-mediated effects during amphibian metamorphosis are still relatively unknown.

1.4.1 TH Synthesis, Regulation, and Metabolism

There are multiple levels at which TH action may be regulated, and thus, multiple levels in which TH action may be perturbed. Primarily, TH release is controlled by the neuroendocrine system and the hypothalamic-pituitary (HPT) axis in both mammals and amphibians (Figure 1.2). Environmental stimulants act on sensory systems to transmit information to the hypothalamus, which releases thyrotropin releasing hormone (Trh) in mammals and corticotropin releasing factor (Crf) in amphibians (Denver, 2013). Crf or Trh stimulates thyrotropes in the anterior pituitary to release thyrotropin or thyroid stimulating hormone (Tsh). Thyrotropin then stimulates the thyroid gland to release TH. Circulating TH can then act *via* feedback loops on the anterior pituitary and the hypothalamus to fine-tune circulating TH levels.

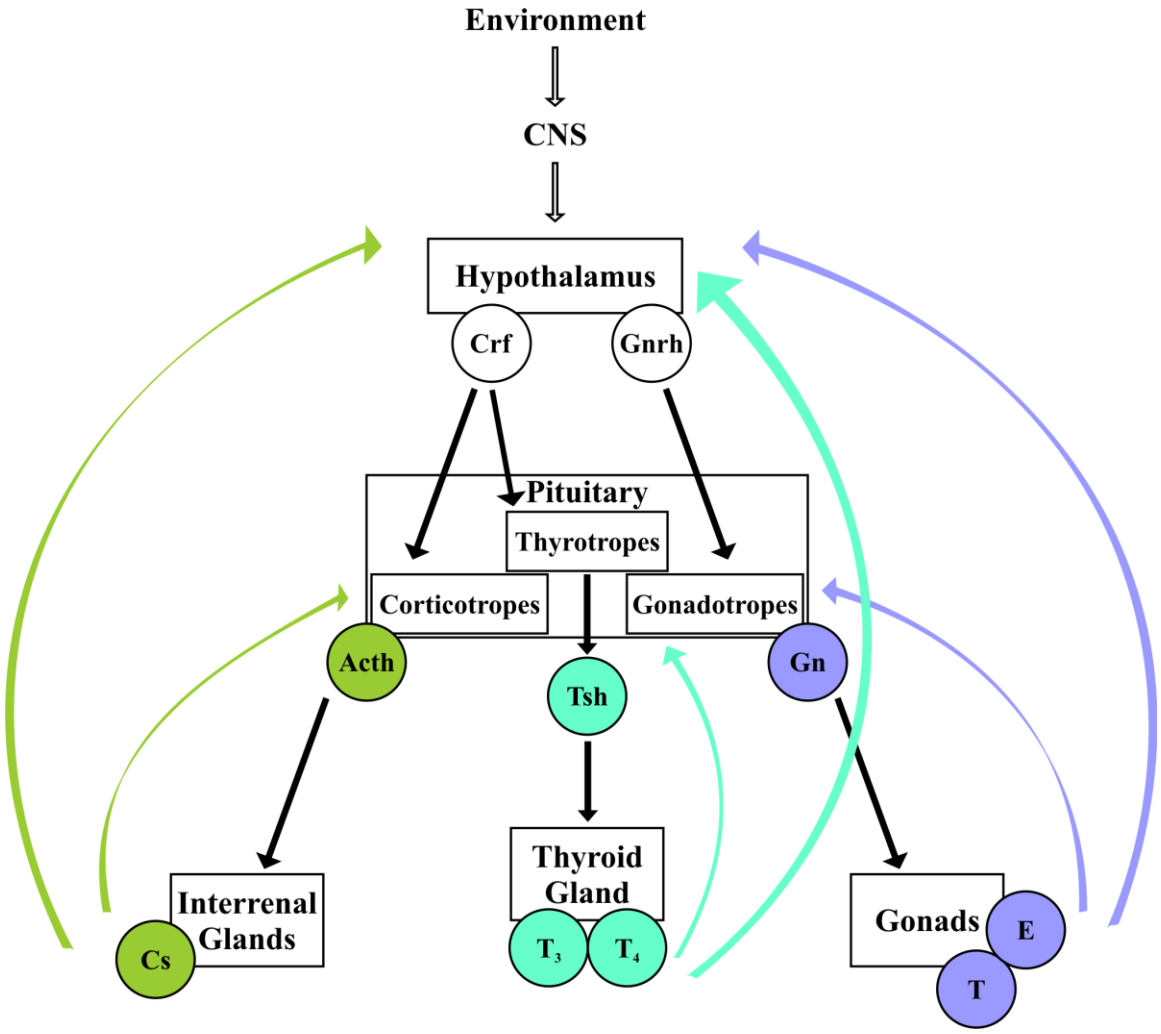

Figure 1.2 Schematic of hypothalamic-pituitary-adrenal (HPA), –thyroid (HPT), and -gonadal (HPG) axes in amphibians. Green: HPA, Blue: HPT, Purple: HPG. Solid black arrows indicate stimulation and coloured arrows indicate feedback. CNS: central nervous systems, Crf: corticotropin releasing factor, Gnrh: gonadotropin releasing hormone, Acth: corticotropin, Cs: corticosteroids, Tsh: thyrotropin, T_3: triiodothyronine, T_4: thyroxine, Gn: gonadotropin, T: testosterone, E: estrogen. Adapted from (Denver, 2013; Vadakkadath and Atwood, 2005).

The HPT axis shares neuroendocrine components with both the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis. In the amphibian HPA axis, Crf can also stimulate corticotropes in the pituitary to release corticotropin (Acth), which stimulates adrenal cortical cells to release corticosteroids, the

primary stress hormones in vertebrates (Denver, 2013). In the HPG axis of mammals and amphibians, the hypothalamus releases gonadotropin releasing hormone (Gnrh) to stimulate the pituitary to produce gonadotropins, which then act on the gonads to produce estrogens (such as E_2) and testosterone (Urbatzka et al., 2010).

At the level of the thyroid gland, TH action can be controlled and altered by multiple steps during both mammalian and amphibian synthesis and secretion of TH (Figure 1.3). The thyroid gland is made up of thyroid follicles, the walls of which are follicular cells or thyrocytes. Thyrocytes take up iodide from the blood *via* a sodium/iodide symporter (NIS) pump. Iodide is then oxidized to iodine as it moves across the thyrocyte to the lumen of the thyroid follicle and is transferred by thyroperoxidase (TPO) to tyrosine moieties of the concurrently secreted thyroglobulin. Monoiodo- and Diiodotyrosines are then covalently coupled to create 3,5,3'-triiodothyronine (T_3) and L-thyroxine (T_4), the two major forms of TH. T_3 and T_4 then re-enter follicular cells along with thyroglobulin, the thyroglobulin is degraded by lysozomes, and the TH is secreted into the blood, predominantly in the form of T_4 (Diamanti-Kandarakis et al., 2009).

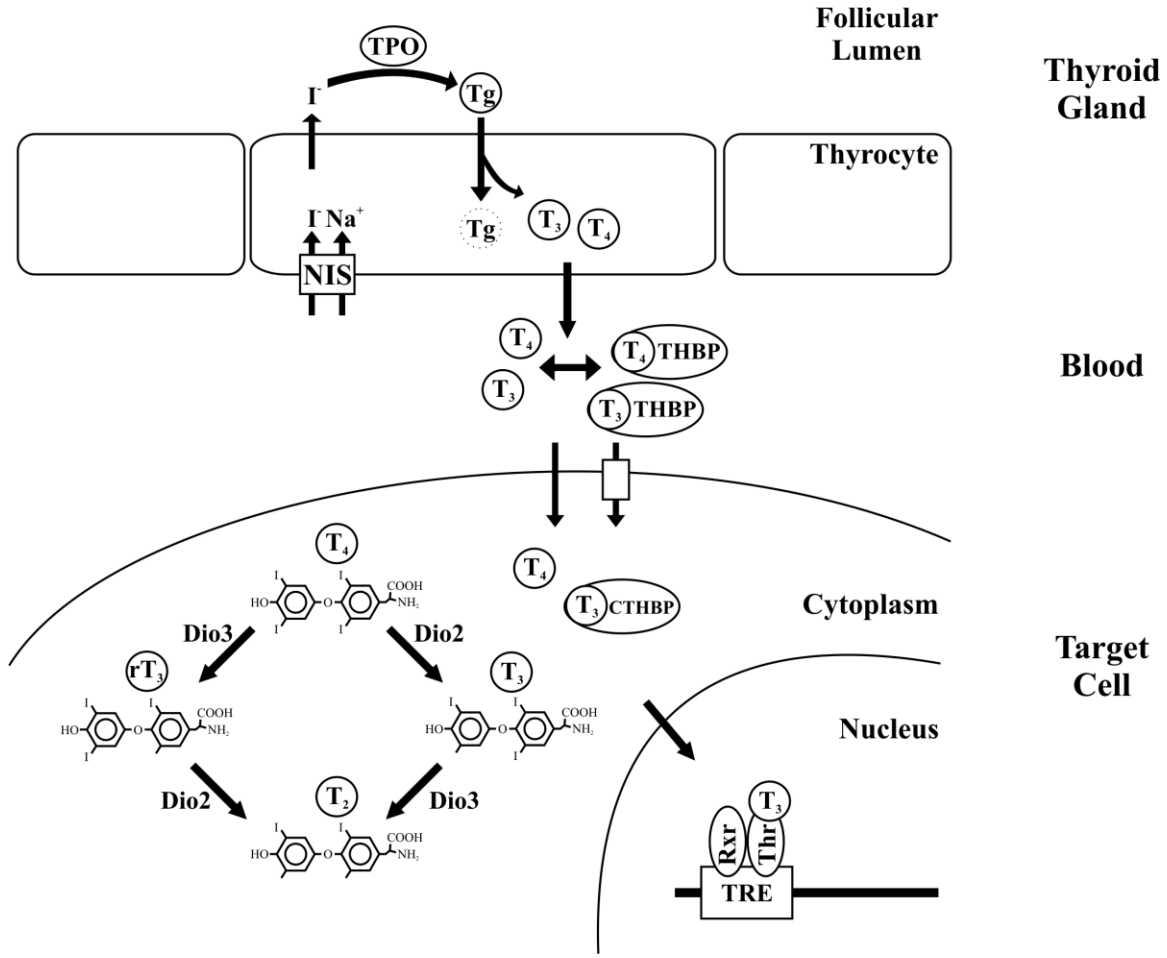

Figure 1.3 Schematic of TH secretion, transport, intracellular metabolism, and genomic action. NIS: sodium/iodide symporter, TPO: thyroperoxidase, Tg: thyroglobulin, T_3: triiodothyronine, T_4: thyroxine, THBP: TH binding protein, CTHBP: cytoplasmic TH binding protein, Thr: thyroid hormone receptor, Rxr: retinoid X receptor, TRE: thyroid hormone response element. Adapted from (Boas et al., 2006; Denver, 2013).

The majority of TH in mammals is reversibly bound to the serum TH binding proteins (THBPs), thyroxine binding globulin (TBG) and transthyretin (TTR). TBG is only found in eutherian mammals and has a high affinity but low capacity for T_4. TTR however, is found in all vertebrates (Power et al., 2000). In mammals, TTR has a higher affinity for T_4 but in amphibians, T_3 binds TTR more strongly than T_4. Albumin also binds TH in

plasma with low affinity and high capacity and is potentially the major form of THBP in amphibians (Denver, 2013).

TH can then be taken up into target cells by active transport *via* three classes of proteins: organic anion transporters (OATCs), monocarboxylate transporters (MCTs), and L-amino acid permeases (LATs). Orthologs of genes of all three forms of proteins have been isolated in frogs but little is known about how they may mediate metamorphosis (Denver, 2013). Once THs enter cells, they can bind to cytoplasmic TH binding proteins (CTHBPs), which have high capacity, low affinity binding sites to limit cell free-TH concentrations as yet another point of TH regulation, or CTHBPs can transport TH to the nucleus (the predominant point of TH action).

Because the TH-response is asynchronous between tissues, but TH-release is controlled centrally, intracellular deiodinase enzymes, which act to convert the "transport form" of TH (T_4) to the "bioactive form" of TH (T_3), are gaining attention as potential tissue-specific mechanisms of TH-pleiotropic effects. There are two fundamental enzymatic reactions that are catalyzed by deiodinases: outer-ring (5'-) monodeiodination, and inner ring (5-) monodeiodination. Three forms of deiodinase enzymes exist in vertebrates to catalyze these reactions: Deiodinase 1 (Dio1) catalyzes both 5'- and 5-deiodination; Deiodinase 2 (Dio2) catalyzes 5'-deiodination; and Deiodinase 3 (Dio3) catalyzes 5-deiodination (St Germain and Galton, 1997). Although the Dio1 gene exists in amphibians, little is known about its expression and activity while Dio2 and Dio3 expression and enzyme activities have been more widely characterized in amphibians (Denver, 2013). Dio2 and Dio3 expression patterns and activities correlate with metamorphic processes. In *Rana catesbeiana* tadpole tailfin, *dio3* mRNA expression and

enzymatic activity increase during prometamorphosis and remain elevated through metamorphic climax (Becker et al., 1997). *dio2* transcript levels and activity, however, are undetectable in *Rana catesbeiana tailfin* before metamorphic climax (Becker et al., 1997). Dio3 is in fact a direct T_3 response gene, and mRNA expression and enzymatic activity are upregulated by exogenous TH in most premetamorphic *Rana catesbeiana* tissues including tailfin (Becker et al., 1995). Dio2 however, is less responsive to TH-induction in *Rana catesbeiana* tailfin as premetamorphic Dio2 activity is low and not enhanced by exogenous TH (Becker et al., 1995).

1.4.2 TH-mediated Transcriptional Regulation

TH exerts its effects predominantly *via* genomic action. In amphibians, as in mammals, there are two types of Thrs, alpha (Thra) and beta (Thrb). The Thrs can undergo alternative splicing to result in multiple isoforms in *Xenopus laevis* (Shi, 2000), although in *Rana catesbeiana* only one isoform of each gene is known. Thrs are part of the nuclear receptor superfamily of transcription factors, which include, among others, estrogen receptor (Esr), retinoid X receptor (Rxr), and glucocorticoid receptor (Nr3c1) (Mangelsdorf et al., 1995). Thr protein structure is highly conserved and consists of four domains: the A/B domain at the N-terminus is involved in transcriptional activation; the C domain is involved in DNA binding; the D domain is the hinge region, and the E domain interacts with the ligand and transcriptional machinery, and facilitates receptor dimerization (Grimaldi et al., 2013). Thrs act both in liganded and unliganded states to activate and repress TH-mediated gene transcription, respectively (Figure 1.4). The dual function model of gene transcription by Thrs proposes that Thrs are always bound to thyroid hormone response elements (TREs) in the promoters of TH-responsive genes. In

the presence of TH, Thrs bind T_3 and activate transcription whereas in the absence of ligand, Thrs repress transcription. Both in the presence and absence of TH, Thrs most strongly bind the consensus TRE: AGGTCA as a direct repeat with a 4-nucleotide space (DR4) (Das et al., 2009). Thrs bind to the TRE as monomers, homodimers, or heterodimers. Heterodimerization with retinoid X receptor (Rxr) alpha, beta, or gamma results in the most efficient T_3-dependent transcription (Wong and Shi, 1995) although Rxr-ligand binding (to 9-cis-retinoic acid) is not required for TH-dependent gene transcription (Grimaldi et al., 2013). During amphibian development, Thr and Rxr expression patterns correlate with metamorphic processes in each tissue (Wong and Shi, 1995; Yaoita and Brown, 1990). In fact, one of the major lines of evidence for the dual function model of TH action is that the expression of amphibian Thra appears before the onset of T_3 production (along with Rxr), suggesting a role for Thra in repressing TH-responsive gene transcription before the onset of metamorphosis. Thrb expression, along with many other direct TH-response genes such as TH basic leucine zipper transcription factor (Thibz) and krüppel-like factor 9 (Klf9), levels increase dramatically with circulating T_3 levels, suggesting a central role in active gene transcription (Shi, 2000). There are, however, a subset of direct TH-response genes that are downregulated by T_3 during amphibian metamorphosis but the mechanisms of TH-mediated gene inactivation or repression are poorly characterized.

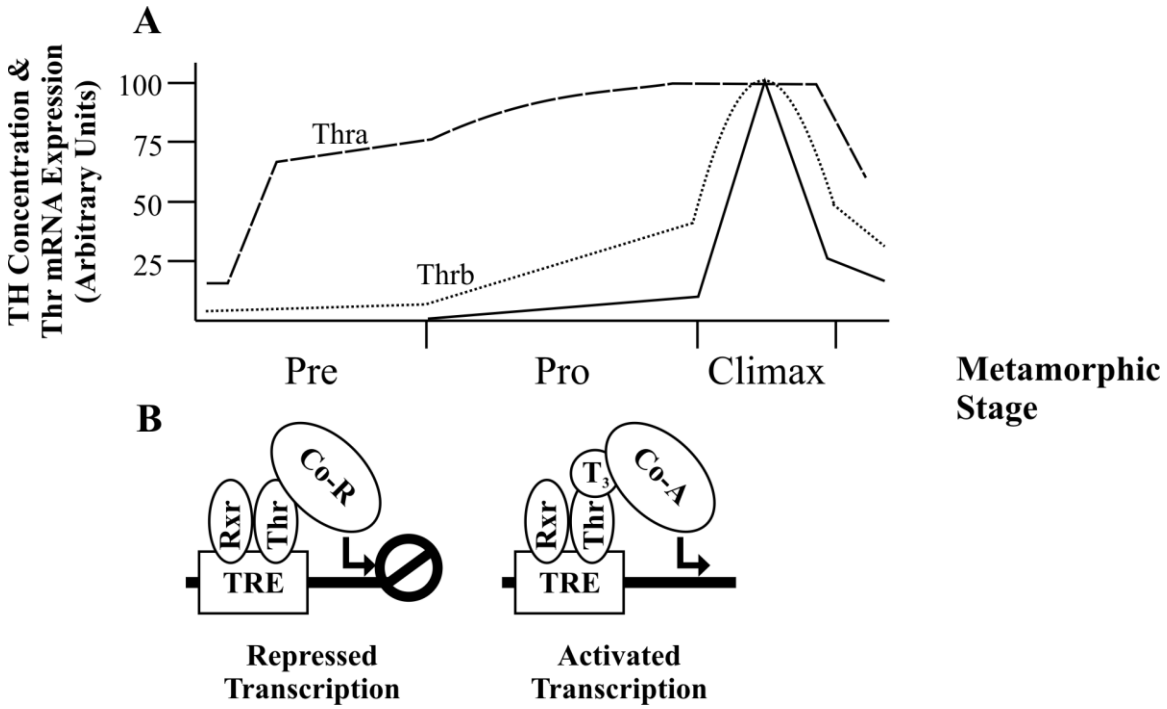

Figure 1.4 mRNA profile of Thrs (dotted lines) and TH concentration (solid line) (A), and Thr dual function model (B) during tadpole development. Thr: thyroid hormone receptor, Rxr: retinoid X receptor, TRE: thyroid hormone response element, Co-R: corepressor complex, Co-A: coactivator complex. Adapted from (Grimaldi et al., 2013).

The mechanisms of gene activation and transcription involve chromatin remodeling. In the absence of ligand, corepressor complexes associate with the Thr/Rxr complexes and associated basal transcriptional machinery (RNA polymerase II and basal transcription factors). Nuclear receptor corepressor (NCoR) and silencing mediator for RAR and TR (SMRT) have been identified as some of the corepressor complexes that associate with the E-region of Thrs to repress transcription in amphibians. These corepressor complexes are also associated with histone deacetylase (HDAC) activity. HDAC activity works to reduce acetylation on N-terminal histone tails, which increases the positively charged histone's affinity for negatively charged DNA which creates a more closed chromatin structure resulting in repressed gene transcription. In the presence of TH, corepressors are

released and coactivator complexes with histone acetyl transferase (HAT) activity such as steroid receptor coactivator (SRC) and p300 are recruited to Thrs (Grimaldi et al., 2013). Histone acetylation has been correlated with T_3-dependent gene expression, Thr binding, and RNA polymerase recruitment (Bilesimo et al., 2011; Grimaldi et al., 2013). Of the subset of genes that are negatively regulated by TH binding (transcriptionally repressed in the presence of ligand), thyroid stimulating hormone alpha (TSHα) is the best characterized. Interestingly, Wang *et al.* (2009) found a similar T_3-induced dissociation of the NCoR-HDAC complex and histone acetylation in the negatively regulated human TSHα as with positively regulated TH-responsive genes suggesting that histone acetylation *per se* cannot be associated with increased transcription.

Although Thrs are necessary and sufficient to mediate metamorphic effects (Das et al., 2010), there are also important non-genomic, or more accurately, non-classical mechanisms of TH-action. Unlike effects from classical nuclear-receptor mediated mechanisms of TH action, which can take hours, non-classical TH-action occurs in a matter of seconds to minutes. Non-classical mechanisms of TH action have been described in mammalian systems *via* mechanisms initiated by TH-cell surface receptors and cytoplasmic Thrs. The integrin αVβ3 is classified as a TH-cell surface receptor and interestingly, has a binding domain for TH that has a higher affinity for T_4 than T_3 (Bergh, 2005). TH-bound αVβ3 activates the mitogen-activated protein kinase (MAPK) signal transduction cascade *via* phospholipase C (PLC) and protein kinase C (PKC) (Cheng et al., 2010). TH-activated MAPK signaling is able to phosphorylate Thrb1 (a mammalian Thrb isoform) and estrogen receptor alpha (Esra – a nuclear receptor which binds E_2 to alter estrogen-responsive gene transcription) in the nucleus and modulate

trafficking of cytoplasmic Thr and Esr into the nucleus (Cheng et al., 2010) thereby indirectly altering transcription. Cytoplasmic proteins are also able to initiate non-classical TH-actions as both classic Thrs and truncated forms have been found in the cytoplasm of mammalian TH-responsive cells (Cheng et al., 2010; Cordeiro et al., 2013) and have caused downstream alteration in specific gene transcription.

In an amphibian context, specifically in *Rana catesbeiana* tailfin, non-classical TH signaling is critical in modulating TH-mediated metamorphic effects. Inhibition of the proapoptotic cyclin dependent kinase 8 (Cdk8) by roscovitine has been shown to inhibit the establishment of the T_3-dependent metamorphic gene expression program (Skirrow et al., 2008). Additionally, T_3-induced tail regression in *Rana catesbeiana* tail tips is inhibited by genistein (a known phytoestrogen in soy products) *via* inhibition of protein kinase C tyrosine phosphorylation (Ji et al., 2007). Both roscovitine and genistein alter TH-mediated transcription of Thrb in *Rana catesbeiana* tailfin (Ji et al., 2007; Skirrow et al., 2008).

1.4.3 Additional Factors That Mediate Amphibian Metamorphosis

1.4.3.1 Stress

Corticosteroids are known to play a role in TH-mediated amphibian metamorphosis. The two major forms of corticosteroids produced in amphibians are corticosterone and aldosterone and their expression levels mirror TH levels during metamorphosis as they are undetectable in premetamorphosis and increase in late prometamorphosis/climax (Shi, 2000). However, corticosteroids do not have the same unidirectional mechanism of action on tadpole development that TH does, as increased corticosteroid exposure during premetamorphosis inhibits tadpole growth and development while exposure during prometamorphosis accelerates metamorphic effects (Denver, 2013). Although the

negative effects of corticosteroids on metamorphosis are not well understood, metamorphic acceleration by corticosteroids and TH is clearer. Corticosterone and TH work synergistically to accelerate *Xenopus laevis* tailfin regression and increase mRNA expression of Thrs. Similarly, although exogenous physiological levels of TH alone could not induce *dio2* mRNA expression to detectable levels in premetamorphic *Xenopus laevis* tailfin cultures, co-treatment with corticosterone caused an increase in *dio2* transcript levels (Bonett et al., 2010).

At the cellular level, reactive oxygen species (ROS) are a major source of stress. ROS are naturally produced as a byproduct of mitochondrial respiration, but too much ROS, or too little of the antioxidant enzymes that work to reduce ROS, can result in oxidative damage. Superoxide dismutase (Sod) and catalase (Cat) are the two predominant antioxidant enzymes (Johnson et al., 2013). Sod catalyzes the reduction of superoxide (O_2^-) to hydrogen peroxide (H_20_2) and oxygen, while Cat converts H_20_2 to water. Thyroxine treatment enhances tail regression partially *via* stimulating mitochondrial respiration and subsequently increasing ROS production (Inoue et al., 2004) leading to apoptosis of cells in the tadpole tail (Ishizuya-Oka, 2011). In *Xenopus laevis*, both Sod and Cat enzymatic activity and gene transcript levels decrease just before tail regression occurs during natural metamorphosis (Johnson et al., 2013; Menon and Rozman, 2007) suggesting a tight regulation of oxidative stress and antioxidant activity. In *Rana* species however, Cat activity in tails is reduced with a concurrent increase in Sod activity (Hanada et al., 1997; Kashiwagi et al., 1999), suggesting that a mechanism for ROS induced tail apoptosis might be an accumulation of H_2O_2 and subsequent oxidative stress leading to apoptosis. Similarly, in cultured tailfins of *Rana catesbeiana* treated with T_3,

cat mRNA transcript levels decrease compared to untreated controls (Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012).

1.4.3.2 Estrogens

Although there are studies of effects of some endogenous estrogens and xenoestrogens on amphibian gonadal development (Hayes et al., 2003; Hogan et al., 2008; Miyata et al., 1999; Oka et al., 2006; Oka et al., 2008; Tompsett et al., 2012; Tompsett et al., 2013), few studies have investigated the effect of E_2 at relevant concentrations on TH-mediated metamorphosis. There is reason to believe, however, that there could be significant crosstalk between estrogen and TH systems. As previously discussed, neuroendocrine regulation of both estrogen and TH release are controlled by common elements, the hypothalamus and pituitary. Although understudied, endogenous sex steroid exposure (at supraphysiological concentrations) on TH-mediated metamorphic events have been previously attributed to neuroendocrine control of the systems as opposed to tissue specific interactions (Gray and Janssens, 1990). There is evidence, however, for estrogen-TH crosstalk at the cellular level as the consensus hormone response elements in both estrogen and TH-responsive gene promoters share a common half-site (Mangelsdorf et al., 1995; Vasudevan et al., 2002). In fact estrogen is able to stimulate human glycoprotein hormone α-subunit (a common subunit of TSH and the gonadotropins produced in the pituitary) and prevent TH-mediated negative regulation due to Esr binding to the TRE (Yarwood et al., 1993). There is also possibility for crosstalk between coregulators of nuclear receptor mediated transcription as both Esr and Thr share coactivators and Esr has been found to cause a reduction in transcriptional activation by Thrs not requiring Esr binding to the TRE (Yen et al., 1995; Zhang et al., 1996). Finally,

non-classical mechanisms of transcription modulation between estrogen and TH have shown crosstalk. As previously described, TH activation of MAPK signaling pathways can cause phosphorylation of Esr that increases recruitment of coactivators to the nuclear receptor to alter transcription (Zhao et al., 2005).

In amphibians, *in vivo* assessments of E_2 effects on TH-mediated metamorphosis are not clear-cut. Oka *et al*. (2006) found that although concentrations as low as 0.1 nM E_2 caused feminization of male *Xenopus laevis* tadpoles exposed during sensitive life stages, subsequent development in clean water resulted in no effects on TH-dependent metamorphic endpoints, such as time to metamorphosis, length, and weight. However, studies at much higher exposure concentrations of E_2 (~ μM concentrations) have shown both agonistic and antagonistic effects on TH-dependent development in *Xenopus laevis* (Gray and Janssens, 1990; Nishimura et al., 1997). At relevant concentrations of E_2, Bauer-Dantoin and Meinhardt (2010) recently found 0.01 – 10 nM E_2 exposures of *Xenopus laevis* caused non-monotonic acceleration of tadpole bone development in conjunction with an increase in developmental stage. Xenoestrogenic exposures of *Rana* species to environmentally relevant concentrations of EE2 have caused delayed metamorphosis and altered sex ratios in *Rana pipiens* (Hogan et al., 2008) and no effects on metamorphic endpoints but similarly altered sex ratios in *Rana sylvatica* (Tompsett et al., 2013). What has become clear from *in vivo* assays of estrogenic effects on TH-mediated metamorphosis is that species specificity, developmental stage of exposure, chemical composition of estrogens, and the concentration of exposure all play a role in altering metamorphic endpoints.

1.5 Assessment Methods for TH-EDCs

As the complexities of TH-action are incredibly multifaceted and critical in all vertebrates, yet the analysis of EDC action is relatively new for environmental regulators, the efficient and accurate assessment of potential chemicals with TH-action is of critical importance. The Organization for Economic Cooperation and Economic Development (OECD) endorses the amphibian metamorphosis assay (AMA) as a Tier 1 standardized assay to determine TH-disrupting compounds. The AMA is currently used as part of the US EPA's endocrine disruptor screening program. The AMA is a premetamorphic *Xenopus laevis* exposure to dilutions of a test chemical for 21 days with morphological TH-mediated metamorphic endpoints such as hind limb length (OECD, 2009). Although the assay has been recently successfully adapted to a more relevant species with additional molecular endpoints (Marlatt et al., 2013), the AMA currently accepted in practice for regulatory purposes does not account for critical molecular effects of complex mixtures of potential EDCs on a North American amphibian species.

Assessments of effects of potential complex mixtures of EDCs in wastewater effluents on amphibians have indicated alterations in TH-mediated development. Searcy *et al.* (2012) examined municipal wastewater effluent exposure on *Xenopus laevis* tadpoles from embryo through metamorphosis and noted increased developmental rates in conjunction with altered mRNA transcript abundance of TH-responsive genes**.** Sowers *et al*. (2009) exposed *Rana pipiens* to a dilution of MWWTP effluents from egg through metamorphosis and noted increased time to metamorphosis and altered thyroid gland morphology in higher concentrations of effluents in addition to alterations in reproductive systems**.** The effects of constructed wetlands, a tertiary MWWTP step, on bullfrogs were considered by Ruiz *et al.* (2010) and although no differences in direct TH-mediated

morphological endpoints were observed, higher frequencies of abnormalities were observed in animals closer to effluent outfalls. Laposata and Dunson (2000) also investigated natural amphibian population exposures to wastewater effluents and found that temporary breeding ponds, which were irrigated by wastewater effluents, had fewer egg masses, reduced hatch success, and reduced larval survival in three different amphibian species as compared to natural non-effluent irrigated ponds.

Molecular assays have also been developed for assessing TH-EDC-like effects and have been used in the context of wastewaters. Assays of human Thra and Thrb have both been used in reporter-gene constructs transfected into mammalian cells (Jugan et al., 2007; Shen et al., 2009), which have later been used to assess TH-disrupting activities in wastewater extracts (Jugan et al., 2009; Shi et al., 2009; Shi et al., 2011; Shi et al., 2012a; Shi et al., 2012b). Although these assays give a quick indication of Thr activity, alternative TH-disrupting mechanisms are not accounted for. Two groups have also developed yeast two-hybrid (Y2H) assays for TH-disruption, which assess the interaction of two proteins. Nishikawa *et al*. (1999) developed a system where the disruption of the ligand binding domain of human Thra interaction with the receptor interacting domain of the Thr coactivator TIF2 was assessed. This assay was used to determine TH-agonistic activities in influent, effluent, and receiving water extracts in Japan (Inoue et al., 2009; Inoue et al., 2011). Li *et al*. (2008) used the interaction of the DNA binding domain of human Thrb with a full-length Thr coactivator (GRIP1/F1) to determine both agonistic and antagonistic TH effects in wastewater samples from influents and effluents of MWWTPs in Beijing (Li et al., 2011). Although both Y2H assays have successfully identified TH-specific action in wastewaters, this screen has a high likelihood of false

negatives as the protein domains chosen for the assays are limited and yeast, a unicellular organism, lacks conserved components of the complex transcriptional machinery of metazoans including the entire complement of the steroid/thyroid hormone receptor superfamily (Rando and Chang, 2009).

Finally there have been a few assays of molecular TH-EDC action in amphibian systems that have been used to assess wastewater effluents. Yamauchi and colleagues have developed a Thr-dependent luciferase gene activation assay in a *Xenopus laevis* cell line (Sugiyama et al., 2005), as well as a competitive T_3-binding assay to *Xenopus laevis* TTR and Thr (Ishihara et al., 2003; Yamauchi et al., 2002). All three of these assays have been used to assess wastewater samples (Ishihara et al., 2009; Murata and Yamauchi, 2008; Yamauchi et al., 2003). Demeneix and colleagues have used whole body fluorescence of transgenic *Xenopus laevis* embryos under the control of *thibz* promoters (Fini et al., 2007) in assessing TH disruption by wastewaters (Castillo et al., 2013). This assay is one of the few to use whole wastewater samples rather than extracts to study the molecular effects of wastewater effluents *in vivo* in amphibians.

1.5.1 The C-fin Assay

Our lab has developed the cultured tailfin (C-fin) assay; a tool to screen for the effects of TH-EDCs and stress in a globally distributed amphibian species by investigating perturbations in transcript levels of key genes of interest (Hinther et al., 2010a). The C-fin assay uses multiple premetamorphic *Rana catesbeiana* tailfin biopsies from one animal cultured in multiple treatment conditions, and thus a repeated measures study design, to reduce animal number and simultaneously gain statistical power in assessment of perturbations to the system (Figure 1.5). The tailfin biopsies are cultured for 48 hours

with or without the presence of T_3 to assess both agonistic and antagonistic effects of chemicals of interest on TH-responsive and stress-responsive gene transcripts by quantitative polymerase chain reaction (QPCR). The TH-challenge allows us to assess the effects of a chemical of interest on both premetamorphic and precociously T_3-induced amphibian tailfin transcript levels. The C-fin has been used to investigate several TH- and stress-disrupting chemicals of interest, but has not been previously used to assess wastewaters (Hammond et al., 2013; Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012). By using the C-fin with whole wastewater samples, we are able to screen a complex mixture of potential EDCs on a complex tissue in a relevant amphibian species. In the present work, the C-fin assay has been used both to screen for potential disruption of amphibian metamorphic processes by wastewaters, as well as to more clearly understand the mechanisms of hormone action in *Rana catesbeiana* tailfin.

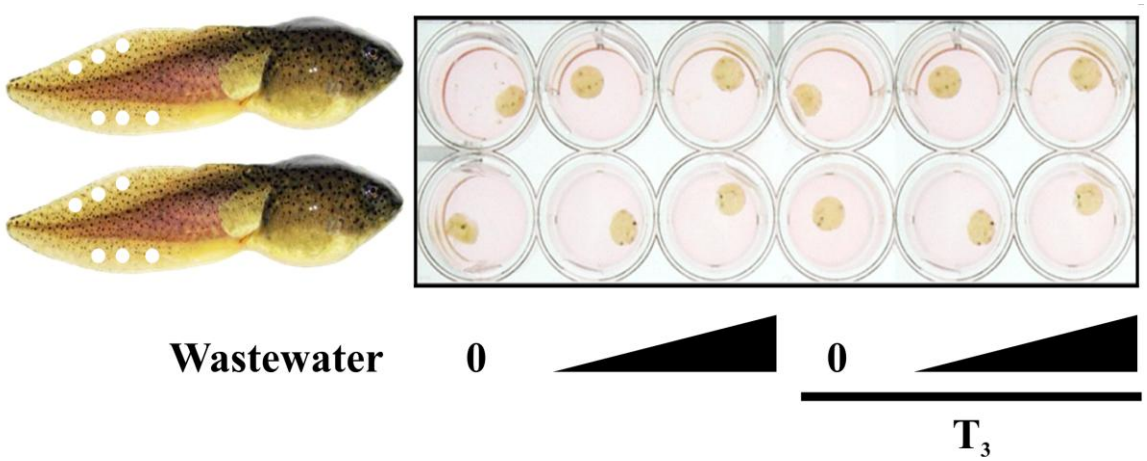

Figure 1.5 Overview of the C-fin assay used for wastewater screening. Biopsies are taken from the tailfin of eight premetamorphic *Rana catesbeiana* (only two animals shown). Six biopsies from one animal are exposed to six different treatment conditions in the presence or absence of 10 nM T_3. The bevel indicates 20% and 50% concentrations of wastewater. Adapted from (Hinther et al., 2010a)

1.6 Research Objectives

Current MWWTPs insufficiently remove chemicals that have the potential to significantly alter highly conserved endocrine systems in all vertebrates. Although much research has focused on xenoestrogenic effects of ECs, compounds that affect the critical TH system have been understudied. Biological assays are needed to aid municipalities in determining which MWWTP systems are the most efficient in removing ECs from municipal wastewaters. The purpose of my research is to determine the ability of the most common municipal wastewater treatment systems to remove potential biological effects of raw wastewater influents on TH function and stress response, and to elucidate some of the complexities of endogenous hormone action in *Rana catesbeiana* tailfin in the process. The C-fin assay is used to first assess the biological effects of successive levels of a conventional full-scale MWWTP (Chapter 2). Finding that secondary treatment is a crucial part of MWWTP processes, the C-fin is then applied to 3 different secondary treatment systems to elucidate the potential differences between treatment configurations and operational conditions on the removal of biological activity (Chapter 3). Chapters 2 and 3 represent work that was part of a larger collaborative project funded by the Canadian Municipal Water Management Research Consortium through the Canadian Water Network, a national group of interdisciplinary researchers interested in characterizing EC removal in Canadian MWWTPs. Finally, Chapter 4 takes advantage of the unique repeated measures design of the C-fin assay to query and challenge the traditional views regarding the biological activity relationship between T_3 and T_4. Moreover, TH- and stress-dependent endpoints are evaluated for their responsiveness to estrogen to determine what influence this hormone may have on TH- and stress-dependent endpoints.

2 Hormone and Stress-inducing Activities of Municipal Wastewater Along Successive Units of a Full-Scale Conventional Activated Sludge Plant

The data presented here are part of a larger collaborative study. Wastewater sampling was done by Emily Austin in the laboratory of Dr. Hongde Zhou at the University of Guelph in Guelph, Ontario. Wastewater extraction and target EC analysis was done by Dr. Ehsanul Hoque and Dr. Tamanna Sultana in the laboratory of Dr. Chris Metcalfe at Trent University in Peterborough, Ontario.

2.1 Introduction

MWWTPs are a significant source of ECs to receiving water bodies. Standard MWWTPs typically consist of three levels of wastewater treatment: a preliminary screen of wastewater influent, a primary settling phase to remove solids, and secondary biological treatment to reduce nutrient loads and biodegrade unwanted compounds (Onesios et al., 2008; Ternes et al., 2004). Although significant improvements to MWWTP technologies have enhanced the qualities of wastewater effluents over the years, downstream of MWWTPs, anthropogenic contaminants at microgram per liter (µg/L) concentrations remain persistent (Blair et al., 2013; Fent et al., 2006; Holeton et al., 2011; Kolpin et al., 2002). These ECs are emerging chemicals of concern not only because they are often recalcitrant to conventional MWWTP systems, but also because many of these contaminants are known EDCs (Lishman et al., 2006; Metcalfe et al., 2013). Much EDC research has concentrated on xenoestrogenic compounds and only in recent years has disruption of other crucial hormone systems such as TH received attention in wastewater effluent receiving waters (Castillo et al., 2013; Ishihara et al.,

2009; Jugan et al., 2009; Kusk et al., 2011; Metcalfe et al., 2013; Murata and Yamauchi, 2008; Searcy et al., 2012; Sowers et al., 2009; Svanfelt et al., 2010). EDCs in municipal wastewater effluents in particular pose an additional layer of complexity to environmental risk management as organisms in receiving water bodies are exposed to more than one compound at a time. Wastewaters are complex mixtures that contain various components that can act as EDCs individually and/or together in ways that are not necessarily predicted by additive or synergistic effects models (Crofton et al., 2005; Kortenkamp, 2007; Rajapakse et al., 2004).

Chemical analyses of the efficacy of removal and fates of select ECs in conventional activated sludge (CAS) systems suggest that a small portion of highly lipophilic compounds are removed via sorption during primary treatment while secondary treatment is the most effective at removing ECs by biodegrading the compounds (Carballa et al., 2004; Onesios et al., 2008; Oulton et al., 2010; Ternes et al., 2004). Biological degradation of compounds however has the potential to create more bioactive metabolites in final effluents (Joss et al., 2004). In addition, although high removal efficacies are found in some MWWTPs, efficient removal rates do not guarantee reduced biological effects (Osachoff et al., 2013).

The present study is part of a larger initiative aimed at determining the efficacy of emerging contaminant removal within existing treatment trains relevant to Canadian operating conditions. The objective of the present study was to examine the performance of a full scale CAS municipal wastewater treatment plant operating in Guelph, Ontario, Canada in the removal of select emerging contaminants and evaluate, at three phases

along the treatment train, the success of reducing bioactive effects on thyroid hormone and stress signaling pathways.

2.2 Materials and Methods

2.2.1 MWWTP Water Sampling and Handling

Wastewater samples were collected as 24 h composite samples from the City of Guelph wastewater treatment plant (ON, Canada) at three points over the span of about 1 year (October 2011, August and November 2012). The MWWTP collects raw wastewater from The City of Guelph and the Township of Guelph/Eramosa and is a mixture of domestic, institutional, commercial, and industrial wastewater. This MWWTP has four parallel treatment trains of which one was selected for intensive study. A schematic of the full-scale MWWTP treatment train selected for study is shown in Figure 2.1. This train included primary sedimentation and secondary conventional activated sludge treatment units. Water samples from the indicated positions along the treatment train (Figure 2.1) were immediately filtered through a 1.5 μm glass microfiber filter (Whatman, Toronto, ON, Canada) and sent overnight on ice to Victoria, BC for C-fin assays, or stored at 4°C before solid phase extraction was conducted.

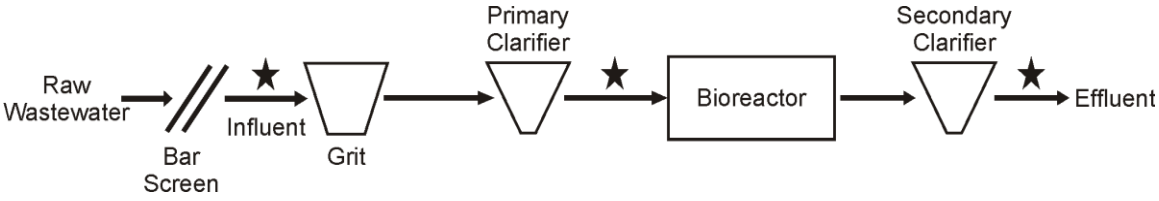

Figure 2.1 Process flow schematic of the full-scale CAS MWWTP treatment train. Stars indicate where composite water samples were collected along the treatment train.

2.2.2 Solid Phase Extraction (SPE) and LC-MS/MS Analyses

SPE was performed as previously described elsewhere (Li et al., 2010). In brief, the SPE cartridges (Oasis® MAX, 6ml, 500mg, Waters Limited in Mississauga, ON, Canada) were preconditioned and loaded with filtered samples. Pharmaceuticals, triclosan, triclocarban and labelled analytes were purchased from Sigma-Aldrich (St. Louis, MO, USA), Toronto Research Chemicals (Toronto, ON, Canada), C/D/N Isotopes (Pointe-Claire, QC, Canada) and Cambridge Isotopes (Andover, MA, USA). Before SPE, each sample was spiked with 0.1 mL of 0.5 mg/L labeled analytes including androstenedione-d3, estrone-^{13}c2, estradiol-^{13}c2, ethynylestradiol-^{13}c2, gemfibrozil-d6, ibuprofen-^{13}c3, triclosan-^{13}c12 and triclocarban-^{13}c13. Target contaminants were then eluted with methanol. Extracts were then evaporated to almost dryness and samples were reconstituted to a final volume of 0.4 ml using methanol for chemical analysis. The SPE extracts were sent to the Water Quality Centre, Trent University, Peterborough, ON, Canada for target EC analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Two separate liquid chromatography and tandem mass spectrometry (LC-MS/MS) methods were utilized to analyze the extracts for pharmaceuticals. Androstenedione was analyzed with positive ion mode in API 3000 LC-MS/MS system with electrospray ionization (ESI) source (AB Sciex, Concord, ON, Canada). API 3000 system was equipped with an autosampler (Series 200 autosampler) from Perkin Elmer (Waltham, MA, USA), and pumps (LC-10AD), degasser (DGU-14A) and system controller (SCL-10A) from Shimadzu (Columbia, MD, USA). Estrone, estradiol, ethynylestradiol, ibuprofen, gemfibrozil, triclosan and triclocarban were measured in negative ion mode using an AB Sciex Q-Trap 5500 instrument with an ESI source. This

system was equipped with an Agilent 1100 series (Mississauga, ON, Canada) LC system that includes an autosampler, pumps and a degasser. MS detection was performed using multiple reactions monitoring (MRM). The mass transitions for analytes and corresponding labelled analytes are listed in Table 2.1.

Table 2.1 Monitored transitions of select ECs in LC-MS/MS analysis

Compounds	Mass transition of m/z
Androstenedione	287.2 / 97.2
Androstenedione-d3	290.2 / 100.2
Estradiol	271.1/145.0
Estradiol-^{13}c2	273.1/146.8
Estrone	269.0/145.0
Estrone-^{13}c2	271.1/147.0
Ethynylestradiol	295.1/145.1
Ethynylestradiol-^{13}c2	297.2/145.0
Gemfibrozil	249.1 / 121.0
Gemfibrozil-d6	255.0 / 121.0
Ibuprofen	205.0 / 161.0
Ibuprofen-^{13}c3	208.0 / 163.0
Triclocarban	312.81/35.0
Triclocarban-^{13}c13	325.9/165.9
Triclosan	287.0 / 35.0
Triclosan-^{13}c12	299.0 / 35.0

In LC-MS/MS methods, analytes were separated chromatographically using a Genesis C18 column of 150 mm long, 2.1 mm ID and 4 µm particle size (Chromatography Specialities Inc., Brockville, ON, Canada) with a guard column (Genesis C18, 10 x 2.1 mm and 4 µm). The mobile phases in gradient elution were (A) water (100%) with 0.1% acetic acid and (B) acetonitrile (100%) with 0.1% acetic acid. For quantification, an internal standard method with a five-point calibration graph covering the range of anticipated analyte concentration in the sample was utilized. The limits of detection

(LOD) and limits of quantitation (LOQ) of the target analytes were 0.1 to 1 ng/mL and 1 to 10 ng/mL, respectively. LODs and LOQs were estimated as the analyte concentrations that produced peaks with signal-to-noise ratio (S/N) of 3 and 10, respectively.

2.2.3 Experimental Animals

Premetamorphic Taylor and Kollros (TK) (Taylor and Kollros, 1946) stage VI–VIII *Rana catesbeiana* tadpoles were caught locally (Victoria, BC, Canada). The care and treatment of animals was in accordance with the guidelines of the Canadian Council on Animal Care under the guidance of the Animal Care Committee, University of Victoria. Animals were housed in the University of Victoria aquatics facility, and maintained in a 100-gallon tank with recirculated water at 14^{o}C with exposure to natural daylight. Tadpoles were fed daily with spirulina (Aquatic ELO-Systems Inc., FL, USA).

2.2.4 C-fin Assays

Tailfin biopsy cultures were prepared according to the methods described previously for the C-fin assay (Hinther et al., 2010a). One C-fin assay was employed per sample from each unit of the treatment train. Each C-fin assay used eight tadpoles. Six circular 4 mm tailfin biopsies (Miltex, Plainsboro, NJ, USA) were taken from each animal in order to test six different treatment conditions outlined below. Biopsies were cultured in individual wells at 25^{o}C in air for 48 h in 96-well multi-well culture plates (BD Falcon, Mississauga, ON, Canada) containing 200 µL of 1X culture medium. This medium was comprised of 70% strength Leibovitz's L15 medium (Gibco – Life Technologies Inc., Burlington, ON, Canada) supplemented with 10 mM HEPES pH 7.5, 50 units/ml penicillin G sodium, 50 ug/ml streptomycin sulfate (Gibco – Life Technologies Inc.), 50

μg/ml neomycin (Sigma-Aldrich, Oakville, ON, Canada), and 2 mM L-glutamine (Sigma-Aldrich).

The six treatment conditions were divided into two sets: three treatments with 10 nM 3,5,3'-triiodothyronine (T_3; #T2752, Sigma-Aldrich) in 400 nM NaOH, and three treatments without T_3 (400 nM NaOH only; T_3-vehicle control). T_3 was prepared as a 10^{-5} M stock in 400 μM NaOH and was applied at 1 μL/mL of media. Within each set, treatments were further subdivided such that each sterilized wastewater sample was tested in the presence or absence of T_3 at 20% and 50% serial dilutions. Samples were sterilized with a 0.2 μm nylon filter (Nalgene – Thermo Fisher, Rochester, NY, USA) before application to the organ cultures. The final concentration of medium components was maintained at 1X. Each individual animal's biopsies were exposed to the six treatment conditions in order to establish 1) that each animal was competent to respond to T_3 alone, 2) how wastewater alone could affect the tissues, and 3) how wastewater could alter the normal T_3 induced response of each animal. At the end of the 48 h incubation period, each biopsy was stored in 100 μL of RNAlater (Ambion – Life Technologies Inc., Burlington, ON, Canada) for 24-48 h at 4°C and then transferred to -20°C until processed for total RNA.

2.2.5 Isolation of RNA and Quantitation of Gene Transcript Abundance

RNA was isolated using TRIzol reagent as described by the manufacturer (Invitrogen – Life Technologies Inc., Burlington, ON, Canada). Mechanical disruption utilized 300 μL TRIzol reagent, a 1 mm diameter tungsten-carbide bead, and a safe-lock Eppendorf 0.5 ml microcentrifuge tube in a Retsch MM301 Mixer Mill (Fisher Scientific, Ottawa, ON, Canada) at 20 Hz two times for 3 min, with the chambers rotated in between cycles.

Twenty μg glycogen (Roche Diagnostics, Laval, QC, Canada) was added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 10 μL diethyl pyrocarbonate-treated (Sigma-Aldrich) RNase-free water and stored at -80°C.

cDNA was synthesized from 5 μL prepared RNA (representing ~1 μg total RNA) as per manufacturer's protocol using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems – Life Technologies Inc., Burlington, ON, Canada). cDNA products were diluted 20-fold prior to QPCR amplification and stored at -20°C.

Transcript abundance of thyroid hormone receptors alpha and beta (*thra* and *thrb*), Cu/Zn superoxide dismutase (*sod*), catalase (*cat*), heat shock protein 30 (*hsp30*), ribosomal proteins L8 (*rpl8*) and S10 (*rps10*), and eukaryotic translation elongation factor 1-alpha (*eef1a*) was analyzed using a MX3005P real-time QPCR system (Agilent Technologies Canada, Inc., Mississauga, ON). The 15 μL multiplex *thra*, *thrb*, *rpl8* and *sod*, *cat*, *hsp30* QPCR reactions contained primers and probes as described in Table A.1 and 0.01% Tween 20, 0.8% glycerol, 10 mM Tris-HCl (pH 8.3 at 20°C), 50 mM KCl, 3 mM MgCl2, 200 μM dNTPs, 69.4 nM ROX reference dye (Invitrogen), one unit of Immolase DNA polymerase (Bioline USA Inc., Taunton, MA, USA), and 2 μL of diluted cDNA. The thermocycle program for multiplex reactions entailed an enzyme activation step of 95°C (9 min) followed by 40 cycles of 95°C denaturation (15 s), 64°C annealing (30 s), and 72°C elongation (30 s). *eef1a* and *rps10* primers were run with SyBr green in 15 μL reactions which contained 0.01% Tween 20, 0.8% glycerol, 10 mM Tris-HCl (pH 8.3 at 20°C), 50 mM KCl, 3 mM MgCl2, 40,000-fold dilution of SYBR Green I

(Molecular Probes, Invitrogen), 200 μM dNTPs, 69.4 nM ROX reference dye (Invitrogen), one unit of Immolase DNA polymerase (Bioline), 2 μL of diluted cDNA, and primers as described in Table A.1. The thermocycle program for *eef1a* and *rps10* were the same as *thra, thrb, rpl8* reactions except for an annealing temperature of 60°C. In accordance with MIQE guidelines (Bustin et al., 2010), amplicon sequences were confirmed and the mRNA levels were normalized to the geometric mean of the transcript levels of *rpl8*, *rps10*, and *eef1a* using the ΔΔCt method (Livak and Schmittgen, 2001). All amplification reactions satisfied the ΔΔCt criteria for efficiency as the slope of the line of $\log_2$ dilutions versus the ΔCt values (between the gene of interest and the normalizer gene *rpl8*) were between -0.1 and 0.1. The three normalizer transcripts were deemed to be suitable normalizers using RefFinder (http://www.leonxie.com/referencegene.php?type=reference). This web-based program uses a combination of software including geNorm (Vandesompele et al., 2002), Normfinder (Andersen et al., 2004), Bestkeeper (Pfaffl et al., 2004), and comparative ΔCt (Silver et al., 2006) to assess normalizer suitability. A representative figure depicting the gene transcript abundance of the three normalizers used for a wastewater C-fin assay is shown in Figure A.1.

2.2.6 Statistical Analysis

Statistical analyses were performed using R-studio software (R-studio Inc., Boston, MA, USA). The data were $\log_2$-transformed and evaluated for normality using a Shapiro-Wilk test. Since the data were nonparametric, a Wilcoxon signed-rank test for repeated measures data was used. Repeated measure comparisons were as follows: first, the ability of the tissues to respond to T_3 treatment was assessed for each animal; second,

wastewater treatment effects were examined relative to the vehicle control (NaOH); third, alteration of the normal response to T_3 by wastewater was assessed. All statistical analyses were performed on $\log_2$ transformed data and were deemed significant at $p<0.05$.

2.3 Results and Discussion

2.3.1 Select Emerging Contaminants

We evaluated the concentrations of select ECs in each of the water sample types (Table 2.2). Contaminants were chosen based on previous indications of EDC activity, impact on hormone-related activities, and prevalence in municipal wastewater effluents (Buser et al., 1999; Han et al., 2010; Hinther et al., 2011; Liu et al., 2009a; Marlatt et al., 2013; Mimeault et al., 2005; Veldhoen et al., 2006). Androstenedione, estrone, estradiol, and ethynylestradiol were below detectable limits in all units of the treatment train. The concentrations of the detectable target contaminants in the influent spanned three orders of magnitude with ibuprofen (IBP) present in the highest concentrations, followed by triclosan (TCS), gemfibrozil (GMF), and triclocarban (TCC). In all cases there was a reduction in the target contaminant concentrations through primary treatment, but the greatest reduction in detectable amounts occurred at the secondary treatment step. IBP and TCS concentrations were reduced by at least 99% and 95%, respectively, through the treatment. GMF concentrations were reduced to between the detection limit and 0.044 ng/L. Although removal rates of emerging contaminants are highly variable depending on the EC and the treatment system, select emerging contaminants were in expected concentration ranges (Lishman et al., 2006).

Table 2.2 Select mean ± standard deviation EC concentrations (ng/L) in wastewater samples. ND: not detected.

		Sample Period	
Chemical	**Treatment Unit**	**August**	**November**
Ibuprofen	Influent	107.56 ± 81.92	58.1
	Primary	40.22 ± 7.58	38.1
	Secondary	0.49 ± 0.03	0.60 ± 0.05
Gemfibrozil	Influent	0.373 ± 0.275	0.3
	Primary	0.305 ± 0.058	0.163
	Secondary	ND	0.044 ± 0.011
Triclosan	Influent	17.11 ± 12.03	27.8
	Primary	7.10 ± 1.50	23.80 ± 11.60
	Secondary	0.50 ± 0.06	1.29 ± 0.11
Triclocarban	Influent	0.168 ± 0.097	0.168 ± 0.097
	Primary	ND	ND
	Secondary	ND	ND

2.3.2 Thyroid Hormone Responsive Gene Transcripts

The repeated measures design of the C-fin assay, along with the internal positive controls in each exposure, allow us to distinguish which animals respond to TH and to what extent. As expected, gene transcript levels for both thyroid hormone receptor alpha (*thra*) and thyroid hormone receptor beta (*thrb*) (Figure 2.2a and 2.2b, respectively) were significantly increased (3- and ~30-fold, respectively) by addition of 10 nM T_3 alone to establish the ability of each individual animal to respond to the hormone and, therefore, to establish competence of the animals to respond to compounds that may alter the inherent TH-response (Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012). In past studies using the C-fin assay, a small percentage of animals

(5-7%) were identified as "non-responsive to T_3 treatment" (Hinther et al., 2010a). In the current work, all animals responded significantly (>2-fold increase in *thrb*) to T_3 and were therefore assessed for perturbations to the TH-response.

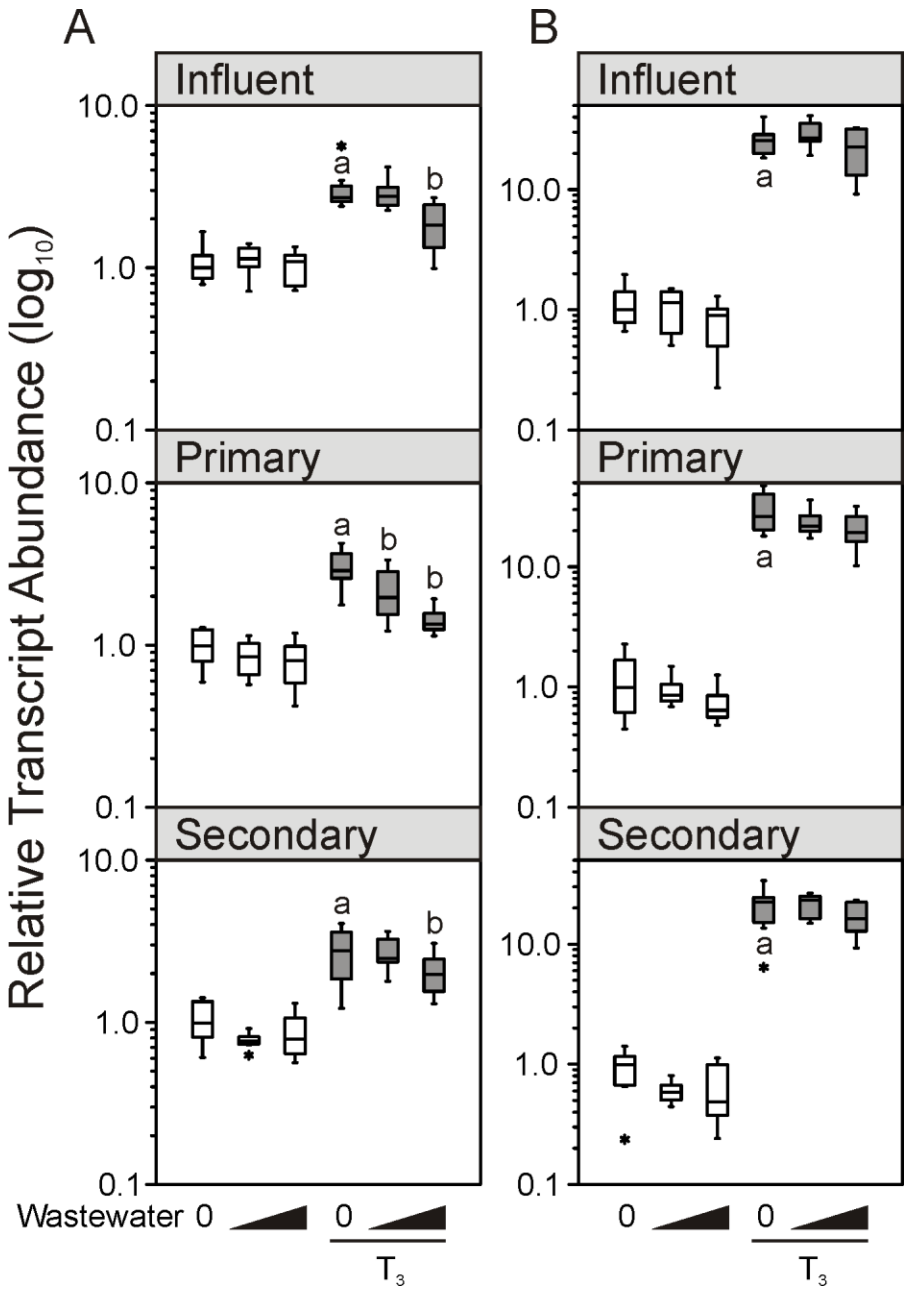

Figure 2.2 Effects of municipal wastewater influent, primary treated wastewater, and secondary treated wastewater on A) *thra* and B) *thrb* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Tissues from eight biological replicates were cultured for 48 h in the indicated wastewaters under two test conditions: with T_3-vehicle control

(400 nM NaOH – white boxplots), and with 10 nM T_3 in 400 nM NaOH (grey boxplots). The bevel indicates 20% and 50% concentrations of wastewater added in each of the two test conditions. Relative transcript abundance is presented in comparison to the geometric mean of transcript levels of ribosomal protein L8, eukaryotic translation elongation factor 1α, and ribosomal protein S10. The median of the data is represented as a solid black line within each box, while the rest of the box indicates the 25th and 75th percentiles, and the whiskers denote the remaining 50% of the data. An asterisk indicates outlier values and an open circle indicates extreme values. "a": significance of 10 nM T_3 (T_3-only control) to 400 nM NaOH (T_3-vehicle control) ($p < 0.05$; Wilcoxon signed-rank test). "b": significance relative to no-wastewater-added control ("0") within a test condition ($p < 0.05$; Wilcoxon signed-rank test).

In the context of the present study, *thra* transcript levels were affected by exposure to municipal wastewater at all stages of the treatment train whereas *thrb* transcript levels were not. Wastewaters attenuated the *thra* response to the internal 10 nM T_3 control by ~20-50% in each of the treatment train units. Secondary treatment however, was only able to remove ~20% of the TH-response-disruptive activity detected in the influent by the *thra* transcript.

Relatively few studies have investigated TH-activity of municipal wastewaters (Castillo et al., 2013; Inoue et al., 2011; Ishihara et al., 2009; Jugan et al., 2009; Metcalfe et al., 2013; Shi et al., 2012b) and none have investigated the effects on *thra* transcript levels specifically. When Ings *et al.* (2011) investigated the same full-scale MWWTP as the present study using caged rainbow trout downstream of an additional tertiary treatment step of the current full-scale CAS MWWTP, liver *thrb* transcript levels were found to be increased by up to 15-fold compared to an upstream reference site. While *thrb* and *thra* function in similar and overlapping roles in amphibians, it has been suggested that *thra* may play a greater role in establishing the animal's competence to respond to TH (Buchholz et al., 2006; Hollar et al., 2011). Although the implications of

the gene-specific TH-perturbations in bullfrog tailfin are unclear, the present data along with the effects of effluents from the same MWWTP on rainbow trout (Ings et al., 2011), suggest that there is potential for thyroid hormone disruption in fully treated municipal wastewater effluents from a CAS system.

2.3.3 Stress Responsive Gene Transcripts

Previous studies have measured oxidative stress endpoints as indicators of effluent exposure in fish and mussels (Carney Almroth et al., 2008; Gagné et al., 2006; Gagné et al., 2011; Ings et al., 2011; Sturve et al., 2008; Veldhoen et al., 2011). In rainbow trout caged downstream of a Swedish MWWTP, in addition to oxidative damage, indications of oxidative stress were identified by altered mRNA and protein levels of genes involved in antioxidant response (Carney Almroth et al., 2008). Little is known, however, about oxidative stress in amphibian species exposed to MWWTP effluents. In the present study, wastewater samples from every treatment train unit caused no major effects on either of the two oxidative stress responsive gene transcripts (*sod* and *cat* – Figure 2.3a and b, respectively). Although our endpoints take into account only the transcript abundance of *cat* and *sod*, the results suggest that, whether the wastewaters are raw or treated, *Rana catesbeiana* tailfin tissues are not significantly affected *via* oxidative stress by ECs in municipal wastewaters in the same way that other aquatic organisms such as rainbow trout (Gagné et al., 2006) might be.

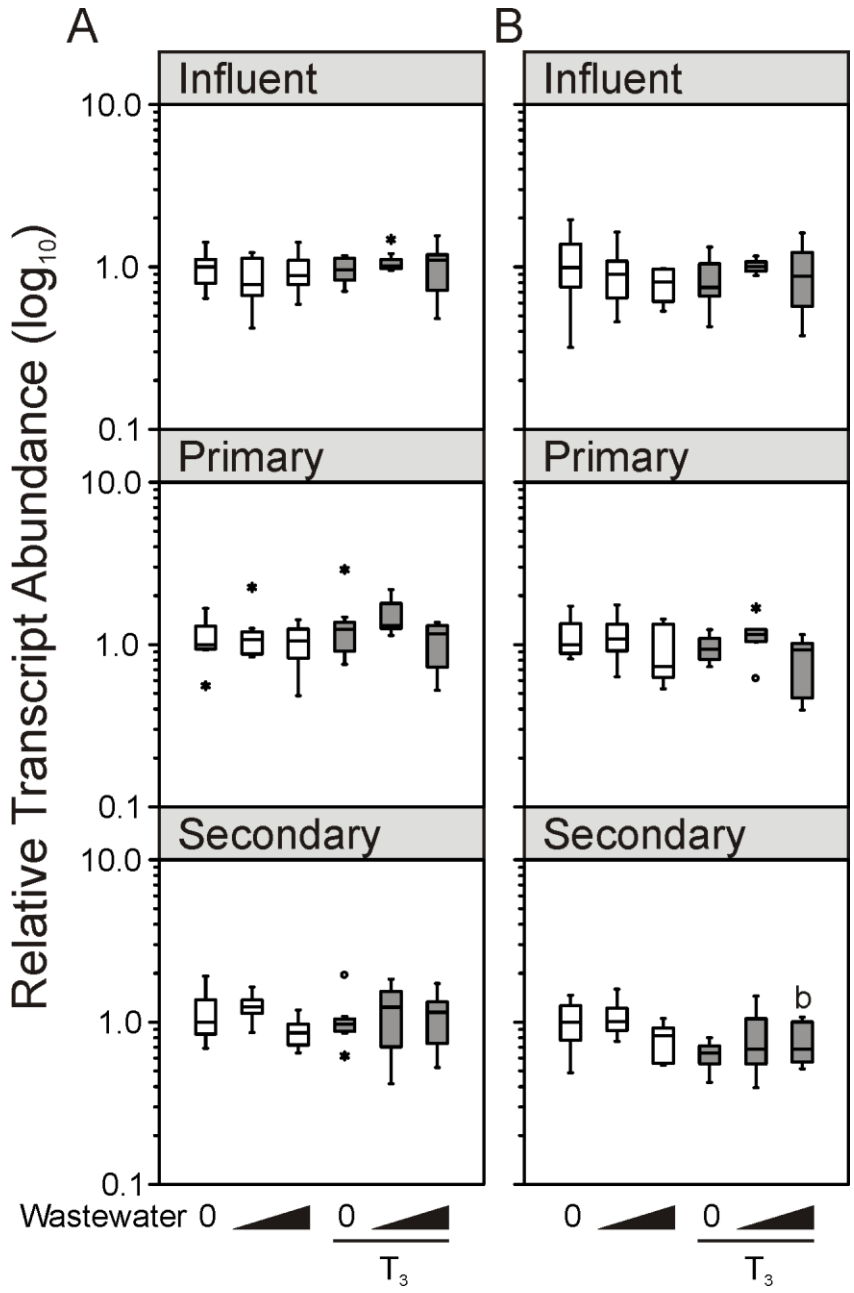

Figure 2.3 Effects of municipal wastewater influent, primary treated wastewater, and secondary treated wastewater on A) *sod* and B) *cat* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Refer to the Figure 2.2 legend for details.

The indicator of general cellular stress (*hsp30*) however, was perturbed by wastewaters (Figure 2.4). In both test conditions (with and without T_3), *hsp30* transcripts showed a notable increase due to influent and primary-treated wastewater exposure. However, this

increase was not evident upon exposure to secondary treated effluents. There was an ~85% reduction of influent perturbations to *hsp30* after secondary treatment suggesting effective removal of this biological activity. The heat shock protein (HSP) family functions under cellular stress conditions to bind and inhibit protein misfolding (Heikkila, 2010). Some members of the HSP family have been implicated in altered energy metabolism in fish exposed to MWWTP effluents (Ings et al., 2011; Ings et al., 2012). Chemical fates of ECs in MWWTPs suggest that primary treatment is expected to minimally remove a small portion of compounds while secondary treatment removes the majority of contaminants via biodegradation (Oulton et al., 2010; Ternes et al., 2004). Secondary treatment of the wastewaters significantly abated the *hsp30* effects induced by raw or partially treated waters suggesting that not only is secondary treatment important in enhanced biological degradation of ECs but it is also crucial in reducing biological effects relating to general stress.

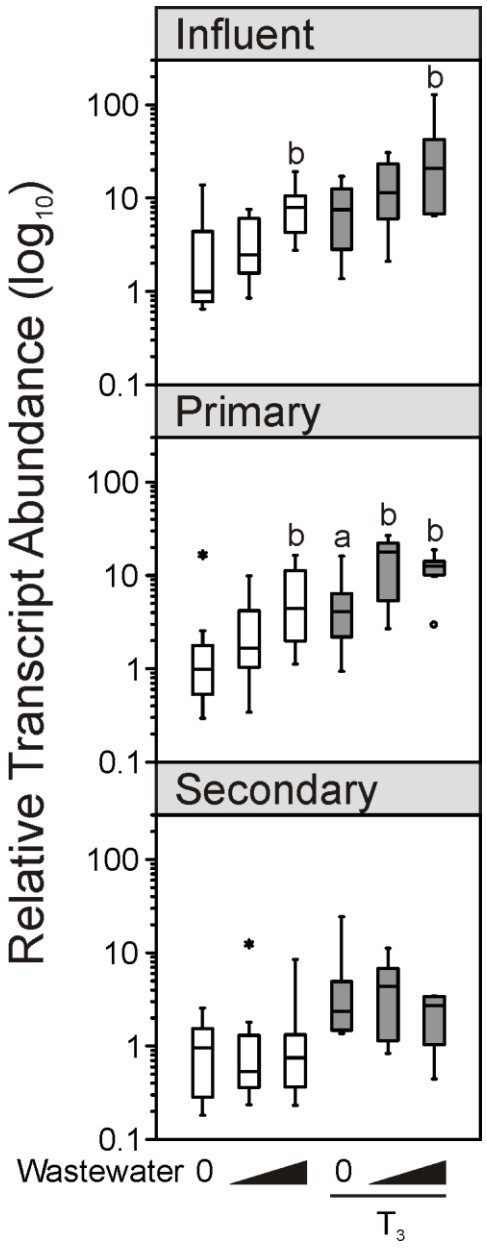

Figure 2.4 Effects of municipal wastewater influent, primary treated wastewater, and secondary treated wastewater on *hsp30* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Refer to the Figure 2.2 legend for details.

The full-scale conventional activated sludge plant in the present study achieved good removal of stress-perturbing activity and select emerging contaminants in successive units of the treatment train analyzed. Nevertheless, the ability to remove endocrine

disrupting biological activities was not necessarily linked to removal of measured contaminants stressing the need to use a combination of tests for evaluating EDC effects in municipal wastewaters.

3 Impact of Wastewater Treatment Configuration and Seasonal Conditions on Thyroid Hormone Disruption and Stress Effects in *Rana catesbeiana* Tailfin

Adapted for this thesis from: Wojnarowicz, P., Ogunlaja, O.O., Xia, C., Parker, W.J., Helbing, C.C. Impact of wastewater treatment configuration and seasonal conditions on thyroid hormone disruption and stress effects in *Rana catesbeiana* tailfin. *Environmental Science and Technology* 47, 23 (2013), pp. 13840-13847.

Pilot wastewater plants were setup and maintained at the Environment Canada Wastewater Technology Centre (WTC) in Burlington, ON by Olumuyiwa O. Ogunlaja a student of Dr. Wayne J. Parker at the University of Waterloo in Waterloo, ON. Wastewater sampling and conventional water quality analysis was done by Chen Xia.

3.1 Introduction

Downstream of MWWTPs, the potential impacts of emerging contaminants on aquatic biota are a growing concern. MWWTPs are major point sources of contaminants such as PPCPs, surfactants, plasticizers, pesticides, and flame-retardants (Kolpin et al., 2002; Lishman et al., 2006; Onesios et al., 2008). Many of these contaminants are known to be EDCs (Servos et al., 2005; Ternes and Daughton, 1999; Ternes et al., 2004). Estrogens and estrogen-active compounds in particular are the most heavily studied EDCs in MWWTP effluents (Miège et al., 2009). In contrast, very little is known regarding the efficacy of removal of THs and TH-active EDCs in MWWTPs (Svanfelt et al., 2010). In a recent study of TH-levels in Finnish waters, Svanfeldt *et al*. (2010) found levels of up to 22 ng/L T_4 in MWWTP effluent; a level that is highly bioactive.

THs are important in all vertebrates to control diverse cellular processes such as cellular differentiation and metabolism (Tata, 2006). In humans, proper TH signaling is important throughout life and is particularly important during the perinatal period for normal growth and development of the central nervous system. In amphibians, THs drive the metamorphosis of a tadpole into a juvenile frog. The fundamental mechanisms of TH action are conserved among vertebrates. T_4 is released from the thyroid gland into circulation where it can be converted to the more bioactive form, T_3, by deiodinase enzymes in target tissues (Galton and Munck, 1981; St Germain and Galton, 1997). T_3 primarily exerts its effects by binding to nuclear Thrs to mediate transcription of TH-responsive genes (Shi, 2009; Shi, 2000). As TH is so crucial in all vertebrates, there is potential for permanent deleterious effects caused by exposure to TH-active compounds in municipal wastewater effluents entering receiving environments, even at parts per trillion levels.

EDCs, in the context of wastewater effluents, pose complex toxicological challenges. Primarily, many EDCs are known to have non-monotonic dose response curves (Kendig et al., 2010; Vandenberg et al., 2012), which can result in unexpected effects at low, environmentally relevant concentrations. Additionally, aquatic organisms are virtually never exposed to one single EDC. Receiving waters are a complex milieu of potential EDCs in dynamic flux dependent on daily influents. Because of these two factors, high dose lab exposures of individual contaminants of concern are of limited utility when extrapolating to expected effects of effluents on organisms in receiving environments.

Chemical analyses as the sole endpoints determining the efficacy of emerging contaminant removal in MWWTPs should be treated with caution. Even in systems that

have a seemingly high percentage of removal of EDCs, effluents are still capable of causing biological effects (Osachoff et al., 2013). Additionally, comparing full scale MWWTPs has proven challenging, as effluents are dependent on influent composition, which fluctuates considerably between cities, seasons, and dates. In order to accurately compare the removal efficiencies of secondary treatment processes and to determine optimal operational parameters of those processes, Miège *et al*. (2009) suggested that pilot studies with carefully controlled influents as well as coordinated operational parameters, such as controlled alterations in sludge retention times (SRTs) and temperature, are necessary.

Limited *in vitro* approaches to studying TH-disruption by wastewater effluent extracts have been reported (Inoue et al., 2011; Ishihara et al., 2009; Jugan et al., 2009; Shi et al., 2009) but methods that study extract effects on protein fragments or synthetic reporter gene expression in non-native systems such as yeast are biologically limited with a high likelihood of false-negatives. A few studies have investigated the effects of wastewater effluents on amphibians (Ruiz et al., 2010; Searcy et al., 2012; Sowers et al., 2009) linking effluent exposures to morphological abnormalities, alterations in mRNA transcript levels, and/or metamorphic rates, but such assays require large animal numbers and are costly and time-consuming. We recently developed the C-fin assay (Hinther et al., 2010a) to rapidly and effectively identify disruption of TH signaling and/or identify a stress response. The C-fin assay assesses gene transcript abundance within standardized tailfin biopsies from the globally-distributed North American bullfrog (*Rana catesbeiana*) tadpole. One tadpole yields multiple biopsies that are individually cultured and exposed to a variety of conditions designed to establish 1) TH-responsiveness on a

per-animal basis, 2) the ability of effluent to affect key gene transcripts important in TH- and stress-related signaling, and 3) the ability of effluent to disrupt a TH-dependent tissue response. Therefore with multiple biological replicates, a powerful repeated measures approach for assessing exposure to MWWTP effluents can be taken. The C-fin approach has successfully been applied previously to a variety of chemical compounds (Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012), but application to complex mixtures has not yet been done.

The present study is part of a larger initiative aimed at determining the efficacy of emerging contaminant removal within existing treatment trains relevant to Canadian operating conditions. Herein, we compare the effluents from three pilot secondary MWWTP process configurations representing three commonly-used, established technologies receiving the same municipal influent by investigating the biological effects of the resultant effluents on *Rana catesbeiana* tadpole tailfin tissues. Gene transcript abundance measurements using QPCR were used to assess the biological impact of wastewater effluent exposure. Two pivotal genes, *thra* and *thrb*, that encode TH receptor alpha and TH receptor beta proteins, have been used as indicators of TH disruption (Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012; Inoue et al., 2011; Searcy et al., 2012). As wastewaters are complex mixtures of pathogens, excessive nutrients, pesticides, heavy metals, pharmaceuticals and personal care products, it is reasonable to expect that stress responses in biota of receiving environments might be significantly affected by effluent exposure. Changes in the abundance of *hsp30* mRNAs are an indicator of general stress (Heikkila, 2004; Heikkila, 2010; Helbing et al., 1996) whereas oxidative stress is frequently indicated by mRNAs

encoding *sod* and *cat* (Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012; Menon and Rozman, 2007). The latter two stress-indicators have been used previously in effluent exposure studies (Gagné et al., 2013; Sturve et al., 2008) but rarely are these endpoints studied in non-fish species.

3.2 Materials and Methods

3.2.1 Pilot Wastewater Treatment Plants

Three pilot wastewater treatment plants situated at the Environment Canada Wastewater Technology Centre (WTC) were employed for the research. The WTC receives authentic City of Burlington municipal wastewater *via* the Burlington Skyway Wastewater treatment plant. This wastewater is considered typical since it is primarily from residential sources with limited commercial, institutional, and industrial inputs. Schematics of the pilot plants used for the study are shown in Figure 3.1, the design parameters in Table 3.1, and operating conditions in Table 3.2.

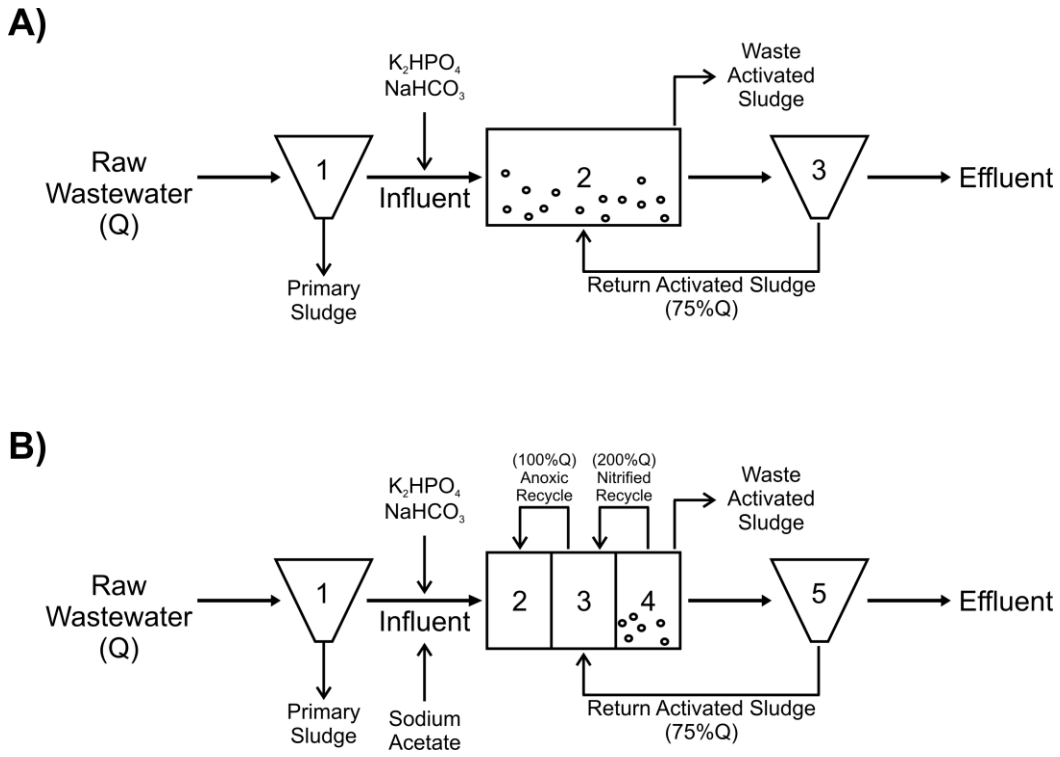

Figure 3.1 Schematic diagram of pilot-scale A) CAS and NAS systems 1) primary settler, 2) aerobic cell, 3) final settling tank; and B) BNR system 1) primary settler, 2) anaerobic cell, 3) anoxic cell, 4) aerobic cell, 5) final settling tank. "Q": raw wastewater volumetric flow rate.

Table 3.1 Pilot plant design parameters

Unit	Parameter	Quantity
Primary settler	Area	0.46 m^2
	Depth	1.56 m
Activated sludge	Pass Volume	6 X 60 L
	Depth	1.28 m
Final settling tank	Area	0.204 m^2
	Depth	1.4 m

Table 3.2 Pilot plant operating conditions

Operating Parameter	Winter Setting	Summer Setting
Flow rate	864 L/d	1282 L/d
Temperature	12 °C	18 °C
SRT	CAS = 6 d	CAS = 3 d
	NAS = 20 d	NAS = 10 d
	BNR = 40 d	BNR = 20 d
Activated sludge DO	CAS = 2 - 2.5 mg/L	
	NAS = 4 - 4.5 mg/L	
	BNR (aerobic) = 4 - 5 mg/L	
	BNR (anoxic) = 1 - 2.5 mg/L	
	BNR (anaerobic) = 0 - 0.2 mg/L	
HRT (Avg nominal)	7 h	
BNR internal recirculation split factor	Anerobic = 2Q L/d	
	Anoxic = Q L/d	

The conventional activated sludge (CAS), nitrifying activated sludge (NAS), and biological nutrient removal (BNR) pilots were all fed from a common primary clarifier that received the raw wastewater. The activated sludge reactors were segmented into six cells (60 L each) to simulate pseudo-plug flow and coarse bubble aerators were used for aeration and mixing (in a cyclic pattern; high air flow at 40 L/min and low air flow at 10 L/min) in the CAS, NAS, and the aerated zones of the BNR. An insulated water jacket with an automatic temperature controller was installed around the bioreactors to control the temperatures of the bioreactors. Di-potassium phosphate (K_2HPO_4) (11 g/L at the rate of 14.4 L/d) and sodium bicarbonate ($NaHCO_3$) (22 g/L at the rate of 14.4L/d) were

added to the clarified influent stream to ensure the system was not phosphorus limited and to provide a pH buffer for the system.

The CAS pilot was a single sludge aerobic system operated at SRTs of 6 and 3 days (winter and summer respectively) to facilitate biochemical oxygen demand removal without nitrification. The NAS pilot was a single sludge aerobic system operated at an SRT of 20 and 10 days during the winter and summer conditions, respectively. These SRTs enable the proliferation of autotrophic nitrifying bacteria for consistent nitrification. The BNR process consisted of anaerobic, anoxic, and aerobic zones and was operated at an SRT of 40 and 20 days during winter and summer conditions, respectively. This mode of operation was designed to achieve nitrification, denitrification, and enhanced biological phosphorous removal. To achieve an appropriate range of chemical oxygen demand to phosphorus (COD/P) ratio that will support a healthy BNR activated sludge, the clarified influent wastewater stream to the BNR bioreactor was augmented with an exogenous source of readily biodegradable organic substrate (34.36 g/L sodium acetate at the rate of 14.4 L/d).

The pilot plants were operated for over 365 days with consistent monitoring for over 180 days to ascertain stable operation in terms of biomass and effluent characteristics so as to enable efficient comparison among the three treatment trains. Stable plant performance was ascertained by monitoring the following parameters: SRT, Temperature, COD, NH_3-N, total phosphorus (TP), soluble PO_4-P, NO_3-N, total suspended solids (TSS), volatile suspended solids (VSS), pH, mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS).

3.2.2 Water Sampling and Handling

Composite samples were collected from all three systems over four dates (November 8, November 22, December 1, and December 9, 2010 – sampling points 1-4) for winter conditions and three dates (June 14, June 23, and July 19, 2011 – sampling points 5-7) for summer conditions. The samples were immediately filtered with a 1.5 μm glass microfiber filter (Whatman, Toronto, ON, Canada), concentrations of ammonia, nitrite, and nitrate were measured as per Standard Methods (APHA, 2005), and sent on wet ice by courier to Victoria, BC for C-fin assays performed the following day. Samples were sterilized with a 0.2 μm nylon filter (Nalgene – Thermo Fisher, Rochester, NY, USA) before dilution and application to the organ cultures.

3.2.3 Experimental Animals

Premetamorphic (TK stages VI-VIII) *Rana catesbeiana* tadpoles were used for the C-fin assays. Care and treatment of the animals was as in Chapter 2.

3.2.4 C-fin Assays

Tailfin biopsy cultures (C-fin assays) were performed according to methods previously described in Chapter 2. In brief, one complete C-fin assay was performed per secondary wastewater treatment type per collection day (n=8 animals/C-fin). Six 4 mm tailfin biopsies (Miltex, Plainsboro, NJ, USA) were taken from each animal and individually cultured in medium for 48 h to test six treatment conditions: three treatments with 10 nM T_3 in 400 nM NaOH, and three treatments without T_3 (400 nM NaOH only; T_3-vehicle control). Each sterilized wastewater sample was tested in the presence or absence of T_3 at 20% and 50% serial dilutions. At the end of the 48 h incubation period, each biopsy was stored in 100 μL of RNAlater for 24-48 h at 4°C and then transferred to -20°C until processed for total RNA.

3.2.5 Isolation of RNA and Quantitation of Gene Transcript Abundance

RNA isolation and cDNA was prepared according to methods described in Chapter 2. Transcript abundance of *thra, thrb, sod, cat, hsp30, rpl8, rps10,* and *eef1a* were determined as described in Chapter 2 with the exception that the *eef1a* and *rps10* primers which were run as a multiplex reaction with primers and probes as described in Table A.1. *eef1a* and *rps10* reaction details were as previously described for *thra, thrb, rpl8,* and the thermocycle program was as previously described for *eef1a* and *rps10* in Chapter 2. The three normalizer transcripts were deemed to be suitable normalizers using RefFinder (http://www.leonxie.com/referencegene.php?type=reference) as in Chapter 2.

3.2.6 Statistical Analysis

Statistical analyses were performed according to methods in Chapter 2.

3.3 Results

3.3.1 Conventional Wastewater Effluent Quality Parameters

The pilot plants were monitored with respect to the removal of conventional wastewater pollutants such as carbonaceous biochemical oxygen demand ($cBOD_5$) and nitrogen species. This data was employed to establish whether the treatment plants were removing conventional contaminants within normally established ranges and to provide insight into the types of microbial metabolism (i.e. aerobic heterotrophic growth, anoxic heterotropic growth, aerobic autotrophic growth) that were active in the bioreactors. Influent median $cBOD_5$ and total kjeldahl nitrogen (TKN) were 45/55 mg/L and 30/34 mg/L (winter/summer conditions), respectively. Considering the inherent variability of processes treating authentic wastewaters, the effluents from the pilot plants were relatively consistent across seasons (Table 3.3). This implied that the biological exposures received samples of consistent conventional pollutant composition over time.

Carbonaceous BOD_5 was consistently removed in all pilot plants in both phases of operation and most final concentrations were less than 10 mg/L. This is indicative of good removal of biodegradable organic matter. The BNR and NAS processes produced the lowest effluent $cBOD_5$ concentrations while the effluent concentrations from the CAS process were slightly elevated (Table 3.3).

Table 3.3 Effluent concentrations of conventional responses. All values are average (standard deviation) in mg/L; TAN: total ammonia nitrogen, TKN: total kjeldahl nitrogen, $cBOD_5$: carbonaceous biochemical oxygen demand.

Winter	TAN	TKN	NO_3-N	NO_2-N	$cBOD_5$
CAS	14.8 (5.6)	18 (5.3)	6.3 (5.2)	0.4 (0.6)	8.2 (9.7)
NAS	0.2 (0.3)	1.8 (0.3)	24.1 (4.2)	0.1 (0.2)	3.8 (3.5)
BNR	0.7 (1.2)	2.4 (1.9)	6.1 (2.7)	0.1 (0.6)	2.9 (1.6)
Summer	TAN	TKN	NO_3-N	NO_2-N	$cBOD_5$
CAS	21.1 (6.4)	21.4 (5.7)	1.7 (4)	0.4 (0.6)	13.3 (10.1)
NAS	0.076 (0.039)	1.2 (0.3)	22 (2.6)	0.013 (0.018)	3.5 (1.7)
BNR	1.3 (2.1)	2.4 (2.3)	5.4 (2.2)	0.3 (0.4)	6.4 (6)

Based upon the operating conditions that were employed in the present study, it was expected that the effluents from the CAS pilot would have elevated TKN concentrations and total ammonia nitrogen (TAN) and low concentrations of NO_3-N and NO_2-N. In winter conditions, the CAS pilot partially nitrified as the concentrations of NO_3-N in the effluent were somewhat elevated at times and there were sporadic events when the TAN decreased (Table 3.3). In summer conditions, with the exception of a short interval at the beginning of the test (data not shown), the NO_3-N concentrations were consistently low

and the effluent TKN and TAN concentrations remained elevated. The NAS pilot was expected to have low concentrations of TKN and TAN and elevated concentrations of NO_3-N in the effluent. These patterns were consistently observed in both running conditions. This indicated that this pilot was nitrifying effectively. The BNR pilot was expected to have low effluent concentrations of TKN, TAN, NO_3-N and NO_2-N. These patterns were consistently observed in both running conditions. The data therefore suggest that this plant was effectively nitrifying and denitrifying. Collectively, the pilots were achieving levels of treatment that are typical of their respective operations at technical scale.

3.3.2 Thyroid Hormone Receptor Gene Transcript Abundance

Transcript abundance of both *thra* (Figure 3.2) and *thrb* (Figure 3.3) in the *Rana catesbeiana* tailfin tissue was always increased by 10 nM T_3 treatment (relative to NaOH vehicle controls) by ~2-fold and ~15-fold, respectively. This important internal test for competence to respond to TH active compounds is highly consistent within the present study and with past C-fin data (Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012).

The concentrations of effluents tested (20 and 50%) corresponded with those concentrations in which no rainbow trout mortalities occurred in concurrent exposure studies (data not shown). *thra* transcript abundance was rarely perturbed by CAS and NAS effluents produced both winter and summer settings (Figure 3.2). BNR effluents produced with winter conditions caused no significant effects on *thra* mRNA levels (Figure 3.2). In contrast, BNR effluent from summer conditions produced five significant perturbations up to 2-fold (Figure 3.2).

thrb was also differentially affected by winter and summer settings (Figure 3.3). In winter settings, CAS effluent exposure resulted in three significant perturbations up to 2-fold in *thrb* transcript abundance, whereas exposure to NAS- or BNR-derived effluents had no effect (Figure 3.3). However, the same pattern observed with the *thra* transcripts upon BNR summer effluent exposure occurred with *thrb* transcripts as well (Figure 3.3) which is highly suggestive of the presence of TH signaling pathway disruptive activity.

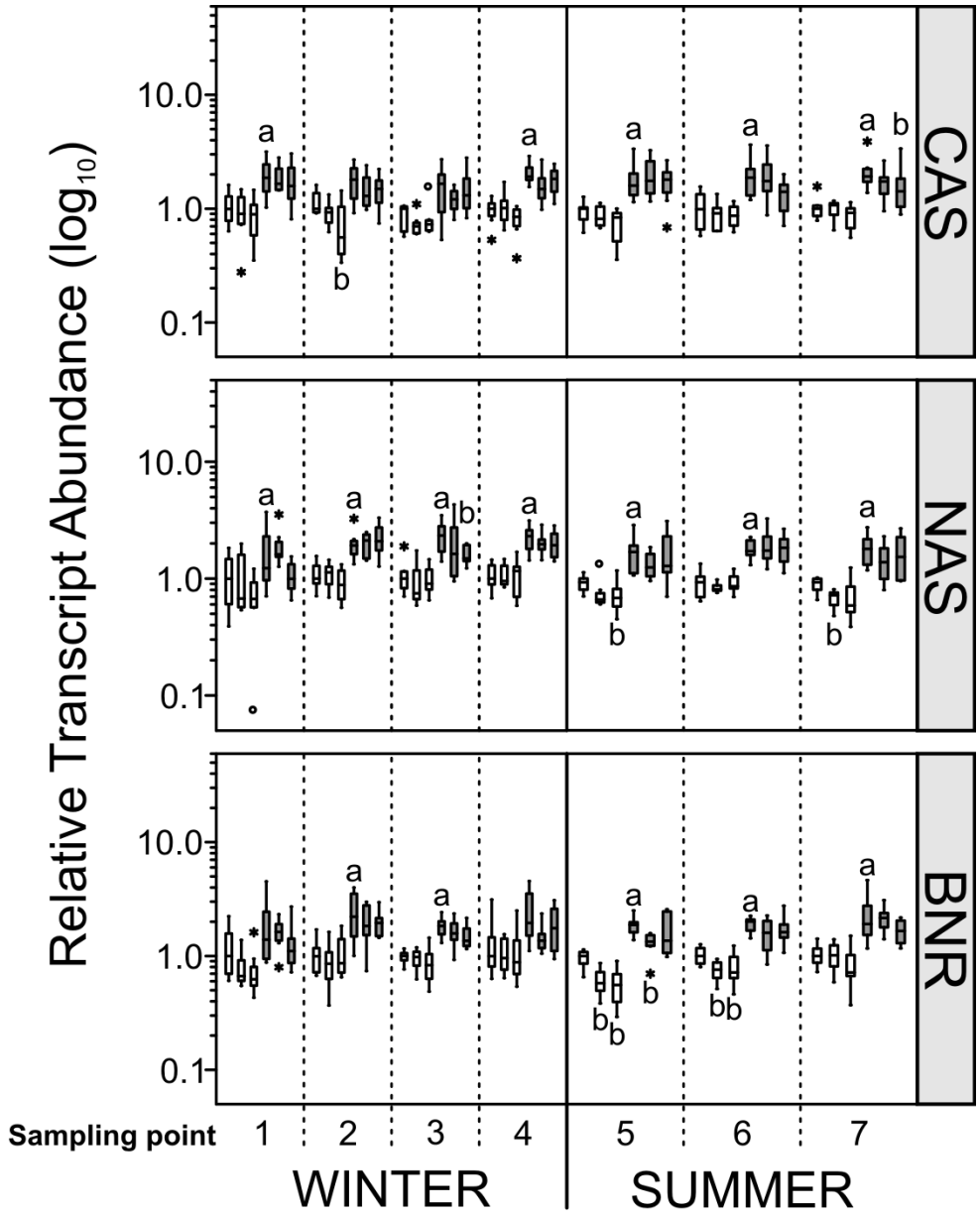

Figure 3.2 Effluent effects from CAS, NAS, and BNR systems run under winter and summer conditions on *thra* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Tissues from eight biological replicates were cultured for 48 h with effluents from each sampling point in two test conditions: with T_3-vehicle control (400 nM NaOH – white boxplots), and with 10 nM T_3 in 400 nM NaOH (grey boxplots). Each test condition includes treatments of 0%, 20%, and 50% concentrations of effluents (left to right). For details regarding the boxplots refer to Figure 2.2. "a": significance of 10 nM T_3 (T_3-only control) to 400 nM NaOH (T_3-vehicle control) ($p < 0.05$; Wilcoxon signed-rank test). "b": significance relative to no-effluent-added control within a test condition ($p < 0.05$; Wilcoxon signed-rank test).

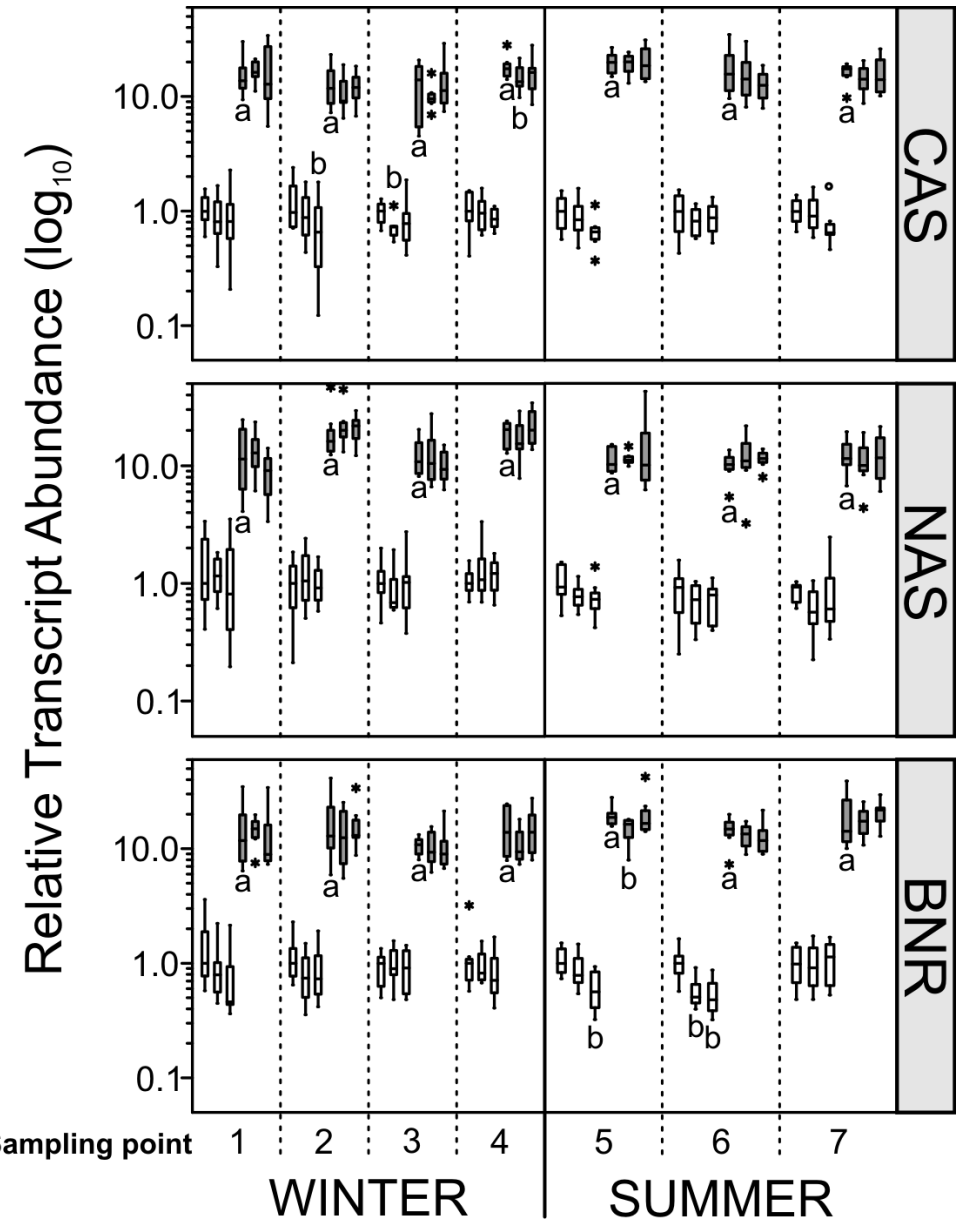

Figure 3.3 Effluent effects from CAS, NAS, and BNR systems run under winter and summer conditions on *thrb* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Refer to Figure 3.2 legend for details.

3.3.3 Stress Responsive Gene Transcript Abundance

Overall, very few impacts with no discernible pattern were observed on stress-related transcripts upon exposure to any of the effluents used in the present study. While *sod* and

cat were essentially not responsive to T_3 treatment in the C-fin assay (Figure 3.4 and Figure 3.5, respectively), the transcript abundance of heat shock protein 30 (*hsp30*), an indicator of general stress, was marginally increased by T_3 treatment compared to the NaOH vehicle control (Figure 3.6) consistent with past C-fin data (Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012).

Very few perturbations of indicators of oxidative stress, superoxide dismutase (*sod*; Figure 3.4) and catalase (*cat*; Figure 3.5), occurred due to wastewater effluent exposures irrespective of whether T_3 was present or not. In summer conditions, there was a slight increase in BNR effluent effects on *sod* as compared to the CAS and NAS effluents (Figure 3.4), but this trend was not paralleled in the winter settings. *cat* gene transcript abundance (Figure 3.5) in both winter and summer settings was minimally affected by all three wastewater effluents.

Wastewater exposure effects on *hsp30* gene transcript abundance were also minimal in both winter and summer wastewater treatment running conditions (Figure 3.6). Exposure to CAS or BNR effluents produced under winter settings resulted in essentially no changes on *hsp30* transcript abundance while the NAS-derived effluent showed some perturbations (Figure 3.6). Effluents produced from the treatments run using summer settings also produced minimal overall effects with no indication of perturbation in NAS-derived effluent (Figure 3.6).

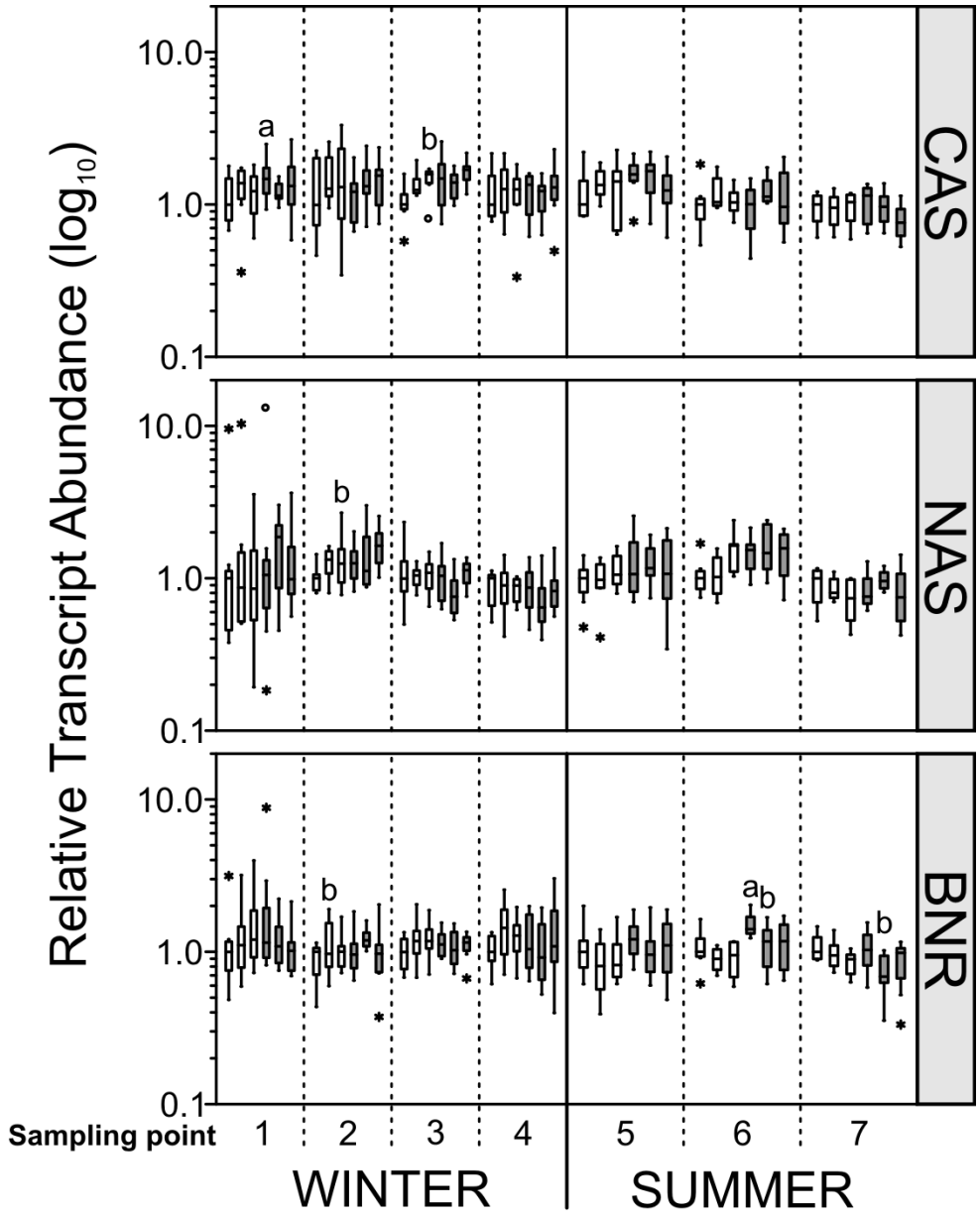

Figure 3.4 Effluent effects from CAS, NAS, and BNR systems run under winter and summer conditions on *sod* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Refer to Figure 3.2 legend for details.

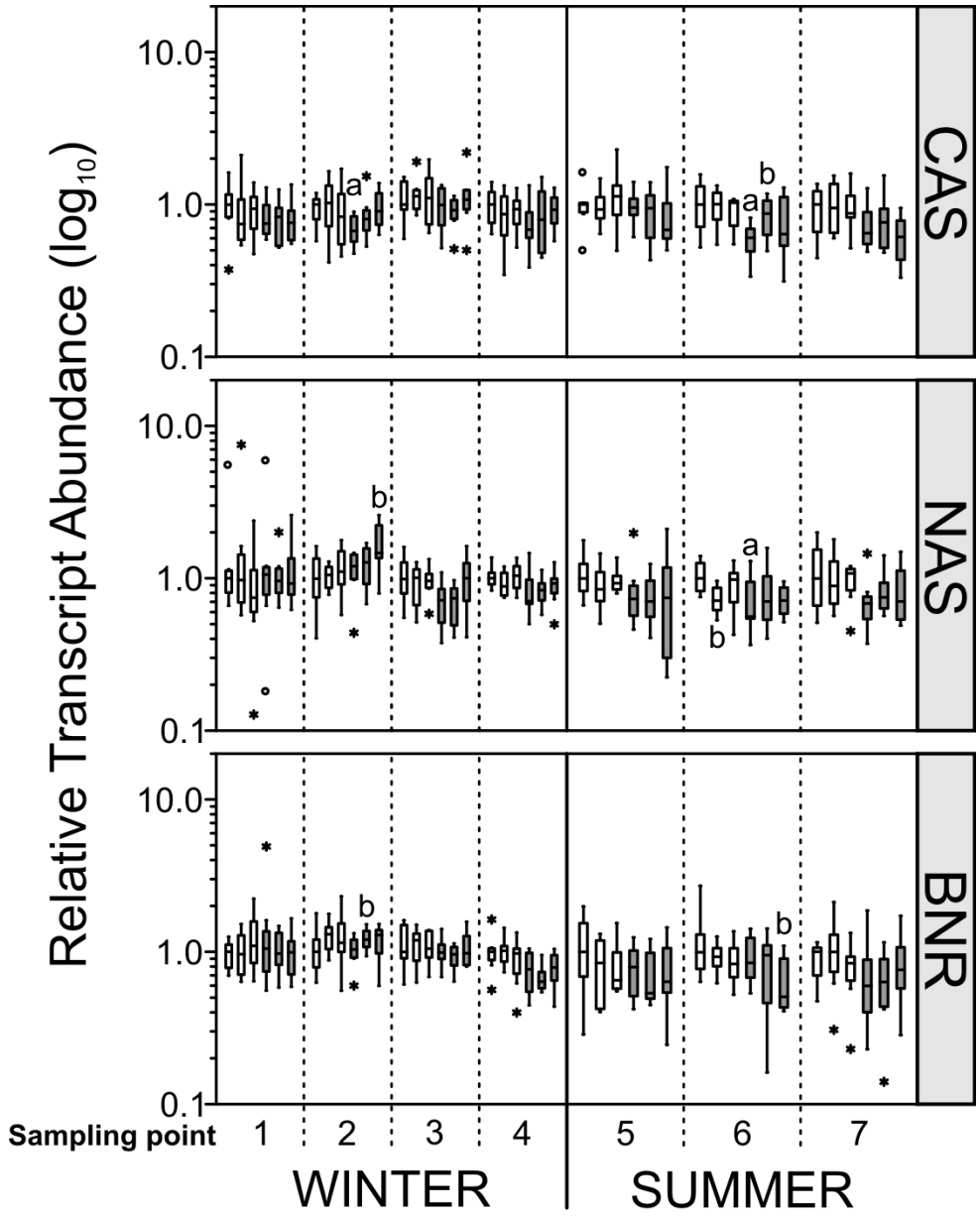

Figure 3.5 Effluent effects from CAS, NAS, and BNR systems run under winter and summer conditions on *cat* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Refer to Figure 3.2 legend for details.

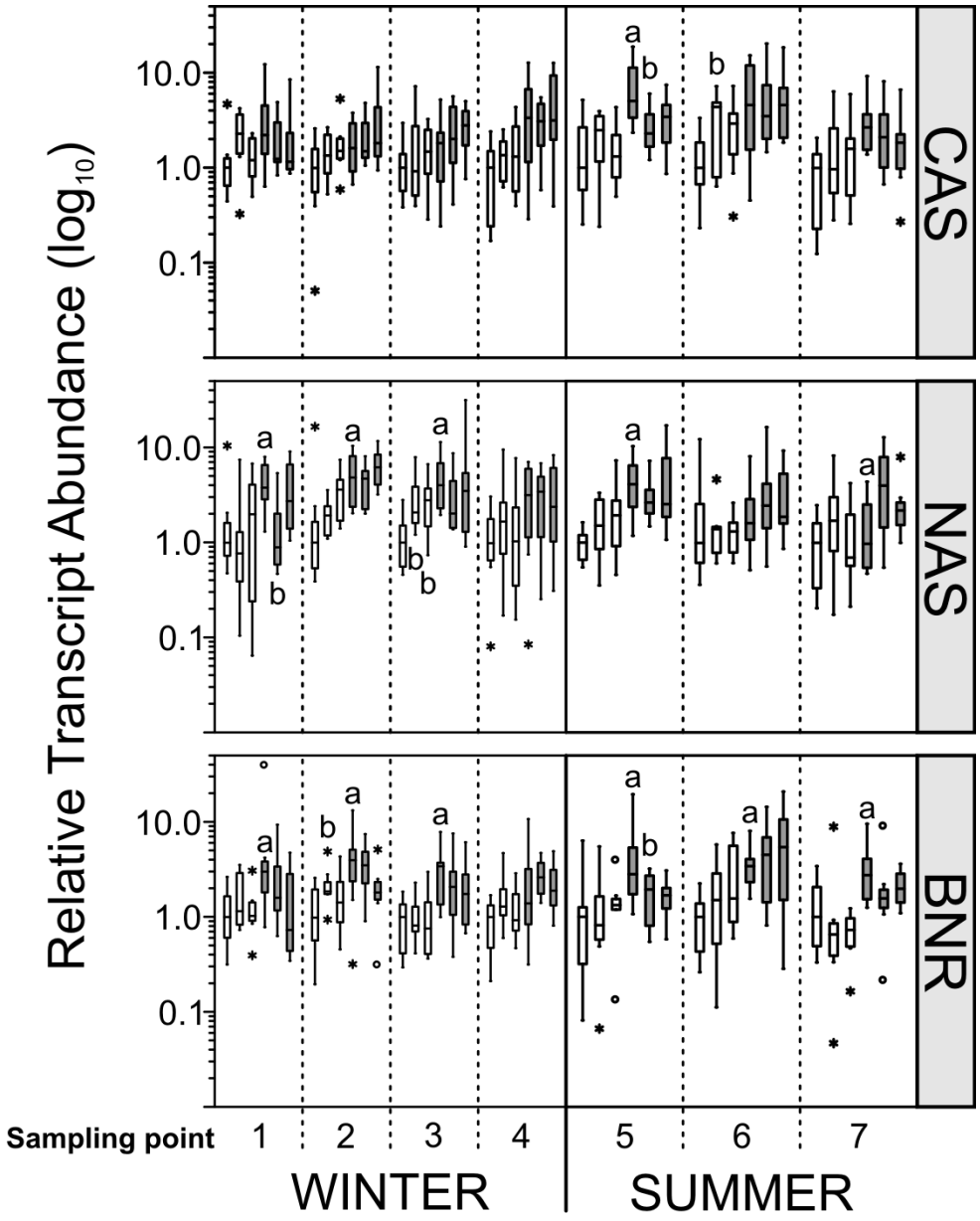

Figure 3.6 Effluent effects from CAS, NAS, and BNR systems run under winter and summer conditions on *hsp30* transcript levels in *Rana catesbeiana* tadpole tailfin biopsies as determined by QPCR. Refer to Figure 3.2 legend for details.

3.4 Discussion

3.4.1 Effects of MWWTP Effluents on Bullfrog Tailfin

Our results indicate a potential for complex effluents from MWWTPs to act more distinctly on hormone systems than stress systems when evaluating effects on amphibian tissue. In the present study, both the general stress indicator (*hsp30*) and oxidative stress responsive gene transcripts (*sod* and *cat*) were seldom perturbed by effluents from all three wastewater treatment processes. Moreover, the high quality of RNA and consistent normalizer transcript data obtained from the biopsy samples also supported the conclusion that the effluents were not overtly toxic.

Tadpole metamorphosis is the most sensitive developmental process dependent upon TH action and for this reason is suggested as a Tier 1 thyroid assay by the OECD (2009). However, the OECD screen depends on a species that is not relevant for most climates and is solely based on morphological endpoints, which are less sensitive than molecular endpoints. The C-fin assay uses a native, "true frog" (*Rana*) species in a format designed to take advantage of the hormone-responsiveness of the tailfin tissue combined with direct internal verification of TH-responsiveness and a method to reduce animal use while maintaining biological complexity. In conjunction with this, rather than screening individual compounds of concern or limited extracted portions of effluents as other *in vitro* methods have, the C-fin is amenable to screening whole, complex mixtures of wastewater effluents which otherwise might be falsely deemed as "safe" for TH systems of animals in receiving environments.

The TH-disrupting effects, in the BNR wastewater effluents in particular, were the most revealing measure of emerging contaminant removal efficiency between all three secondary treatment processes tested in the present study. The BNR system consistently

removed the widest variety of conventional contaminants in the present study (as compared to the CAS and NAS systems). Although removal efficiencies of EDCs were not determined (as their identities are currently unknown), the present data adds to the mounting evidence that higher MWWTP removal efficiencies do not necessarily result in lower endocrine-activity of effluents. As Osachoff *et al*. (2013) recently observed in a CAS system, although known estrogen-active contaminants in a synthetic sewage were efficiently removed (40-99.6%) as determined by chemical analysis, vitellogenin (a biomarker of estrogenic activity) transcript and protein levels in rainbow trout livers were not significantly decreased in effluents as compared to influents, while transcripts of stress pathways were less affected in general by the EDC-spiked synthetic sewage. Gonzalez-Rey and Bebianno (2012; 2013) also found a higher health impact on mussels by estrogenic endocrine disruption (as indicated by alkali-labile phosphate levels) as compared to the effects on superoxide and catalase activities in mussels exposed to two common emerging contaminants (ibuprofen and fluoxetine). Taken together, these low-dose response indicators of sublethal EDC effects (altered transcript, protein, and enzyme activity levels) by emerging contaminants in treated effluents suggest a potential for hormone disruption of biological systems below levels which engage the organism's first line of defense – stress.

3.4.2 Comparing Secondary Wastewater Treatment Configurations and Operational Parameters

As previously indicated, the effluents produced from the CAS, NAS, and BNR systems did not reveal one single MWWTP system as the preferred choice for year-round reduction in biological effects. In fact, opposing conclusions emerged regarding the extent of TH-disrupting activities from each treatment system depending upon which

seasonal conditions were run. This was most evident with the BNR system which, under winter conditions (40 day SRT at 12°C), performed best out of the three treatments with no evidence of TH-disrupting activities. However under summer conditions in the BNR (20 day SRT at 18°C), there were clear indications of alterations in abundance of both Thr-encoding transcripts.

It has been suggested that SRT is the major design parameter that can be used in evaluating the efficacy of removal of emerging contaminants from MWWTPs (Clara et al., 2005a). SRT was the major difference in design between the CAS and NAS systems in the present study (both pilots were completely aerobic reactors running at different SRTs). If SRT were the major design parameter affecting removal efficiency, we would expect the same pattern of effects between the CAS and NAS effluents independent of running temperatures. Both *hsp30* and *thrb* responses did not support this hypothesis as the relationship between CAS and NAS effluent effects changed dramatically depending on the temperature. Additionally, when taking into account MWWTP configuration, we would also expect the relationship of effects between the three systems to remain consistent at all running temperatures. As noted previously however, the BNR system in warmer temperatures performed the worst of the three systems in its ability to reduce TH-disrupting effects.

Previous studies of removal efficiencies of specific EDCs in MWWTP effluents were higher in winter and lower in summer (Sui et al., 2011; Vieno et al., 2005; Yu et al., 2013). However, our BNR effluent effects results (as measured by the C-fin assay) appear to contradict this seasonal pattern. As the enzymatic reaction rates and growth of activated-sludge bacteria increase with increased temperature, it is reasonable to expect

increased degradation of emerging contaminants in MWWTPs during warmer temperatures. All of the aforementioned studies however, used chemical analyses of wastewater as the sole evaluation of emerging contaminant removal efficiency. A study by Fernandez *et al*. (2008), using biological effects endpoints (a yeast estrogen receptor binding screen), found results similar to the present study when examining a full-scale BNR system, as warmer temperatures in the BNR reactor created a buildup of more bioactive free-estrogens. Although these results do not fully explain what may be causing an increase in TH-activity in the summer effluents of the BNR system in the current study, this indicates that a BNR plant may less efficiently remove EDCs across temperature fluctuations and that biological effects endpoints to evaluate MWWTP systems are necessary.

To determine which MWWTP system is best at removing emerging contaminants, operational parameters such as SRT cannot practically be the sole defining factor for efficacy of removal. Although chemical analyses of removal efficiencies of EDCs often suggest better removal in warmer months, biological data is necessary to determine seasonal effects of MWWTP effluents as higher removal efficiencies do not always result in fewer biological effects. Additional studies to clarify fates and effects of TH-active EDCs in MWWTP effluents are needed in order to accurately define and manage risk to aquatic receiving environments.

4 Defining *Rana catesbeiana* C-fin-derived Transcript Profiles of Stress- and TH-responsive Genes in Response to the Endogenous Hormones T_3, T_4, and E_2

4.1 Introduction

TH is a critical modulator of vertebrate development and has been known to cause amphibian metamorphosis for over a century (Gudernatsch, 1912), yet the central question of how one hormone can cause so many diverse effects in one organism still remains. During amphibian metamorphosis, TH alone causes effects as disparate as *de novo* organogenesis of limbs to apoptosis and resorption of organs such as the tail. Studies of additional endogenous regulators such as stress or crosstalk with other steroid hormones, including estrogens, have been implicated in altering TH-effects in amphibians (Hayes, 1997; Kashiwagi et al., 1999; Kulkarni and Buchholz, 2012; Vasudevan et al., 2002) but few clear distinctions in the mechanism of action of TH, the sole hormone necessary for amphibian metamorphosis, have been elucidated to define TH pleiotropy.

The diverse biological effects of TH may primarily be attributed to the two different main TH forms: T_4 and T_3. In the classical view, T_4 is often referred to as the prohormone that is released from the thyroid gland and distributed to peripheral target tissues (Gereben et al., 2008). Once in the cytoplasm, outer ring deiodinase enzymes remove a 5'-iodine from T_4 to produce T_3. It is this form that is translocated to the nucleus and alters TH-responsive gene transcription by binding to Thrs that interact with TREs in the promoters of TH-responsive genes (Cordeiro et al., 2013; Fondell, 2013; Grimaldi et al., 2013). In the absence of ligand (without TH), transcription is repressed by Thrs through TRE binding. In the presence of TH, Thrs activate transcription (Bernal and Morte,

2013). Because of the dependence upon outer ring deiodinase (Dio2) conversion, T_4 is often referred to as a T_3-prohormone and the biological effects of TH are presumed to be mediated through this conversion. However, studies examining nuclear binding affinities of T_3 and T_4 to nuclear receptors suggest that T_3-Thr affinity is approximately 5-10 times that of T_4-Thr (Cheek et al., 1999; Frieden, 1981; Gagnon et al., 1992; Latham et al., 1976). Furthermore, T_3 was ~5 times more potent at inducing direct-response gene transcript abundance compared to T_4, but this observation was tissue-dependent (Zhang et al., 2006). This raises questions as to the relative importance of T_4 directly acting on Thrs *versus* Dio2-mediated conversion of T_4 to T_3 before Thr binding. The latter view has been favoured as the mechanism of TH-mediated amphibian metamorphosis because, with some notable exceptions, there is widespread 5'-deiodinase activity distribution in tadpole tissues (Becker et al., 1997; Shi, 2000), and an inhibition of deiodinases can inhibit T_4-induced metamorphic effects (Becker et al., 1997). Interestingly, Becker *et al*. (1997) found that in *Rana catesbeiana* tadpoles treated to block endogenous TH synthesis as well as deiodinase activity (with methimazole and iopanoic acid, respectively), metamorphic processes were rescued by administration of exogenous T_3 but not T_4. These experimental results have been interpreted as indicative that the conversion of T_4 to T_3 by deiodinase is necessary for bioactivity. However, iopanoic acid treatment can also inhibit high affinity, membrane-bound solute carrier proteins required for the transport of T_4 into the cell (Westholm et al., 2009) providing an alternative mechanism for iopanoic acid. This observation, coupled with the knowledge that non-classical TH signaling pathways exist that discriminate between T_3 and T_4 involving cell surface or mitochondrial membrane receptors (Cheng et al., 2010) and the observation that T_4

replacement in hypothyroid patients does not alleviate all symptoms (Bunevicius et al., 1999) raise the question to what extent are T_3 and T_4 biologically interchangeable.

The North American bullfrog (*Rana catesbeiana*) tadpole undergoes a precocious metamorphosis upon exposure to exogenous TH and C-fin assays have recently been used to identify substances capable of disrupting TH signaling at the tissue level (Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012). The repeated measures experimental design of the C-fin assay facilitates the direct assessment of responses to multiple treatments with the same biological background. Notably, however, *Rana catesbeiana* tailfin does not have detectable Dio2 activity until metamorphic climax (Becker et al., 1997). Undetectable levels of T_4 to T_3 enzymatic conversion in premetamorphic *Rana catesbeiana* tailfin therefore make the C-fin assay an ideal system to challenge the assumption that T_4 is solely a T_3-prohormone.

In the present study, we compared the molecular responses evoked by T_3 and T_4 in the context of the C-fin assay by examining select TH- and/or stress-responsive gene transcripts. With limited Dio2 in the tailfin tissue, we hypothesized that conversion of T_4 to T_3 is likely minimal. If the T_3 and T_4 act primarily through Thr binding, then a 5X T_4 concentration (to account for ligand-binding affinity) should elicit an equivalent T_3 response on gene transcript abundance. If T_4 acts solely as a T_3-prohormone in a system without Dio2 conversion, then 5X T_4 treatments should cause far less than equivalent responses to their paired T_3 treatments. Additionally, E_2 treatment was assessed to determine specificity of the TH-mediated responses. Thr and Esr, which binds the endogenous ligand E_2, are structurally alike and recognize similar hormone response elements on the DNA (Vasudevan et al., 2002; Zhu et al., 1996).

The TH-responsive gene transcripts examined are part of the early response to the hormones: *thra* and *thrb* (Das et al., 2010), TH basic leucine zipper transcription factor (*thibz*), (Bilesimo et al., 2011; Buchholz et al., 2005), and krüppel-like factor 9 (*klf9*) (Bagamasbad et al., 2008). These represent up-regulated genes. We also examined an apparent early response gene transcript whose expression is substantially decreased by TH, *Rana* larval keratin I (*rlk1*) (Domanski and Helbing, 2007). Transcripts encoding deiodinase 2 and 3 (*dio2* and *dio3*) were also evaluated. The Dio3 enzyme removes outer-ring iodines from T_3 and T_4, inactivating them to create T_2 and rT_3, respectively (Becker et al., 1995). Stress-response gene transcripts included *sod*, *cat*, and *hsp30* that have previously been implicated in the TH-mediated response. The results demonstrate that T_3 and T_4 affect this tissue in a manner predicted by their differences in Thr affinities for some but not all TH-responsive genes tested revealing differential modes of TH action.

4.2 Materials and Methods

4.2.1 Experimental Animals

Premetamorphic (TK stages VI-VIII) *Rana catesbeiana* tadpoles were used for the C-fin assays. Care and treatment of the animals was as in Chapter 2.

4.2.2 C-fin Assays

Tailfin biopsy cultures were prepared according to the methods described previously for the C-fin assay (Chapter 2). One C-fin assay was performed for TH-dilution series and a second C-fin was performed for the E_2 dilution series. For T_3/T_4 dilution series, thirteen circular 4 mm tailfin biopsies from each animal were exposed to thirteen different treatment conditions in 96-well multi-well culture plates (BD Falcon) containing 200 µL of 1X culture medium. The thirteen treatment conditions were 0.01, 0.1, 1, 10, 25,

and 50 nM T_3 (#T2752, Sigma-Aldrich) and 0.05, 0.5, 5, 50, 125, and 250 nM T_4 (#T2501, Sigma-Aldrich) in 800 nM NaOH. T_3 and T_4 were prepared as a 1000X stock in 800 μM NaOH and were applied at 1 μl/ml of media. For the E_2 dilution series, eight circular 6 mm tailfin biopsies from each animal were exposed to eight different treatment conditions in 24-well multi-well culture plates (BD Falcon) containing 1 mL of 1X culture medium. The eight treatment conditions were 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 nM E_2 (#E4389, Sigma Aldrich) in deionized water. E_2 was prepared as a 10^{-3} M stock and serially diluted in media. Biopsies were cultured for 48 h and were subsequently stored in 100 μL of RNAlater for 24-48 h at 4°C and then transferred to -20°C until processed for total RNA.

4.2.3 Isolation of RNA and Quantitation of Gene Transcript Abundance

RNA extraction from tissues and cDNA synthesis was performed as described previously in Chapter 2. Transcript abundance of *thra*, *thrb*, *sod*, *cat*, *hsp30*, and *rpl8* was determined as described in Chapter 2, while *rps10* and *eef1a* were determined according to methods in Chapter 3. Transcript abundance of TH-induced basic leucine zipper transcription factor (*thibz*), Krüppel like factor 9 (*kfl9*), and *Rana* larval keratin 1 (*rlk1*) was determined using conditions as described for SYBR reactions in Chapter 2 and primers as described in Table A.1. The thermocycle program for *thibz*, *klf9*, and *rlk1* was as described in Chapter 2 with the exception of an annealing temperature of 62°C for *thibz* and *klf9* and an annealing temperature of 55°C for *rlk1*. Deiodinase 3 primers were previously published by Mochizuki *et al.* (2012); reactions were optimized and verified for C-fin tissues. We had attempted to use multiple *dio2*-specific primers. Despite successful validation in multiple tissues with known Deiodinase 2 activity (Veldhoen et

al., 2013), we were unable to reliably detect specific *dio2* signal in tailfin tissue. This is consistent with the previous observation that *Rana catesbeiana* tailfin has very low levels of deiodinase 2 activity (Becker et al., 1997).

4.2.4 Statistical Analysis

Nonparametric, repeated measures statistical analyses were performed using R-studio software (R-studio Inc., Boston, MA, USA). A Wilcoxon signed-rank test was used to compare each treatment to the vehicle control to assess alterations in transcript levels of each gene of interest due to hormone treatment. For the TH dilution series, each T_3 treatment was also compared to its five-times-concentrated T_4 to assess whether the relative transcript abundance was statistically different between the paired doses. Statistical analyses were performed on $\log_2$ transformed data and were considered significant at $p<0.05$. Trendlines through individual-specific gene transcript responses to T_3 and T_4 were plotted using Microsoft Excel 2011 software.

4.3 Results

4.3.1 Thyroid Hormone Receptor Gene Transcript Profiles

Transcript abundance of both *thra* and *thrb* mRNA increased with exposure to increasing concentrations of T_3 and T_4 (Figure 4.1A). Gene expression profiles of both transcripts had monotonic dose response curves. Each T_3 concentration compared to its matched (five-times-concentrated) T_4 concentration caused the same fold-change difference in *thra* transcript abundance (Figure 4.1A). *thrb* transcript abundance responses were similarly equivalent in T_3 and 5X T_4 matched treatments except at the two highest concentrations (25 nM and 125 nM T_4; 50 nM T_3 and 250 nM T_4; Figure 4.1A). Both *thra* and *thrb* mRNA transcripts were not affected by exposure to increasing

concentrations of E_2 (Figure 4.1B) with the exception of *thrb* transcript abundance at one concentration (1 pM E_2; $p = 0.03$).

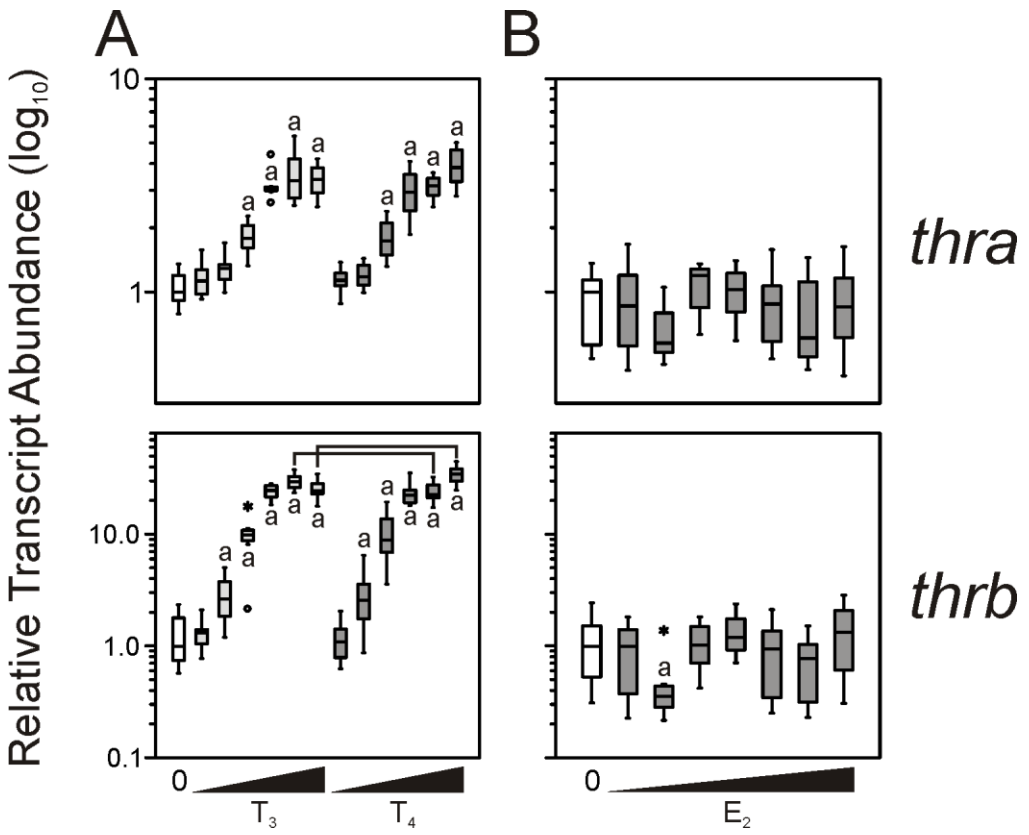

Figure 4.1 Effects of A) T_3 or T_4, and B) E_2 concentration gradients on *thra* and *thrb* transcript levels in *Rana catesbeiana* tailfin biopsies as determined by QPCR. Eight biological replicates per C-fin assay were cultured for 48 h with indicated hormones or vehicle control (0). One C-fin assay was performed for the TH series (T_3 concentrations: 0.01, 0.1, 1, 10, 25, and 50 nM T_3; T_4 concentrations: 0.05, 0.5, 5, 50, 125, and 250 nM T_4). A separate C-fin assay was performed for the following E_2 concentrations: 0.1 pM, 1 pM, 10 pM, 0.1 nM, 1 nM, 10 nM, and 100 nM E_2. For details regarding the boxplots see Figure 2.2. "a": significance of hormone treatment relative to vehicle control ("0") ($p < 0.05$; Wilcoxon signed-rank test). Square bracket: significance of T_3-treatment relative to its five-times-concentrated T_4 paired dose ($p < 0.05$; Wilcoxon signed-rank test).

4.3.2 Additional TH-Responsive Gene Transcript Profiles

The additional TH-responsive gene transcripts tested (*thibz*, *klf9*, *rlk1*, and *dio3*) were also monotonically responsive when the tailfin tissue was exposed to increasing concentrations of both T_3 and T_4 (Figure 4.2A). Statistically significant departures from apparent equivalent responses between the paired 5X T_4 and T_3 concentrations were evident for *thibz* transcripts (Figure 4.2A). The extremes of the T_4 dose ranges (0.05 nM T_4 and 250 nM T_4) caused a higher transcript level abundance than with T_3 matched treatments (0.01 nM T_3 and 50 nM T_3). Whereas one T_3 treatment in the middle of the concentration range (1 nM T_3), caused a higher level of transcript abundance than its 5X partner (5 nM T_4). E_2 did not cause any effect on the additional TH-responsive gene transcripts tested at any concentration (Figure 4.2B).

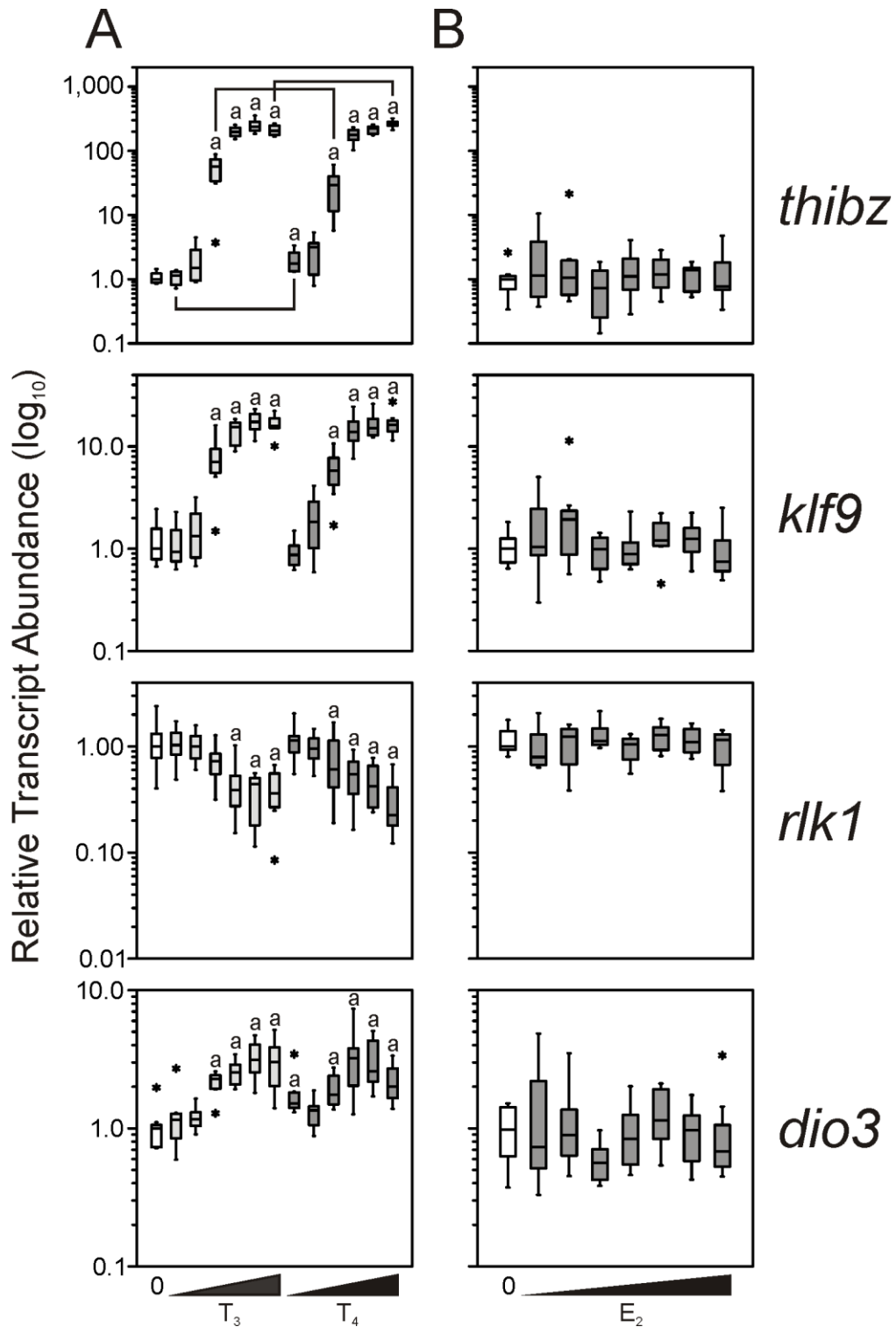

Figure 4.2 Effects of A) T_3 or T_4, and B) E_2 concentration gradients on TH-responsive basic leucine zipper transcription factor (*thibz*), krüppel-like factor 9 (*klf9*), *Rana* larval keratin 1 (*rlk1*), and deiodinase 3 (*dio3*) transcript levels in *Rana catesbeiana* tailfin biopsies as determined by QPCR. Refer to the Figure 4.1 legend for details.

4.3.3 Stress Responsive Gene Transcript Profiles

Stress responsive gene transcripts, which have been used for previous C-fin assays assessing effects of environmental contaminants, had less clear dose-response relationships than TH-responsive genes (Figures 4.1A and 4.2A) when treated with T_3 or T_4 (Figure 4.3A). TH induced an overall increase of the general cellular stress indicator *hsp30* but oxidative stress endpoints were relatively unaffected by TH treatment. Exposure to E_2 at all concentrations caused no significant change in any of the stress responsive gene transcripts (Figure 4.3B).

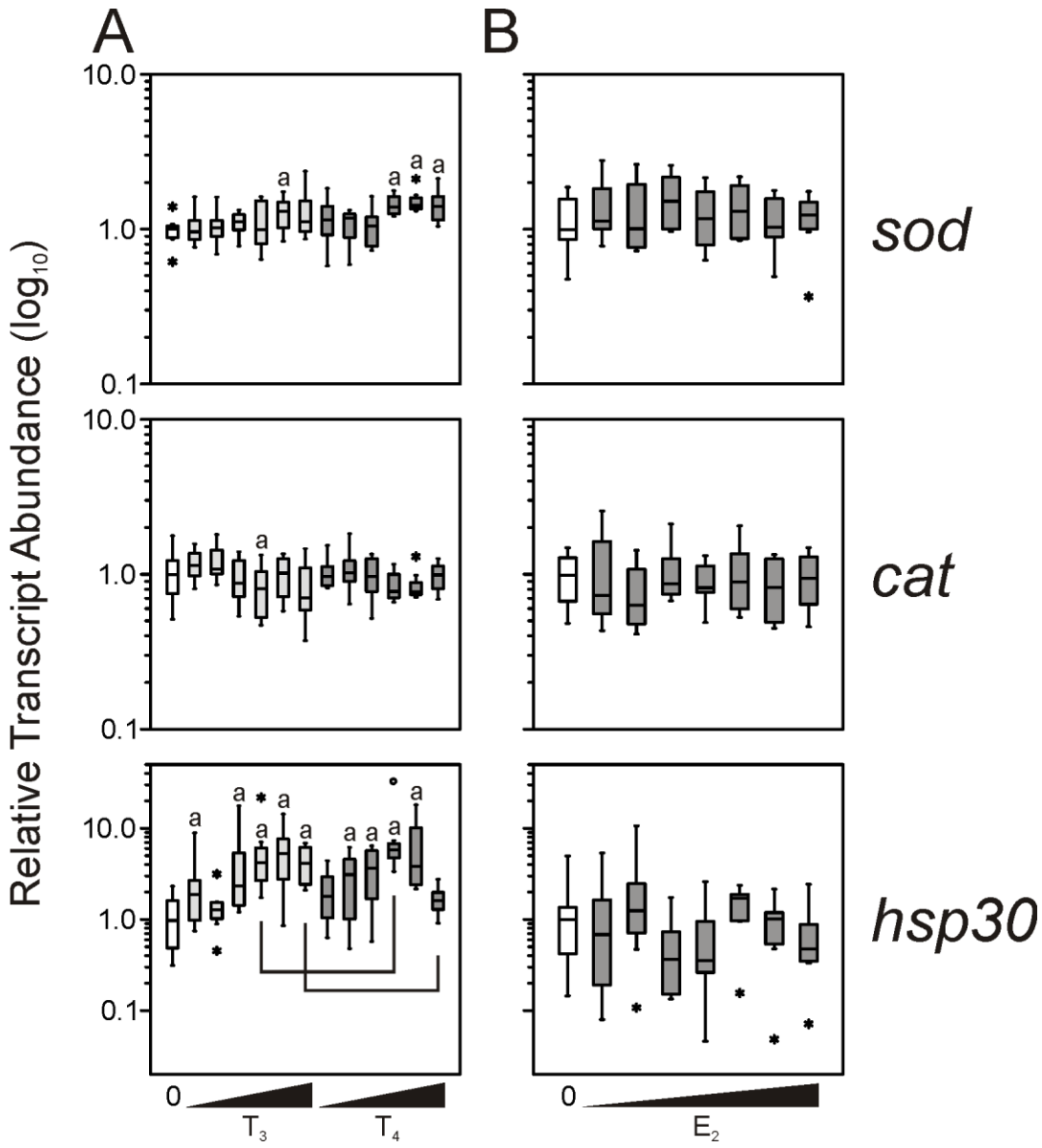

Figure 4.3 Effects of A) T_3 or T_4, and B) E_2 concentration gradients on *sod*, *cat*, and *hsp30* transcript levels in *Rana catesbeiana* tailfin biopsies as determined by QPCR. Refer to the Figure 4.1 legend for details.

4.3.4 Individual-Specific T_3 and T_4 Gene Transcript Responses

The repeated-measures design of the C-fin assay permits the comparison of the response of an individual animal to multiple treatment conditions. We therefore were able to directly examine the relative response of the biopsies on a "per animal" basis and compare the individual's response to T_3 and T_4. Figure 4.4 represents the relative

transcript abundance change from biopsies exposed to T_3 along the x-axis and biopsies from the same animal exposed to the corresponding 5X T_4 paired dose along the y-axis. If the relative gene transcript abundance responds with the same magnitude to the 5X T_4 as it does to T_3, the responses should lie along a line with a slope = 1.

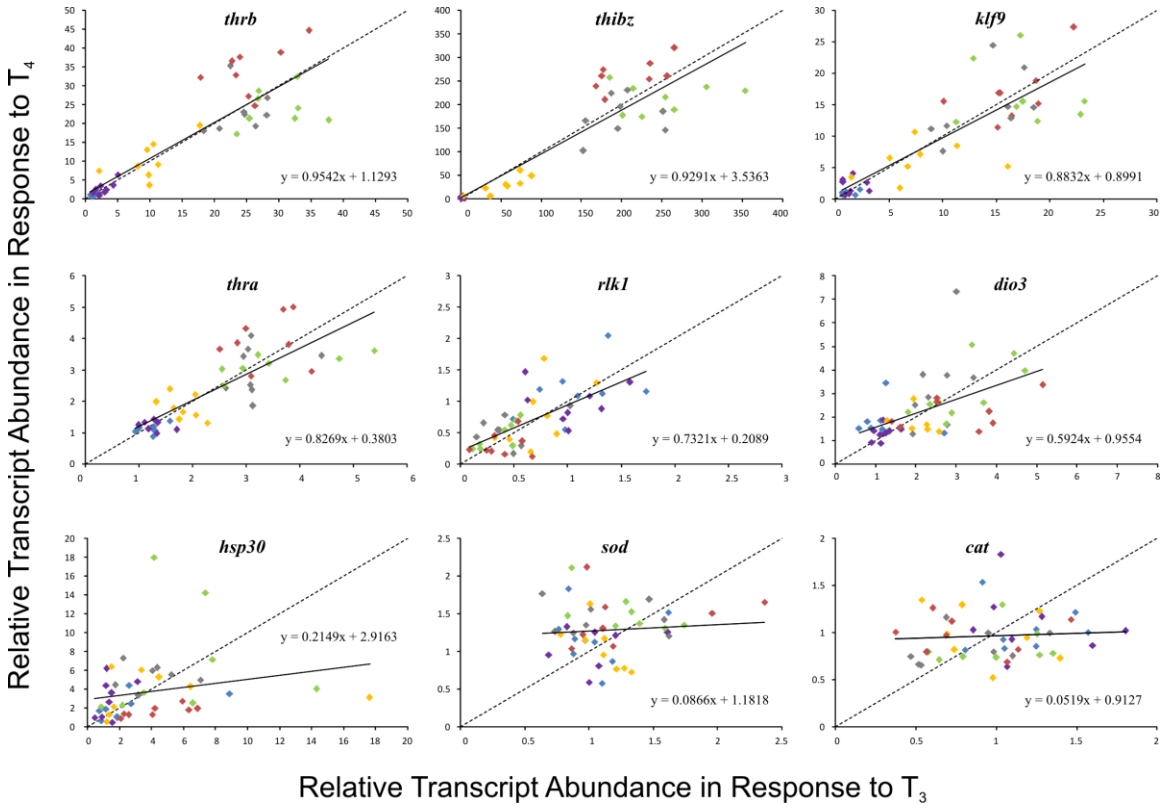

Figure 4.4 Distribution of relative transcript abundance in response to a concentration range of T_3 (x-axis) and 5X T_4 (y-axis) treatments in tailfin tissue biopsies within each *Rana catesbeiana* animal as determined by QPCR. To test the assumption that T_3 is five times more bioactive than T_4, transcript abundance data relative to the control from the same animal matched through specific pairings were plotted. Each animal has every pairing represented as follows: blue: 0.01 nM T_3/0.05 nM T_4; purple: 0.1 nM T_3/0.5 nM T_4; orange/yellow: 1 nM T_3/5 nM T_4; grey: 10 nM T_3/50 nM T_4; green: 25 nM T_3/125 nM T_4; red: 50 nM T_3/250 nM T_4. Data from eight animals are presented on each graph for the indicated transcript. The dashed line represents the ideal line with a slope = 1 where a given concentration of T_3 elicits the same response as 5X T_4. The solid line is a trendline through the data with the corresponding slope equation shown.

As these data denote two independent points from one animal, a trendline of the data is represented rather than a linear regression model with a corresponding coefficient of determination (R^2). A trendline through the data of the most TH-responsive genes: *thrb*, *thibz*, and *klf9* revealed slope values close to 1: 0.95, 0.93, and 0.88, respectively. In all three of the most TH-responsive genes, distributions of the individual responses followed this idealized line with a slope = 1 (dashed line) closely at lower concentrations with greater scatter at the higher concentrations (Figure 4.4). Specifically, a linear relationship was less evident above 10 nM T_3/50 nM T_4 for *thrb* and *thibz* (Figure 4.4). A higher degree of variation about the line was evident for *klf9* transcripts (Figure 4.4).

The TH-responsive gene transcripts, *thra*, *rlk1*, and *dio3*, were generally less responsive to THs (Figures 4.1 and 4.2) and had lower slope values (0.83, 0.73, and 0.59, respectively). Distribution of the individual responses for *thra* transcripts followed a similar trend to the most TH-responsive genes (Figure 4.4). The down-regulated *rlk1* transcript demonstrated some clustering around the idealized line but with a high degree of scatter (Figure 4.4). There was a very poor relationship between the idealized line and the relative responses of *dio3* to T_3 and T_4 (slope of trendline = 0.59; Figure 4.4). The stress-responsive gene transcripts *hsp30*, *sod*, and *cat* had slope values far less than the ideal slope = 1: 0.21, 0.09, 0.05, respectively, with essentially random distribution of data points (Figure 4.4).

4.4 Discussion

4.4.1 T_3 vs. T_4 Effects

Although T_4 is often referred to as a T_3-prohormone, our data indicate that, in the context of the C-fin assay, T_4 is acting less like a prohormone to T_3, and rather more

within the capacity of a nuclear receptor ligand. In general, the 5X T_4 treatments caused equivalent transcript abundance changes compared to their paired T_3 treatments in the TH-responsive gene transcript profiles (Figures 4.1A and 4.2A - with exceptions in *thrb* and *thibz* at specific doses; discussed below). Additionally, when the TH-responsive gene transcripts were plotted to show each individual animal's T_3/T_4 responses across the 6 paired concentrations, the more TH-responsive gene transcripts *thrb*, *thibz*, and *klf9* displayed the closest responses to the expected T_3/T_4 relationship (Figure 4.4). Although Dio2 activity levels were not measured in the present study, previous work has found that Dio2 activity is low in premetamorphic *Rana catesbeiana* tailfin (Becker et al., 1997). These observations were consistent with the fact that transcript levels of *dio2* were undetectable in the present C-fin biopsies. Taken together, the current observations indicate that the less-bioactive T_4 itself may be acting independently of potential Dio2-mediated T_4 conversion to T_3 to alter TH-responsive gene transcript levels.

Because of the repeated measures design of the C-fin assay, gene transcript abundance changes in one animal due to exposure to two different TH forms could be observed for the first time. By examining one paired concentration set (one coloured set of points in Figure 4.4) across different genes of interest, it is clear that within one animal, each gene is responding differently to the same TH challenge. A particularly striking example is the 1 nM T_3 and paired 5 nM T_4 treatments (orange/yellow). The relative transcript abundance of *thrb* lies evenly distributed about the dashed ideal line with a slope of 1, demonstrating the expected T_3/T_4 relationship. Almost all of the equivalent points in the *thibz* transcript abundance graph, however, lie below this dashed ideal line suggesting

that the response to T_3 is disproportionately stronger compared to the matched T_4 concentration.

In contrast, the highest concentration pair tested (50 nM T_3/250 nM T_4) shows a clear tendency toward a disproportionately stronger response of T_4 compared to the matched T_3 concentration for both *thrb* and *thibz* transcripts (Figures 4.1A, 4.2A, and 4.4). *thibz* and *thrb* both have well-characterized promoters and Thr binding has been extensively examined in the laboratory model species *Xenopus laevis*. These promoters have distinct relative Thr affinities and differential recruitment of Thrb upon T_3 exposure (Buchholz et al., 2005; Helbing et al., 2011). The *klf9* gene transcript shared a roughly similar T_3/T_4 relationship profile compared to *thrb* and *thibz* (Figure 4.4). However, a higher degree of scatter about the idealized line was observed suggesting a more complex relationship between T_3 and T_4 signaling for this gene. Similarly, *rlk1*, whose gene structure is currently unknown, and *dio3* exhibited a more complex relationship in transcript responses between these two hormones. Taken together, these observations indicate that, outside of Thr ligand binding affinity, T_3 and T_4 are not necessarily equivalent in their ability to alter TH-responsive gene transcripts and suggest that TH identity could provide a further gene-specific modulation of the hormone response. The mechanisms for these differences include classical (*via* nuclear receptors) or non-classical means. Non-classical pathways could include signaling *via* a plasma membrane receptor and activation of signaling cascades. The plasma membrane receptor αVβ3 has a much higher affinity for T_4 compared to T_3 in mammals (Bergh, 2005; Davis et al., 2008). Comprised of integrins whose ligands include fibrinogen, fibronectin, and matrix metalloproteinase 2 (MMP2), this receptor likely has a high degree of homology to frog species (Johnson et al., 2009),

however this remains to be determined. Additionally, two lines of evidence support the notion that non-classical signaling of TH has an important role in the apoptosis and resorption of *Rana catesbeiana* tailfin. First, inhibition of proapoptotic cyclin dependent kinase 8 activity prevents the establishment of the T_3-dependent metamorphic gene expression program (Skirrow et al., 2008) and, second, protein kinase C-mediated tyrosine phosphorylation is required for T_3-induced tail regression, which contributes to the post-translational modification of Thr and altered gene expression in cultured *Rana catesbeiana* tail tips (Ji et al., 2007).

The C-fin assay has previously been used as a screen for xenobiotic effects on transcript levels of stress-responsive genes. Although TH can act on stress-responsive gene transcripts (Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012), in the present study, *cat* and *sod* levels were not affected by THs (Figure 4.3). The lack of TH-responsiveness was recapitulated in the animal-specific T_3/T_4 representation of the data as both *cat* and *sod* plots exhibited a random relationship (Figure 4.4). A significant general increase of *hsp30* transcript levels due to TH exposure occurred but the classic monotonic dose-response curve of the natural ligands (T_3 or T_4) to their receptors was not paralleled in the *hsp30* gene transcript profile or in the animal-specific T_3/T_4 representation of the data. *hsp30* transcript levels do increase with TH exposure (Helbing et al., 1996; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012) but the present study indicates that this response is likely more complex than a single ligand-receptor interaction. The mechanisms for this phenomenon may be an indirect consequence of TH exposure and requires further examination.

4.4.2 E_2 Effects and Implications for C-fin Xenobiotic Screens

Because nuclear hormone receptors for TH and E_2 are structurally conserved (Vasudevan et al., 2002) and because xenobiotics such as genistein are able to act both as estradiol analogs and TH-signaling disruptors (Ji et al., 2007), our investigation into the specificity of TH-responsive genes in the C-fin assay is not unwarranted. E_2 treatment has caused both agonistic and antagonistic effects on TH-mediated amphibian metamorphosis (Hayes, 1997; Shi, 2000) but E_2-TH crosstalk studies more often suggest that E_2 (albeit at μM concentrations) may act on TH-effects by interfering at systemic levels, such as at the hypothalamic-pituitary axis, rather than on tissue- or cell-specific systems (Gray and Janssens, 1990). Nevertheless, the question of how estradiol might affect *Rana catesbeiana* tailfin tissues in the context of the C-fin assay has previously not been addressed. E_2 exposure in the present study caused virtually no perturbations on any of the TH-responsive or stress-responsive gene transcripts of interest. Therefore the possible contributory effect on the direct modulation of these transcripts by E_2 in the context of the C-fin assay appears to be minimal.

4.5 Conclusions

Previous C-fin assays have been used as screens for TH-specific EDCs (Hinther et al., 2010a; Hinther et al., 2010b; Hinther et al., 2011; Hinther et al., 2012). In that context, a TH-challenge of 10 nM T_3 is applied to the premetamorphic tissues to determine if the xenobiotic in question is able to disrupt normal TH-signaling in the tissue. Previous work indicated that the 10 nM T_3 dose is an appropriate concentration to maintain a replicable and statistically significant positive control with room for agonistic and/or antagonistic perturbations in *thrb* and *rlk1* transcript levels (Hinther et al., 2010a). The current data added to our fundamental understanding of the TH-sensitive system by investigating

another TH-form (T_4) in addition to the previously unexamined TH-responsive gene transcripts *thra, klf9, thibz,* and *dio3*. With this data, although the 10 nM T_3 dose is nearing the plateau of the sigmoidal dose-response curves of many of the TH-responsive gene transcript profiles investigated, there is still room for detection of potential TH-agonists in addition to a 10 nM T_3 dose. Future C-fin assays may benefit from a slightly lower concentration of T_3 to more effectively challenge the battery of TH-responsive gene transcripts.

Thyroxine plays a larger role in TH-responsive gene transcription than simply as a pro-hormone to T_3. Thyroxine itself, in a tissue with low Deiodinase 2 activity, is able to alter TH-responsive genes of interest at a five times higher concentration than T_3; a concentration reflecting the difference in binding affinities to Thr. *thrb* and *thibz* exhibit some exceptions to this rule at some concentrations tested. Non-classical actions of TH may play a role in these observations but further research is needed. The TH-responsive genes of interest are TH-specific, as the natural estrogen, E_2, does not affect any of the gene transcripts studied. In the context of the C-fin assay, stress-responsive transcripts previously used as indicators of xenobiotic exposure do not have distinct monotonic responses to the endogenous hormone exposures. In TH-specific EDC research however, the 10 nM T_3 concentration currently used in assessing perturbations of TH-action may be slightly high but does not negate effects of potential EDCs observed previously.

5 Conclusions and Future Directions

TH-activity, although vital for all vertebrates, is an understudied system in the growing field of endocrine disruption. This thesis employs a novel assay developed by our lab, the C-fin assay, to further practical research in managing the major point sources of aquatic EDCs (MWWTPs), in addition to developing and challenging current understandings of the basic molecular mechanisms of endocrine action in the *Rana catesbeiana* tadpole tailfin.

The data presented herein suggest that there are TH-active agents in fully treated Canadian MWWTP effluents. Although levels of TH in wastewater effluents have only been studied in one case in Finland (Svanfelt et al., 2010), perturbations of TH-responsive gene transcripts in the tadpole tailfin were observed after secondary treatment of Canadian wastewaters in the present work in both assessments of successive levels of a full-scale conventional MWWTP and more specific analyses of pilot-scale secondary conventional MWWTP configurations and operating conditions. Although chemical studies of ECs in MWWTPs suggest that biodegradation of EDCs during secondary treatment is the most efficient point of managing EDC release, most of these studies have been conducted on estrogenic compounds. TH-EDC fates in MWWTPs have been ignored in the efforts to improve EC removal efficiencies in MWWTPs but my research makes the case that, even beyond basic stress-inducing activity of wastewater effluents, TH-active effluents are a problem for managing MWWTP effluent qualities. The TH-disruption seen in tailfin biopsies exposed to fully treated wastewater effluents highlight the need for further investigations into TH-EDCs and their potential fates in MWWTPs. Without such investigations, discharge of TH-activity from MWWTP effluents, as seen

in this thesis, will be inefficiently managed and continue to cause risks to a critical endocrine system not only in receiving biota, but also in organisms with highly conserved systems, including humans.

The integration of biological endpoints is an essential part of managing effluent water qualities in the efforts to improve EC removal efficiencies in MWWTPs. Conventional assessments of MWWTP efficiencies have found high removal rates of some specific known ECs. Biological assays, however, reveal that high removal rates do not necessarily result in low biological effects in wastewaters. Partially because conventional analytical methods have limits of detection and the complex milieu of wastewaters is potentially comprised of many unknown EDCs in addition to EDCs that are known to be poorly removed, there is a need for biological assays of MWWTP effluents. With the C-fin assay we were able to assess a more complete picture of the efficacy of removal of biological activity on a complex tissue as compared to the more myopic conventional chemical analysis of EC removal in effluents.

Since targeted chemical analyses are the standard assessment of MWWTP efficiencies, this thesis questions conventions established by chemical analyses of MWWTP mechanisms of EDC removal and optimal conditions for such removal. Secondary treatment (a process expected to best degrade ECs), longer SRT times and increased temperature (both shown to cause improved biodegradation), and more advanced MWWTP configurations did not cause clear improvements in the indicators of TH-disruption in the C-fin assays assessed in Chapters 2 & 3. The biological endpoints in the C-fin assay are potentially painting a more complex picture of MWWTP EC removal than standard analytical chemistry may suggest. Because the C-fin assay measures

perturbations of select transcript levels of interest in one amphibian species, additional biological endpoints and other species are necessary for a more complete picture. As these assays were performed in the context of collaborators' work, a more conclusive decision on the best MWWTP system or optimal operating conditions may be revealed upon analysis of additional biological and chemical endpoints.

Although indications of TH-endocrine disruption remained in fully treated Canadian MWWTP effluents, my research does not disagree with the greater body of evidence that suggests increased municipal wastewater treatment results in improved effluent water quality. Such data is the basis for the Canadian Council of Ministers of the Environment (CCME) guidelines, which have mandated Canadian municipalities to improve wastewater effluent quality to a minimum of secondary treated levels in the near future (CCME, 2009). Conventional activated sludge secondary treatment of wastewaters in this thesis resulted in a significant reduction in perturbations of cellular stress (as indicated by *hsp30*) in the C-fin assay. The CCME's federal mandate has become controversial in some municipalities facing considerable investments in infrastructure, as financial inputs may seem larger than potential environmental benefits. Although this cost-benefit analysis is for each municipality alone to debate, the data presented herein, along with countless other chemical analyses of MWWTP removal efficiencies, suggest that the CCME proposal is not unreasonable; secondary treatment improves the quality of MWWTP effluents.

Interestingly, my investigation into disruption of TH activity in *Rana catesbeiana* tailfin by wastewaters highlighted an important differential effect between the two critical Thr-encoding gene transcripts. The wastewater exposed tailfin transcript profiles,

contrary to expectations, showed few perturbations in the more TH-responsive *thrb* gene transcript compared to the less TH-responsive *thra*. Although the two Thrs play crucial roles in amphibian metamorphosis, the gene-specific mechanisms of action remain poorly characterized. The present data not only necessitate the further investigation of gene-specific action of Thrs, but also suggest an important caveat in current TH-EDC screens: one TH-responsive endpoint cannot comprehensively represent TH endocrine disruption. These results are particularly worrisome because of the current propensity of classic toxicologists and environmental regulators to use EDC screens that are even more simplified than the C-fin assay. An efficient primary screening tool should tend toward false positives rather than false negatives in efforts to protect sensitive aquatic biota. In screening assays that use yeast expression of portions of human nuclear receptors to assess potential EDC-like effects, or artificially expressed nuclear receptor controlled reporter-gene constructs, the biological complexity of an incredibly multifaceted system is reduced to one molecular interaction. The C-fin data suggest that multiple endpoints of the complex TH-system are necessary for a complete assessment of TH-EDCs implying the same may be true for the more common screening of estrogenic-EDCs.

By using the C-fin assay's repeated measures design, which enabled the simultaneous evaluation of a single animal's response to both forms of TH, we were able to challenge the current understanding of T_4 as simply a T_3-prohormone and determine that T_4 can act independent of this conversion. The differential ability of T_3 and T_4 to modulate some TH-responsive transcript levels while showing equivalency in other TH-responsive transcripts in the tadpole tailfin has wide implications for not only amphibians but also for all vertebrates. Human patients with TH-related symptoms are often treated with T_4

and further research into the mechanisms of T_3 *versus* T_4 transcriptional regulation may be exploited to more clearly understand the mechanisms of treatment and to potentially reduce adverse side effects of TH treatment. Furthermore, specificity of TH-responsive gene transcripts was revealed in the investigation of the impact of E_2 on TH- and stress-responsive transcripts. Interaction of TH and estrogen systems is poorly understood but highly likely in most contexts, and of high priority in a screen for endocrine disruption. As EDCs are often assessed for estrogenic activity, E_2 effects were examined with the C-fin screen and were found to not affect the TH-responsive genes. Future work however, necessitates the development of tools for assessing E_2 responsive genes not only as a positive control for the E_2 exposure but also for added clarity of potential TH/E_2 crosstalk in the *Rana catesbeiana* tailfin.

Finally, several potential improvements for future C-fin assays were revealed by my work. Although the 10 nM T_3 dose is a sufficient dose to consistently upregulate previously assessed TH-responsive gene transcripts in the C-fin assay, a slightly reduced dose could improve TH-agonistic response sensitivity. This was partially determined by assessing additional TH-responsive gene transcripts, previously known to be important in the TH-mediated amphibian metamorphic program, but almost always ignored in the context of TH-EDC assessments. Additional genes of interest with distinct monotonic TH-response curves assessed in Chapter 4, such as *klf9* and *thibz*, are strong candidates for future studies of TH-disruption in the tadpole tailfin. Additionally, the current results consistently displayed poor TH response in *sod* and *cat*, gene transcripts known to be important in TH-signaling pathways in amphibians and important indicators of effluent exposure in fish species. Such results beg the question of whether or not the *Rana*

catesbeiana species is as sensitive to stress responses as might be necessary for such a screen or perhaps whether simply alternative stress-responsive gene transcripts (perhaps more integrally connected to the HPA axis, such as *nr3c1*) might benefit the C-fin assay in future screening of xenobiotics.

As the relatively new study of xenobiotic endocrine disruption has become a hotly debated topic of regulation due to significant financial incentives, the appropriate development of scientifically-sound screening tools for complex endocrine systems is a high priority. Because TH-EDC studies specifically are now gaining more attention in the field of endocrine disruption, the basic understanding of mechanisms of TH-action, as investigated in this thesis, are necessary to ensure accurate and complete development of TH-EDC assays. For Canada specifically, the research presented herein is a timely investigation into much-needed TH-responsive biological endpoints of the efficacy of conventional MWWTP systems, as the impending upgrade of many Canadian municipal wastewater treatment systems will require significant investments.

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Appendix

Table A.1 Gene-specific DNA primers and probes for QPCR

Chapter Reference	Gene Target	Oligo Name	Oligo Sequence[a]	Amplicon Size (bp)	pmol per reaction	ΔΔCT Criteria
Ch.2-4	*rpl8*	AMM1	5'-AGGCAGGTCGTGCNTACCA-3'	89	1.5	107%[b]
		AMM2	5'-GGGATGTTCTACAGGATTCATAGC-3'		1.5	
		AMM3	5'-Cy5-AAACTGCTGGCCACGTGTCCGT-IABk-3'		1.5	
Ch.2-4	*thra*	AMM4	5'-TGATAAGGCCACAGGRTACCACTA-3'	141	4.5	0.037[c]
		AMM5	5'-CGGGTGATCTTGTCGATRA-3'		1.5	
		AMM6	5'-FAM-ACTATCCAGAAGAACCTGCACCCCTC-IABk-3'		4.5	
Ch.2-4	*thrb*	AMM7	5'-CTCATAGAAGAAAACAGAGAAAARAGA-3'	237	4.5	0.016 [c]
		AMM8	5'-GAAGGCTTCTAAGTCCACTTTTCC-3'		1.5	
		AMM9	5'-HEX-CATGTGGCCACCAATGCACAGG-IABk-3'		4.5	
Ch.2-4	*hsp30*	AMM13	5'-GCCTCCACCAGACTTACCA-3'	238	4.5	0.057 [c]
		AMM14	5'-TCTGTCTCCCTTTTCTTGTCG-3'		1.5	
		AMM15	5'-HEX-CCACCGCCCCTCAAGACAAATC-IABk-3'		4.5	
Ch.2-4	*cat*	AMM16	5'-GAATGGTTACGGCTCACACA-3'	176	1.5	0.056 [c]
		AMM17	5'-TGGCAATGGCTTCATACAGAT-3'		1.5	
		AMM18	5'-Cy5-CAGGGCATCAGGAATCTGACGGT-IABk-3'		1.5	
Ch.2-4	*sod*	AMM19	5'-CGAGCAGGAAGAAGATGGA-3'	323	4.5	0.078 [c]
		AMM20	5'-CGCCTTTTCCCAAGTCATC-3'		1.5	
		AMM21	5'-ATTTCAACCCCCAAGGCAAGACC-3'		4.5	
Ch.2	*eef1a*	AMM43	5'-GCTGCTGGTGTTGGTGART-3'	257	5	0.033 [c]
		AMM44	5'-AGCATGTTGTCACCRTTCC-3'		5	
Ch.2	*rps10*	TAX7up	5'-TTTGCYTGGCGKCACTTTT-3'	213	5	0.018 [c]
		TAX7dn	5'-ARCRGCACTGCGYCTGTA-3'		5	

Ch.3-4	*eef1a*	AMM43	5'-GCTGCTGGTGTTGGTGART-3'	257	1.5	0.069 [c]
		AMM44	5'-AGCATGTTGTCACCRTTCC-3'		1.5	
		AMM45	5'-HEX-TACATCAAGAAGATTGGTTACAACCC-IABk-3'		1.5	
Ch.3-4	*rps10*	AMM37	5'-GCYTGGCGTCACTTTTACTG-3'	289	1.5	0.101 [c]
		AMM38	5'-CACGTCCAAAYCCTCCTCTAA-3'		1.5	
		AMM39	5'-FAM-AAGGCTGAGGCTGGWGCTGGAG-IABk-3'		1.5	
Ch.4	*klf9*	TAX16a up	5'-CYGCTCAGTGTCTGGTGT-3'	250	5	0.038 [c]
		TAX16a dn	5'-ARGGGCCGGTACTTGTTT-3'		5	
Ch.4	*thibz*	TAX15a up	5'-ASCTCCRCAGAAYCAGCA-3'	354	5	0.140 [c]
		TAX15a dn	5'-TCACGTACCAGGCCAAAA-3'		5	
Ch.4	*rlk1*	DDKerF3	5'-GTTGGCGTTGGTGTTAGCGC-3'	336	5	0.080 [c]
		DDKerRQ	5'-GGCACTGCTTCTTGCAACTTG-3'		5	

[a]Underlined sequence indicates the presence of an internal ZEN quencher

[b]Amplification efficiency

[c]Absolute value of slope of the ΔΔCt curve relative to *rpl8* normalizer

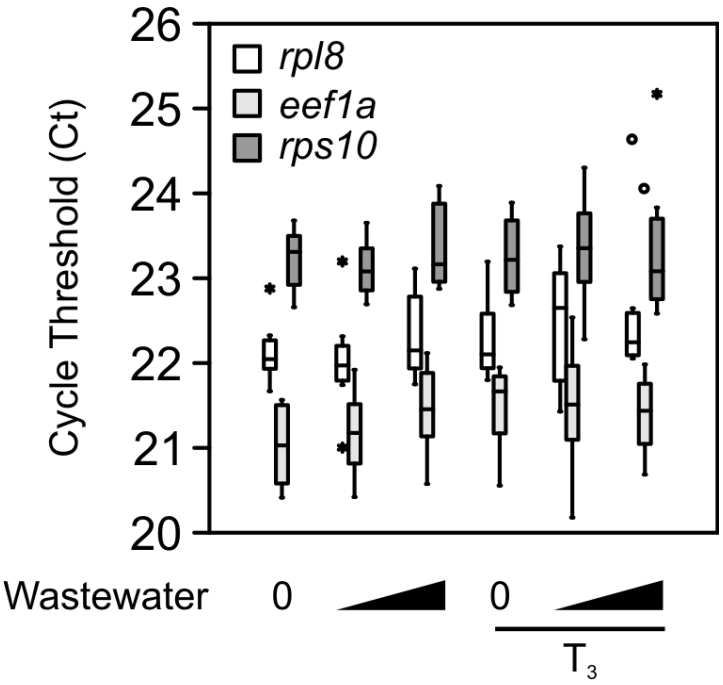

Figure A.1 Normalizer gene transcript abundance in a representative C-fin assay of wastewater exposure. Boxplots represent cycle threshold (Ct) levels of ribosomal protein L8 (*rpl8*), eukaryotic translation elongation factor 1α (*eef1a*), and ribosomal protein S10 (*rps10*). For details regarding the boxplots and treatments refer to Figure 2.2.