The thyroid hormone response profile of olfactory epithelium and its potential toward molecular bioindication of endocrine disruption in aquatic systems

by

Kevin William Jackman
A.Sc. Biology, Snow College, 2012
B.Sc. Biotechnology, Utah Valley University, 2015

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

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in the Department of Biochemistry and Microbiology

Supervisory Committee

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Dr. Leigh Anne Swayne (Division of Medical Sciences)
Outside Member

Abstract

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Rana (Lithobates) catesbeiana is a sentinel species for xenoendocrine disruption in aquatic and semi-aquatic environments. These anurans equip an olfactory system that requires extensive and dramatic restructuring to allow for successful transition from aqueous to semi-aqueous environments, and for a dietary lifestyle that transitions from herbivorous to carnivorous. This transformation is complex and driven principally by the action of the thyroid hormones (THs), L-thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3). Little is known about the genes involved in this change in the olfactory system, nor about how endocrine disrupting chemicals (EDCs) in the environment may interfere molecularly or behaviorally. *R. catesbeiana* tadpoles were exposed to either physiologically relevant concentrations of T_4, T_3, or 17-beta-estradiol (E_2), or environmentally relevant concentrations of treated municipal wastewater effluent from two different contemporary treatment systems for 48 h. Effluent was prepared from either anaerobic membrane bioreactor (AnMBR) or membrane enhanced biological phosphorous removal (MEBPR), where municipal wastewater feed stocks for each reactor were split into two separate treatment trains per system, with each feed stock receiving either a cocktail of personal care products (PPCP) or a vehicle control. Tadpoles were evaluated for olfactory-mediated avoidance responses following these

exposures. Significant disruptions to typical avoidance behavior were observed among the tadpoles exposed to T_3 and treated effluent, but not to T_4 or E_2. qPCR analysis of the olfactory epithelium (OE) and the olfactory bulb (OB) was performed on the animals involved in the behavioral assays, as well as on parallel groups exposed to the same conditions. Transcript abundance in *thra*, *thrb*, and *thibz* was significantly greater in the T_3-exposed behavioral group than that of the T_4. E_2 exposures exhibited no transcript response of these genes, whereas *thibz* exhibited an increase in transcript levels when tadpoles were exposed to either type of municipal wastewater effluent, regardless of addition of the PPCP cocktail, indicating presence of TH-mimic activity. A transcriptomic analysis was performed on the OE from T_3-, T_4-, and E_2-exposed tadpoles compared to their respective controls using RNA-seq. While the overall number of contigs identified were comparable between hormone treatments, the OE was ~100X more sensitive to TH than E_2. While many contigs were in common between T_3- and T_4-treated tadpoles, the T_3-exposed hormone group contained over 20% more significant contigs than the T_4-exposed group relative to their respective controls. Gene ontology (GO) analysis showed a stronger response in the T_3-exposed tissue toward detection of chemical stimulus involved in sensory perception compared to T_4-exposed tissue; a finding that is consistent with the difference in behavioural response to an avoidance cue. Using the results of the transcriptomic analysis, new qPCR tools were designed to additional TH-responsive transcripts and applied to OE from tadpoles exposed to effluent. Substantial removal of known and suspected EDCs was observed in both treatment systems, but molecular evidence of EDC activity remained. Further analysis of the OE as a means for bioindication of endocrine disruption is justified.

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Acknowledgments

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Dedication

to my cat (the grey one, not the other one)

List of Abbreviations

Use of capitalization and italics for gene transcripts and proteins follow the scheme derived from http://www.xenbase.org/gene/static/geneNomenclature.jsp.

Transcript = *thibz*, protein = thrb.

AnMBR	Anaerobic membrane bioreactor
AOP	Adverse outcome pathway
BART	Bullfrog Annotation Resource for the Transcriptome
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
bp	base pair
BQL	Below quantitation limit
cDNA	Complementary DNA
CNS	Central nervous system
Co-A	Coactivator
Co-R	Corepressor
CRF	Corticotropin-releasing factor
Ct	Cycle threshold
D1/dio1	Type I iodothyronine deiodinase
D2/ dio2	Type II iodothyronine deiodinase
D3/dio3	Type III iodothyronine deiodinase
D4	Octamethylcyclotetrasiloxane
D5	Decamethylcyclopentasiloxane

DE	Differential expression
DEHP	diethylhexyl phthalate (Bis(2-thylhexyl) phthalate)
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E1	Estrone
E2	Estradiol
EDC	Endocrine disrupting compound
EE2	17α-ethynlestradiol
eef1a	Eukaryotic translation elongation factor 1
GO	Gene ontology
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HPT	Hypothalamus-pituitary-thyroid
IBF	Ibuprofen
kbp	kilobase pair
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LC/MS-QTOF	Liquid chromatography-mass spectrometry-quad time of flight
LOD	Limit of detection
MAD	Median absolute deviation
MAPK	Mitogen activated protein kinase
MEBPR	Membrane enhanced biological phosphorous removal process
MHC	Major histocompatibility complex

MIQE	Minimum information for publication of quantitative real-time PCR experiments
MMP11	Matrix metalloproteinase-11
mRNA	Messenger RNA
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ncRNA	Non-coding RNA
ND	Not determined
NF	Nieuwkoop and Faber developmental stage
NSAID	Non-steroidal anti-inflammatory drug
OB	Olfactory bulb
OE	Olfactory epithelium
OMP	Olfactory marker protein
OR	Olfactory receptor
ORF	Open reading frame
OSN	Olfactory sensory neurons
PESC	Pacific Environmental Science Centre
PCA	Principle component analysis
PCR	Polymerase chain reaction
PPCP	Pharmaceutical and personal care products
PVDF	Polyvinylidene difluoride
qPCR	Real-time quantitative polymerase chain reaction

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Rxr	Retinoid X receptor
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-seq	High-throughput RNA sequencing
rpl8	Ribosomal protein L8
rps10	Ribosomal protein S10
SEM	Standard error of the mean
SENP5	Sentrin-specific peptidase 5
st3	Stromelysin-3
TAD	Topologically Associating Domain
T_3	3,5,3' -triiodothyronine
T_4	Thyroxine
TCC	Triclocarban
TCS	Triclosan
TH	Thyroid hormone
thibz	Thyroid hormone-induced basic leucine zipper-containing protein
thra	Thyroid hormone receptor alpha (α)
thrb	Thyroid hormone receptor beta (β)
TK	Taylor and Kollros developmental stage
TMP	Transmembrane pressure
TR	Thyroid hormone receptor, either isoform
TRα	Thyroid hormone receptor alpha
TRβ	Thyroid hormone receptor beta

TRE	Thyroid response elements
TRH	Thyrotropin-releasing hormone
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
trpv1	Transient receptor potential cation channel subfamily V member 1
TSA	Transcriptome shotgun assembly
TSH	Thyroid stimulating hormone
UBC	University of British Columbia
UCT	University of Cape Town
UTR	Untranslated region
VTG	Vitellogenin
WAS	Waste biomass
XEMA	*Xenopus laevis* Metamorphosis Assay
YAS	Yeast Androgen Screen
YES	Yeast Estrogen Screen
ZNF567	Zinc finger protein 567

Thesis Format and Manuscript Claims

This thesis is presented in the format of a manuscript. The first chapter provides a general background and introduces the rationale of the thesis. Chapters two and three are written in a manuscript style containing an Abstract, Introduction, Materials and Methods, Results, and Discussion. The fourth chapter provides a synthesized conclusion of the major findings of the data chapters and suggests future directions that subsequent investigations might pursue.

Chapter 2: Heerema J*, **Jackman KW***, Miliano RC, Li L, Zaborniak TSM, Veldhoen N, van Aggelen G, Parker WJ, Pyle GG, Helbing CC. 2017. Behavioral and molecular analyses of olfaction-mediated avoidance responses of *Rana (Lithobates) catesbeiana* tadpoles: Sensitivity to thyroid hormones, estrogen, and treated municipal wastewater effluent. **Hormones and Behavior. pii: S0018-506X(17) 30331-8. doi: 10.1016/j.yhbeh.2017.09.016.** Caren C. Helbing, Greg G. Pyle, Graham van Aggelen, and Nik Veldhoen designed the exposures. Jody Heerema and Rachel C. Miliano performed the behavioral exposures. Wayne J. Parker and Linda Li produced the treated effluents. Kevin W. Jackman and Tristan S.M. Zaborniak performed RNA isolation and qPCR analysis. Jody Heerema, Kevin W. Jackman, Caren C. Helbing, and Greg Pyle wrote the manuscript.

Chapter 3: **Jackman KW**, Veldhoen N, Miliano RC, Roberts B, Li L, Khojasteh A, Zheng X, Zaborniak TSM, van Aggelen G, Lesperance M, Parker WJ, Hall ER, Pyle GG, Helbing CC. 2018. Transcriptomic analysis of the *Rana (Lithobates) catesbeiana* tadpole olfactory epithelium in response to thyroid hormones, estrogen, and treated municipal

wastewater effluent exposure. **In Preparation**. Caren C. Helbing, Greg G. Pyle, Graham van Aggelen, and Nik Veldhoen designed the exposures. Wayne J. Parker, Linda Li, Eric R. Hall, Azadeh Khojasteh, and Xiaoyu Zheng produced the treated effluents. Kevin W. Jackman and Tristan S.M. Zaborniak performed RNA isolation and qPCR analysis. Kevin W. Jackman prepared and analyzed RNA samples for RNA sequencing. Bonnie Roberts and Mary Lesperance designed the bioinformatics pipeline and performed RNA sequencing analyses. Kevin W. Jackman and Caren C. Helbing wrote the manuscript.

1 Introduction

1.1 Thyroid hormones

The thyroid is a gland in the vertebrate endocrine system and is a component of the hypothalamus-pituitary-thyroid (HPT) axis. The structure and function of this axis is generally conserved across all vertebrates (Brown and Cai, 2007). Glands in the endocrine system produce hormones: a class of signaling molecules that are able to regulate physiology and behavior. There are three general structural classes of hormone: eicosanoids, steroids, and amines/amino acids. Hormones of the thyroid belong to the amino acid-derived class and are synthesized from tyrosine. The two most biologically active isoforms of thyroid hormones (THs) secreted by the thyroid are 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4).

This neuroendocrine process is initiated when environmental cues trigger activation of specific hypothalamic neurons (**Figure 1.1**). This induces hypothalamic release of corticotropin-releasing factor (CRF) (thyrotropin-releasing hormone (TRH) in mammals). The downstream activation of CRF receptors in the pituitary elicit the production of thyroid stimulating hormone (TSH) which, in turn, stimulates the thyroid to begin the production of T_3 and T_4 (**Figure 1.1;** Nussey and Whitehead, 2001). The ratio of production of T_4:T_3 is generally around 4:1 (Bianco et al., 2002). A negative feedback mechanism serves to homeostatically balance the plasma levels of these THs (**Figure 1.1**).

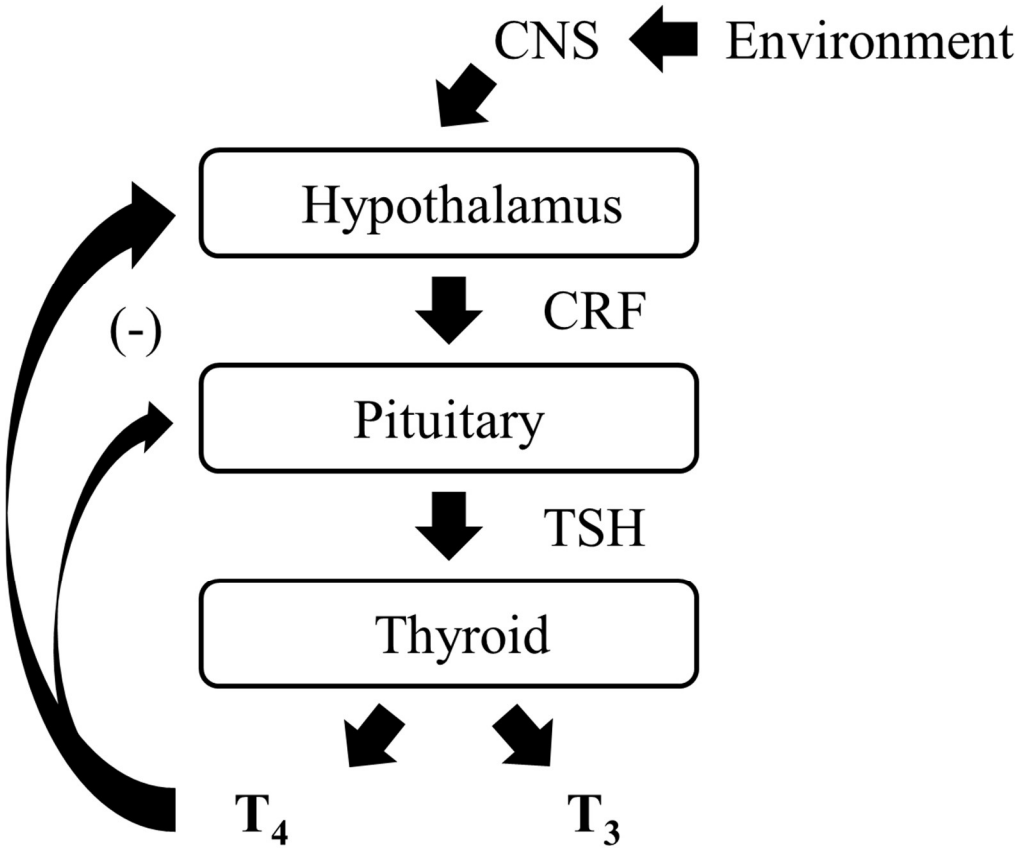

Figure 1.1 Hypothalamus-pituitary-thyroid axis in Amphibia. Adapted from Nussey and Whitehead (2001).

These circulating THs are able to regulate anabolic and catabolic pathways to maintain homeostasis in metabolism, thermogenesis, cell proliferation, and tissue differentiation (Cordeiro et al., 2013; Sirakov et al., 2013). T_4 is generally carried into destination tissues around the body and then converted within the tissue to T_3 by intracellular type I and type II 5'-deiodinases (dio1 and dio2, respectively; **Figure 1.2**), though recent work has demonstrated that T_4 can also act directly on gene expression in the absence of deiodinases (Maher et al., 2016).

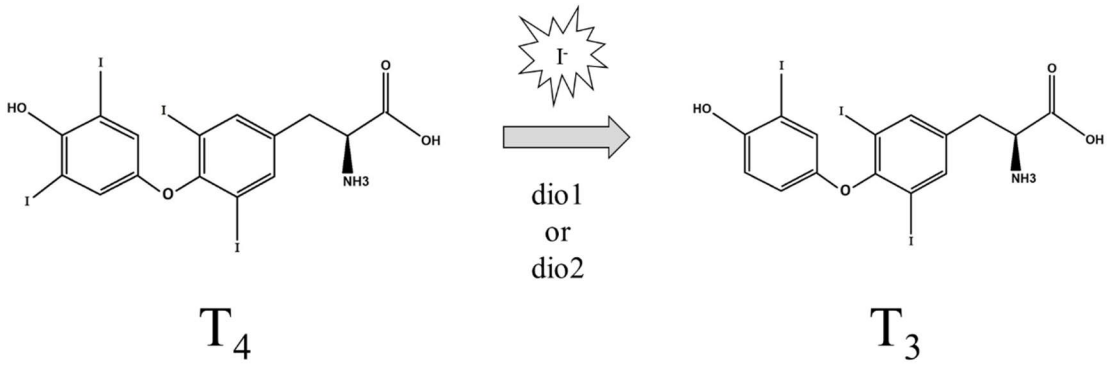

Figure 1.2 Structures of T_4 and T_3, and simplified representation of their 5' deiodination.

1.2 Mechanisms of TH-modulated gene expression

The THs predominantly modulate gene expression through interaction with thyroid hormone receptors (TRs) alpha (thra) and beta (thrb). These receptors most commonly form heterodimers with the retinoid X receptor (Rxr) on TH response elements (TREs) typically located upstream of the transcription start site (**Figure 1.3**). Both Rxrs and TRs belong to the steroid and thyroid hormone receptor superfamily (Evans, 1988; Ward and Weigel, 2013), which also includes receptors for steroid hormones such as estradiol (E_2). When bound to the TRE in the absence of ligand, TRs associate with corepressors (Co-R) and histone deacetylases (HDACs) and promote a repressed transcriptional state (Paquette et al., 2014). TH binding results in a conformational change in the TR allowing association of coactivators (Co-A) and histone acetylases (HATs) such as steroid receptor co-activator (SRC) and p300 (Grimaldi et al., 2013; Shi, 2013) to promote gene expression (**Figure 1.3**). Repression of gene transcription plays an important role during development. In frogs, *thra* is expressed prior to the production of THs by the thyroid, allowing TRα to repress TH-responsive gene transcription prior to the beginning of

metamorphosis. In contrast, *thrb* expression patterns correlate to those of a TH-responsive transcription factor, *thibz*, wherein they dramatically increase with circulating levels of THs to facilitate TH-responsive transcription programs (Shi, 2000).

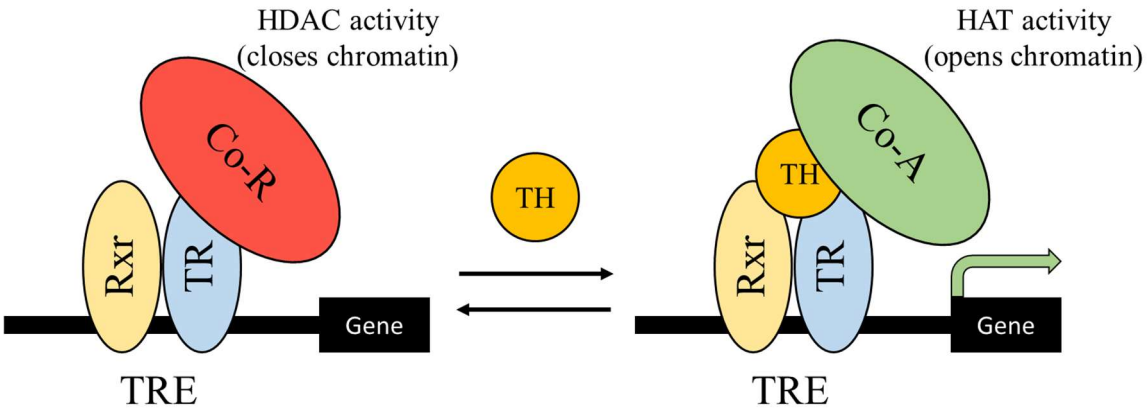

Figure 1.3 Simplified model of activation of gene transcription by binding of a TH to a TR. The TR is heterodimerized with Rxr and bound to a TRE in the nucleus upstream of the target gene. Co-repressors (Co-R) prevent transcription. In the presence of TH, co-activators (Co-A) are recruited that enable transcription. Adapted from Grimaldi et al., 2013.

The above classical genomic route of TH-response modulation is not the only means by which THs regulate gene expression. Non-genomic mechanisms involving TH binding to cell membrane-associated integrins and signal transduction pathways such as mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways depending upon the cellular context (Cheng et al., 2010;Cao et al., 2005; Lei et al., 2008).

1.3 Anuran metamorphosis

Thyroid hormones are the principal driving force of anuran metamorphosis. This process induces massive physiological changes across the entire organism as it transitions from an herbivorous, aquatic larval tadpole into a carnivorous terrestrial juvenile froglet, occupying a vastly different ecological niche. The larval tadpoles can be divided into stages of metamorphic development, and multiple staging programs have been implemented such as Taylor and Kollros (TK), Gosner (Gs), and Nieuwkoop and Faber (NF) (Gosner, 1960; Nieuwkoop and Faber, 1956; Taylor and Kollros, 1946). The TK system used in the present study is often used for *R. catesbeiana*, while NF is used for staging the African clawed frog, *Xenopus laevis*. The premetamorphic stages (TK I-X) are characterized by the presence of a thyroid gland, but no detectable circulating TH (**Figure 1.4**). As THs begin to circulate, visible metamorphic changes occur and the tadpole enters the prometamorphic stages (TK XI-XIX; **Figure 1.4**). Metamorphic climax occurs at TK stage XX, wherein the highest levels of circulating THs in the body are recorded and the most significant changes occur across the organism (Shi, 2000). The tail fully reabsorbs by TK XXV, and metamorphosis is considered complete.

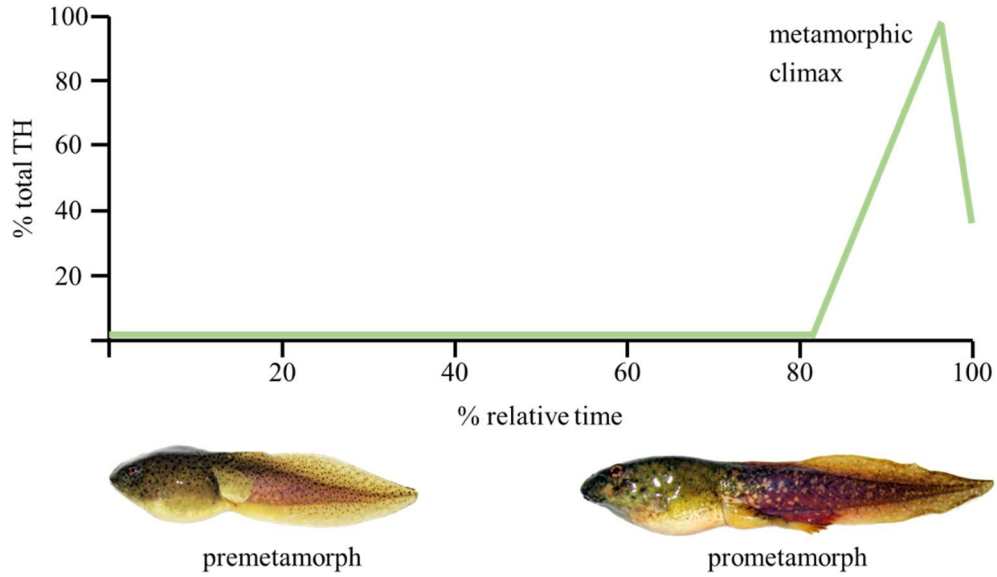

Figure 1.4 Percentage of circulating total thyroid hormones as compared to relative time of metamorphosis. Premetamorph is represented by TK stages I-X, and prometamorph is represented by TK stages XI-XIX.

Exposure of premetamorphic tadpoles to exogenous TH in the aqueous environment induces precocious metamorphosis (Regard et al., 1978). This allows controlled induction of metamorphosis in different conditions and the opportunity for subsequent laboratory molecular analysis of gene expression.

The gene expression networks involved in this process are understandably quite complex and remain largely unknown. Investigations have separated these programs into an initiation phase and an execution phase by blocking transcription and protein translation and observing which genes were able to produce transcripts upon TH exposure. Genes that showed an mRNA increase without requiring protein translation were deemed part of the metamorphic initiation phase, and those that required proteins to be translated fell into the execution phase (Buckbinder and Brown, 1992; Das et al.,

2009; Kanamori and Brown, 1993). The initiation phase is complete within 24 hours of exposure, while the execution phase occurs within 48 hours of exposure.

Though extensive research has been performed on *X. laevis* due to their convenience in captive breeding (Parker et al., 1947), as *X. laevis* has a natural range limited to Africa, it can be argued that *R. catesbeiana* is a better candidate for ecological observations in North America. As a true frog that fully transitions out of an aqueous environment, unlike *X. laevis*, *R. catesbeiana* provides a stronger parallel in human and mammalian development, wherein the aforementioned TH peak occurs during perinatal development in preparation for transition from an aqueous amniotic environment to a terrestrial one. With both anuran metamorphosis and mammalian perinatal development being driven by the same thyroid hormones - T_3 and T_4 - reasonable parallels from experimental findings in *R. catesbeiana* physiology to similar developments in perinatal humans can be made.

1.4 The olfactory system

One of the tissues that goes through drastic change during anuran metamorphosis is the olfactory epithelium (OE; **Figure 1.5**). It has been observed that there is massive cell death in this tissue during metamorphosis and that the majority of larval sensory neurons are replaced (Dittrich et al., 2016; Higgs and Burd, 2001). Changes in the olfactory system are not limited to just the exposed epithelium. Neurons within the olfactory bulb (OB) - the component of the brain that receives the stimuli from the epithelium and formulates a behavioral response – exhibit increased axonal growth, and that there is evidence that this tissue is re-specified throughout metamorphosis, and not turned over as in the epithelium (Higgs and Burd, 2001; **Figure 1.5**). These changes occur during this transition to adulthood due to the drastic shift in ecological niche described previously in

section 1.2. The adult anuran olfactory system needs to not only be able to properly detect airborne stimuli as compared to solely aqueous, but the response to these stimuli needs to be modified as well. An odorant that previously signalled the presence of a "predator" to an herbivorous prey, may now represent a food source to a newly transitioned carnivorous frog. These contextual changes in olfactory-mediated stimulus responses are critical for the fitness of this organism to survive into adulthood.

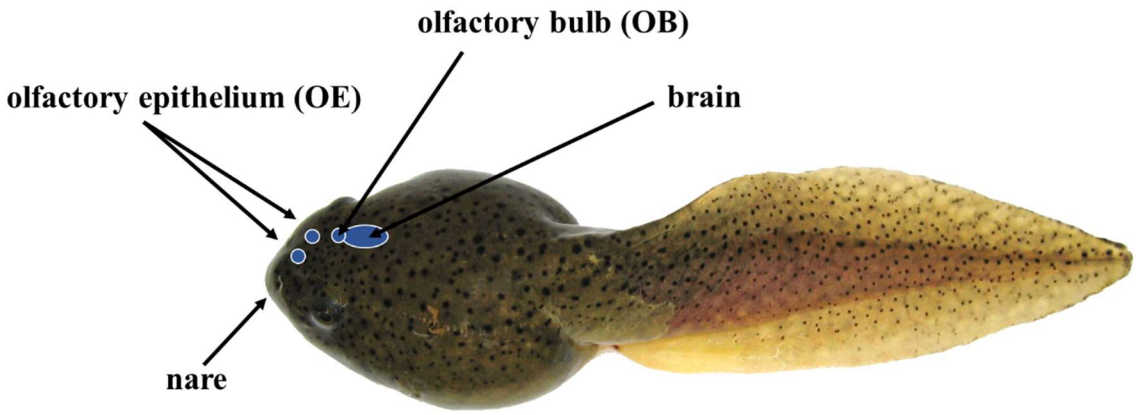

Figure 1.5 Anatomical locations of the olfactory epithelium (OE) and the olfactory bulb (OB). Neither tissue is externally visible, but the OE is exposed to the environment through the nare.

Because this process is driven by THs, it is likely that a disruption in the natural processes of thyroid hormone function could result in disruption to development, behavior, and ecological fitness. Recent studies have found that exposure to toxicants have reduced anti-predator responses to alarm stimuli in fish (Azizishirazi et al., 2014; Dew et al., 2014) and tadpoles (Ehrsam et al., 2016; Ferrari et al., 2007), but the question remains as to what the extensive tadpole gene expression profile in the OE looks like

when exposed to THs, and where in this profile the evidence of disruption may be occurring.

1.5 Municipal wastewater treatments

Wastewater treatment plants were implemented in the 1950s and were mainly designed with the aim of biological oxygen demand (BOD) removal (Ternes et al., 2004). Several innovations have taken place since then, and the contemporary aims of modern water treatment now include mitigation of discharge of phosphorous and ammonia load into the environment (Ternes et al., 2004). The mitigation of anthropogenic pollution into ecosystems has been substantial since the implementation of these facilities nearly seventy years ago, but the technologies have been strained to keep up with the massive influx of pharmaceuticals and personal care products (PPCPs).

Initial separation of wastewater occurs when solid waste is separated from liquid, and the liquid is passed through a filtration membrane. With membrane pores as small as 0.04 μm, excrement, bacteria, and larger organisms are easily excluded from the effluent, but free chemical compounds are not. A typical treatment facility degrades chemical compounds in the wastewater through an activated sludge process, wherein specific bacteria are introduced into the separated solid waste and the liquid is run across it with the intention of adsorption and metabolism by the degradative bacteria. Of the thousands of anthropogenic chemicals introduced into municipal wastewater, some may be partially metabolized, completely mineralized, or entirely untouched by the complement of bacteria that reside in the activated sludge of the treatment plant (Ternes et al., 2004). Therefore, there are compounds and unknown metabolites of compounds that are making their way through these treatment facilities and into the environment.

1.6 Endocrine disrupting chemicals (EDCs)

Among these compounds that survive water treatment facilities are those that have known or suspected abilities to disrupt the normal function of the endocrine systems of both humans and animals, called endocrine disrupting chemicals (EDCs; Bergman et al., 2013). EDCs do not share any easily predictable structure or chemical action, as they can be present in PPCPs, pesticides, plastics, and industrial surfactants, and have been found to have effects on reproduction, cancer, metabolism, obesity, the cardiovascular system, and the thyroid axis (Diamanti-Kandarakis et al., 2009).

A unique characteristic of many EDCs is that they exhibit nonmonotonic dose-response curves, wherein effects are observed at a low dosage that do not directly translate to a similar response at a higher dosage. These low-dosage responses occur at environmentally-relevant concentrations and cannot be predicted by linear regression of higher dose exposures (Vandenberg et al., 2012). Corresponding with this phenomenon, EDCs have been found to induce biological responses in organisms when exposed to concentrations below a limit of detection (LOD) of traditional toxicological studies (Kusk et al., 2011; Sifakis et al., 2017; Wojnarowicz et al., 2014). Molecular and transcriptional changes are occurring at these environmentally and biologically relevant levels of exposure, and very little is known about how far-reaching these effects actually are, despite recent confirmations that exposure to these EDCs in the environment are associated with human diseases and disabilities (Vandenberg et al., 2012).

A further complication that has severely limited research into the biological effects of individual EDCs is that in wastewater and environmental waters, these chemicals exist as complicated mixtures. Mixtures of contaminants have demonstrated additive, less than

additive, and synergistic effects occurring in mixtures of different chemicals and concentrations (Crofton, 2008; Kortenkamp, 2007; Rajapakse et al., 2004).

The disruption of endocrine processes during the developmental and growth phases of organisms is of utmost concern and can have effects that do not occur in the fully developed adults exposed to the same concentration of chemical. When evaluating disruptions to the thyroid system, the visibly striking thyroid hormone-modulated process of metamorphosis of a premetamorphic tadpole into a juvenile froglet is a strong model for observation.

1.7 Current methods of screening for EDCs

The global scientific field of screening for and determining relevant toxicity of known and suspected EDCs is enormous and complex. The term endocrine disruptor came into use only as recently as 1991, and still today there is still no definitive risk assessment tool for EDCs (Futran Fuhrman et al., 2015). Futran Fuhrman et al., (2015) claims in a review on the global state of EDC risk assessment that there are many challenges obstructing a more straightforward approach to EDC research, with one of the most critical being that extrapolations between EDC studies at different levels of organization (i.e., cell-to-human, inter-species, sub-chronic-to-chronic/long-term) has considerable uncertainty.

Several screening programs have been implemented that approach the EDC problem from different angles. The Yeast Estrogen Screen (YES) assay is employed by recombining the DNA sequence of the human estrogen receptor into the yeast (*Saccharomyces cerevisiae*) genome in a plasmid with estrogen-responsive sequences that control the expression of the *lacZ* reporter gene. The presence of estrogens induce synthesis of β-galactosidase and the medium visibly changes from yellow to red

(Routledge and Sumpter, 1996). A similar screen, called YAS for Yeast Androgen Screen, was developed to test for androgen activity to find evidence of androgenic activity in specific synthetic compounds (Blüthgen et al., 2013; Fic et al., 2014; Mertl et al., 2014), and for screening breeding sites of aquatic animals such as the common toad (Pickford et al., 2015). Other commonly used *in vitro* assays such as the E-Screen (Schilirò et al., 2011; Wagner and Oehlmann, 2011) for estrogen activity and the T-Screen (Gutleb et al., 2005; Ren et al., 2013; Schriks et al., 2006) for thyroid hormone activity use cell culturing assays: a human carcinoma cell line and a rat pituitary cell line, respectively. These *in vitro* assays are enormously helpful for basic determination of presence of hormone activity, but are limited in the applied interpretation of results; the effects may be entirely lost *in vivo*, and no organ, organismal, or population effects can be monitored or confidently implied.

The Organization for Economic Cooperation and Development (OECD) has endorsed several assays to detect EDC activity. Notably, the *Xenopus laevis* Metamorphosis Assay (XEMA) was developed to evaluate TH active chemicals (OECD, 2015) and similar assays were subsequently developed for some native frog species (Helbing, 2012). As these assays solely rely on morphological endpoints, they lack the required sensitivity needed for EDC identification.

Other assays under development use transgenic amphibian and fish embryos to quantify endocrine activity of synthetic substances (Lillicrap et al., 2016), and can monitor thyroid and androgenic active compounds using the Xenopus Embryonic Thyroid Assay (XETA) and the Rapid Androgen Disruption Animal Replacement (RADAR), respectively. These assays offer more reliable organismal effects than *the in*

vitro assays and are less labor intensive than previous OECD test guidelines (Spirhanzlova et al., 2017), but still do not give information on which specific genes are being affected, or by what mechanism.

A systemized approach has recently been organized in an attempt to combine all of these different approaches and collections of data-types into a transparent collaborative record to most effectively approach EDC risk assessment by linking an exposure event with adverse effects. This approach, termed the Adverse Outcome Pathway (AOP), is defined as a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization relevant to risk assessment (**Figure 1.5**) (Ankley et al., 2010; Futran Fuhrman et al., 2015).

The AOP concept allows for enhancement of across-chemical extrapolation and support for prediction of mixture effects, while facilitating the use of molecular biomarkers for forecasting chemical impacts on individuals and populations. While there are a few established AOPs for estrogenic substances, there is a conspicuous lack of AOPs for THs. The present thesis attempts to address this problem by linking TH-dependent gene expression in frog OE to olfactory system-linked behaviour.

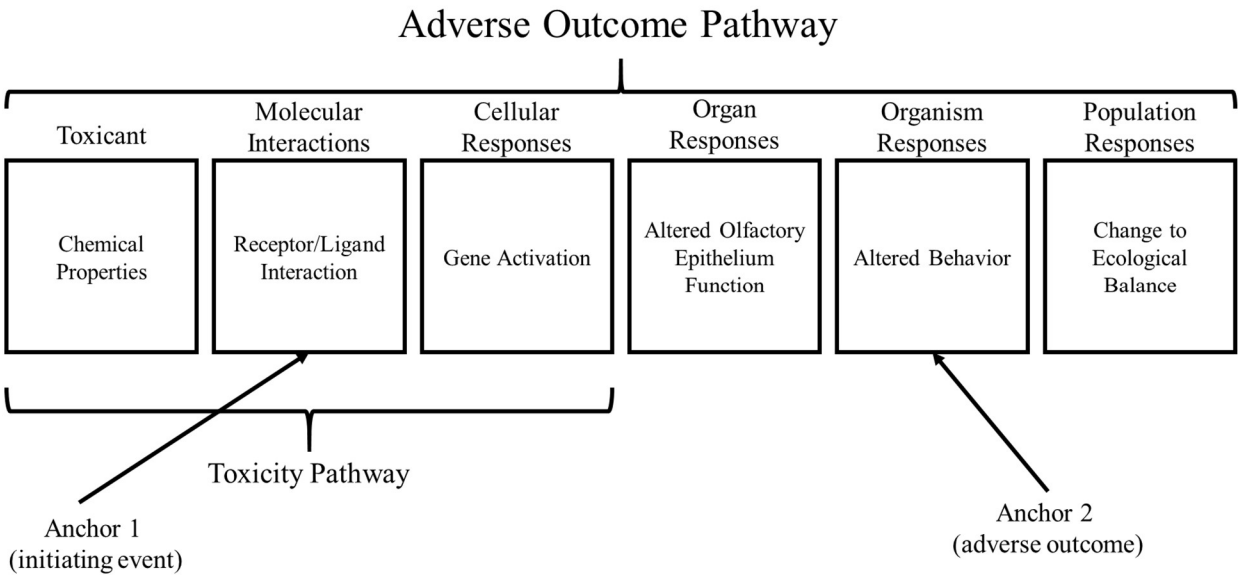

Figure 1.6 Conceptual diagram of key features of the adverse outcome pathway (AOP) using examples pertaining to this thesis. An AOP begins with a molecular initiating event in which a chemical interacts with a biological target (anchor 1) leading to a sequential series of higher order effects to produce an adverse outcome with direct relevance to a given risk assessment context (e.g., altered behavior, change to ecological balance; anchor 2). Adapted from Ankley et al., 2010.

1.8 Hypothesis and thesis objectives

1.8.1 Hypothesis

TH-responsive gene transcripts in a sensitive, environmentally-exposed frog tissue, such as the olfactory epithelium, can be utilized for monitoring xenoendocrine TH activity in aquatic systems and their abundance levels can be linked to observable changes in behavior.

1.8.2 Thesis objectives

The main objectives of this thesis are:

1) to evaluate whether molecular responses of multiple classical thyroid hormone-responsive transcripts can be correlated to olfactory-mediated behavioral responses to a predatory cue

2) to investigate the transcriptome-wide response of the olfactory epithelium to thyroid hormones to identify novel hormone-responsive transcripts that can be utilized as bioindicators of endocrine activity in environmental discharge of treated municipal wastewaters

The first data chapter addresses the first objective and the second data chapter addresses the second.

2 Behavioral and molecular analyses of olfaction-mediated avoidance responses of *Rana (Lithobates) catesbeiana* tadpoles: Sensitivity to thyroid hormones, estrogen, and treated municipal wastewater effluent

Abstract

Olfaction is critical for survival, facilitating predator avoidance and food location. The nature of the olfactory system changes during amphibian metamorphosis as the aquatic herbivorous tadpole transitions to a terrestrial, carnivorous frog. Metamorphosis is principally dependent on the action of thyroid hormones (THs), L-thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3), yet little is known about their influence on olfaction during this phase of postembryonic development. We exposed Taylor Kollros stage I-XIII *Rana (Lithobates) catesbeiana* tadpoles to physiological concentrations of T_4, T_3, or 17-beta-estradiol (E_2) for 48 h and evaluated a predator cue avoidance response. The avoidance response in T_3-exposed tadpoles was abolished while T_4- or E_2-exposed tadpoles were unaffected compared to control tadpoles. qPCR analyses on classic TH-response gene transcripts (*thra*, *thrb*, and *thibz*) in the olfactory epithelium demonstrated that, while both THs produced molecular responses, T_3 elicited greater responses than T_4. Municipal wastewater feed stock was spiked with a defined pharmaceutical and personal care product (PPCP) cocktail and treated with an anaerobic membrane bioreactor (AnMBR). Despite substantially reduced PPCP levels, exposure to this effluent abolished avoidance behavior relative to AnMBR effluent whose feed stock was spiked with vehicle. *Thibz* transcript levels increased upon exposure to either effluent indicating TH mimic activity. The present work is the first to demonstrate differential TH responsiveness of the frog tadpole olfactory system with both behavioral and molecular alterations. A systems-based

analysis is warranted to further elucidate the mechanism of action on the OE and identify further molecular bioindicators linked to behavioral response disruption.

2.1 Introduction

Extensive changes occur to the larval anuran body plan during metamorphosis with almost all of these changes initiated by the thyroid system (Brown and Cai, 2007). Overall, the anuran thyroid system is comparable to thyroid systems in other vertebrates (Furlow and Neff, 2006). In classic thyroid hormone (TH) production, the thyroid gland secretes thyroxine (T_4), which is transported to target tissues and converted to 3,5',3-triiodothyronine (T_3) through the action of 5' deiodinases. T_3 has historically been referred to as the bioactive form of TH while T_4 is referred to as a prohormone (Brown and Cai, 2007). However, recent evidence indicates that T_4 has tissue-dependent biological activity independent of deiodinase status (Maher et al., 2016).

Metamorphosis is mediated by two nuclear TH receptors (TRs), TRα and TRβ. Thyroid hormones bind to these receptors to facilitate the expression of TH-responsive genes to trigger the remodeling of the entire anuran body plan (Brown and Cai, 2007). Premetamorphic tadpoles have an inactive thyroid gland and no endogenous THs in circulation (Tata, 2006). These TH levels increase naturally and are responsible for the induction of the metamorphic processes. Although there are no THs in circulation during these premetamorphic stages, TRα and TRβ are present in tissues at low levels (Grimaldi et al., 2013; Tata, 2006). Premetamorphic tadpoles are thus equipped to respond to THs, and exposure to exogenous THs has induced precocious metamorphosis in premetamorphic tadpoles in a number of studies (Brown and Cai, 2007; Maher et al., 2016; Tata, 2006).

One of the systems that undergoes major remodeling during anuran metamorphosis is the olfactory system (Hansen et al., 1998; Wang et al., 2008). The olfactory system is generally comprised of paired olfactory cavities that are lined with olfactory epithelia. Ciliated or microvillous olfactory sensory neurons (OSNs) project into the apical surface of the OE and express odor receptors (ORs). Odorants bind to ORs and a signal is propagated from the OSN to the olfactory bulb (OB) through axonal projections. The signal is processed in the OB and can result in behavioral responses to the odorant (Ache and Young, 2005; Gascuel and Amano, 2013).The metamorphic changes in the anuran olfactory system are not restricted to structure alone. Previous studies have measured changes in olfactory acuity to olfactory stimuli during the metamorphosis of *Xenopus laevis* tadpoles (Manzini and Schild, 2004). THs may play a role in triggering both structural and functional changes in the anuran olfactory system during metamorphosis, although no direct link has been reported (Dittrich et al., 2016; Reiss and Burd, 1997). This is especially interesting, as prior to frog metamorphosis, the olfactory system is equipped for aquatic environments exclusively, in which the premetamorphic tadpole maintains a vegetarian diet. Upon completion of metamorphosis, the organism becomes carnivorous. Although *Xenopus laevis* continues breathing air, it maintains an aquatic lifestyle while ranid species emerge on land.

The influence of EDCs which may be found within a multitude of everyday consumer products including pharmaceuticals, pesticides, plasticizers, personal care products, and flame-retardants on olfactory acuity may also have important implications for ecological health. These chemicals accumulate in wastewater and typically undergo treatment. Unfortunately, EDCs are persistent in treated municipal wastewater effluent and

discharged into receiving waters such as rivers and reservoirs and have been detected across the globe at concentrations as high as µg/L (Boyd et al., 2003; Kolpin et al., 2002). Although the bulk of past EDC research has focused on the effects of these chemicals on the reproductive system (Jobling et al., 2002; Scott and Sloman, 2004), some EDCs share similar structures with T_3 and T_4. These can interfere with the normal function of the thyroid system (Crofton, 2008), and studies have reported accelerated or delayed metamorphosis in tadpoles as a result of EDC exposure (Crump et al., 2002; Sowers et al., 2009; Veldhoen et al., 2006, 2014b). Detection of olfactory stimuli informs aquatic organisms about the location of both potential threats and sources of food, and therefore influences behavior (Laberge and Hara, 2001). The effects of TH on tadpole behavior are largely unstudied, however there is evidence for TH disruption leading to changes in behavior in fish (Zhou et al., 2000).

The purpose of the present study was two-fold: (1) to characterize the effects of exposures to physiologically relevant concentrations of THs on olfactory-mediated avoidance responses in *Rana (Lithobates) catesbeiana* tadpoles and to try to link those responses to classic thyroid hormone-response endpoints and (2) to investigate the effects of treated municipal wastewater effluent using the same behavioral and molecular endpoints.

Premetamorphic tadpoles were exposed for 48 h to each of T_3, T_4, 17β-estradiol (E_2), and two municipal wastewater effluents produced from parallel Anaerobic Membrane Bioreactor (AnMBR) treatment trains, wherein the municipal wastewater feed stock was spiked with a pharmaceutical and personal care product (PPCP) cocktail of known and suspected EDCs (effluent 2) or vehicle alone (effluent 1). Following exposure, olfactory-

mediated avoidance responses were measured. Previous studies have used behavioral endpoints such as activity and refuge use (Ferrari et al., 2007; Garcia et al., 2012). In the present study, olfaction was measured by quantifying tadpole chemosensory-mediated responses in a linear trough-style maze (I-maze). Estrogenic compounds are often measured in effluent receiving waters (Kolpin et al., 2002), but have not been shown to have direct effects on the thyroid system. In the present study, E_2 exposure served to determine whether effects on the olfactory system are specific to THs. After behavioral tests were completed, classic TH-response gene transcript levels were determined in the OE tissue. Significant differences were observed in both the behavioral and the molecular analyses between the different chemical exposures.

2.2 Materials and methods

The present study was conducted in two separate experimental locations. The model chemical exposures using T_3, T_4, and E_2 were conducted at the University of Lethbridge, Lethbridge, AB. The municipal wastewater effluent exposures were conducted at the Pacific Environmental Science Centre (PESC), North Vancouver, BC.

2.2.1 Experimental animals

Premetamorphic *R. catesbeiana* tadpoles of mixed sex were caught locally in Victoria (BC, Canada) and staged according to Taylor and Kollros (TK) (Taylor and Kollros, 1946). Tadpoles were fed daily with *Spirulina* (Aquatic ELO-systems, Inc., FL, USA) and housed at the University of Victoria Outdoor Aquatics Unit in 100 gallon covered fiberglass tanks containing recirculated dechlorinated municipal water at 15±1°C, pH 6.8 and 96-98% dissolved oxygen (DO). Depending upon the experiment, tadpoles were sent either to the University of Lethbridge or to PESC. The care and treatment of animals was

in accordance with guidelines established by the Canadian Council on Animal Care and approved by the Animal Care Committees of the Universities of Victoria and Lethbridge.

Tadpoles sent to Lethbridge for the model chemical experiment were housed at the University of Lethbridge in the Aquatic Research Facility on a re-circulatory system. Tadpoles were fed *Spirulina* flakes (Nutrafin Max, Rolf C. Hagen, Montreal, PQ, Canada) *ad libitum* daily, and were held on a light: dark 16: 8 h photoperiod. Prior to running experiments, tadpoles were acclimated to 24 °C for 24h.

Tadpoles sent to PESC for the wastewater experiment were housed at PESC, North Vancouver, British Columbia in a covered outdoor facility. Tadpoles were brought indoors 48 h prior to the start of the experiment, fed *Spirulina* flakes *ad libitum* daily, and housed at 19°C under a light: dark 16:8 h photoperiod.

2.2.2 Experimental exposures

2.2.2.1 Model chemicals

Tadpoles were exposed to physiologically relevant concentrations of T_3 (Sigma-Aldrich, Oakville, ON; Catalog #T2752, CAS 55-06-1), T_4 (Sigma, Catalog #T2501, CAS 6106-07-6), or water-soluble E_2 (Sigma, Catalog #E4389, PubChem Substance ID: 329799056) at 24 °C for 48 h (Maher et al., 2016). T_3 and T_4 sodium salts were solubilized with an 800 nM NaOH vehicle, and dechlorinated water was used for the water-soluble E_2. For the T_3 exposure set, tadpoles were exposed to one of the concentrations of 0.1, 1, or 10 nM T_3 (equivalent to 0.065, 0.65, and 6.5 µg/L, respectively), 800 nM NaOH vehicle control, or dechlorinated water. For the T_4 exposure set, tadpoles were exposed to one of the concentrations of 0.5, 5, and 50 nM T_4 (equivalent to 0.078, 0.78, and 7.8 µg/L, respectively), 800 nM NaOH vehicle control, or dechlorinated water. The concentrations of T_4 to which tadpoles were exposed were 5

times greater than the T_3 concentration series due to the ~5 times greater biological activity and TR binding affinity of T_3 in comparison to T_4 (Maher et al., 2016). For the E_2 exposure set, tadpoles were exposed to one of the concentrations of 0.1, 1, or 10 nM water-soluble E_2 (equivalent to 0.027, 0.27, and 2.7 µg/L, respectively) or dechlorinated water. Detailed tadpole morphology for each exposure group is reported in **Appendix A**. All exposures were conducted in aerated 15 L polypropylene buckets (Home Depot Canada, North York, ON, Canada) at a ratio of 7.5 L per tadpole (2 tadpoles per bucket). Water quality parameters were tested regularly for each treatment and are reported in **Appendix B**. Actual hormone measurements in the treatment water were measured by liquid chromatography – tandem mass spectrometry (LC-MS/MS) for the THs or liquid chromatography – mass spectrometry – quad time of flight (LC/MS-QTOF) for E_2 (**Appendix C**) and were found to be similar to the nominal concentrations. Therefore, nominal concentrations for the model chemicals are used throughout the manuscript.

2.2.2.2 Municipal wastewater effluents

An anaerobic membrane bioreactor (AnMBR) was utilized to treat municipal wastewater at the University of Waterloo (Waterloo, ON). The AnMBR combines an anaerobic biological process for biodegradation of contaminants with an ultrafiltration membrane to produce an effluent with low particulate concentrations while using a low energy input. A detailed description of this wastewater treatment process is provided in **Appendix D**. Raw sewage was collected every other day from nearby sanitary sewers and spiked with either the PPCP cocktail or a vehicle control (0.0017% methanol, 0.0080% ethanol). PPCP cocktail composition details are in **Appendix E**. To create the PPCP cocktail, fifteen known or suspected endocrine disrupting chemicals that are

commonly found in municipal wastewater were obtained from Sigma-Aldrich (Oakville, ON, Canada) with exception of butylparaben (Acros Organics, Geel, Belgium), and tonalide (Santa Cruz Biotech, Mississauga, ON, Canada). Most of the PPCP cocktail constituents were previously used to evaluate the treatment of a synthetic wastewater and the concentrations were chosen to reflect typical amounts found in municipal wastewater (Osachoff et al., 2014). This cocktail was prepared as a 10,000x concentrate at the University of Victoria and shipped to the University of Waterloo by overnight courier on ice.

Two benchtop treatment plants were run in parallel, with one processing vehicle-spiked feed stock to produce effluent 1, and the other processing the PPCP cocktail-spiked feed stock to produce effluent 2. AnMBR operation was monitored over a two-month period with consistent addition of spiked material to the feed stock to ensure consistent performance. Orthophosphate, ammonia, and biochemical oxygen demand (BOD) results are in **Appendix F**. Effluent was collected over the course of approximately four days and stored at 4 °C before being shipped by overnight courier in coolers to PESC.

Concentrations of effluent tested were determined with a range-finder test. Tadpoles were exposed for 48 h at 16°C at a ratio of 3.5 L per tadpole in aerated aquaria (3-4 tadpoles/aquarium). Tadpoles were exposed to a geometric dilution series of effluent for 48 h during which mortality was measured. The highest concentration of effluent to cause no mortality was utilized in the present study plus an additional dilution thereof. Tadpoles were exposed to one of (1) well water, (2) 7.5% effluent 1, (3) 15% effluent 1, (4) 7.5% effluent 2, or (5) 15% effluent 2. Detailed tadpole morphology for each exposure group is reported in **Appendix G**. Water samples were collected from exposure aquaria on the

first and last experimental days. Water quality and PPCP analyses were performed using standard analytical procedures at PESC (**Appendices H and I**).

2.2.3 Behavioral experiments and tissue collection

The avoidance response was measured using an I-Maze. Photographs of the experimental setup are shown in **Appendix J**. An individual tadpole was placed into an acclimation chamber and subjected to a 20-minute acclimation period in clean water prior to the start of each test. An amino acid mixture that elicited an avoidance response simulating a predator cue (Castilla, 1972; Rehnberg et al., 1985; Shamushaki et al., 2011; Sola et al., 1993) was simultaneously administered to one end of the I-maze and dechlorinated water (blank) to the other and the amount of time spent in each arm of the maze was recorded via webcam (Logitech, Romanel-sur-Morges, Switzerland) and viewed remotely on a laptop computer (MacBook Air, Apple, Cupertino, CA, USA). The researcher was blind to which arm of the maze that the amino acid cue or blank was administered. The amino acid mixture was comprised of L-alanine (USP grade, Sigma-Aldrich, Oakville, ON, Canada), L-serine (USP grade, VWR, Radnor, PA, USA), and glycine (proteomics grade, AMRESCO, Cleveland, OH, USA) and was prepared fresh in dechlorinated water on the days of behavioral experiments. Concentrations of amino acid mixture were ultimately determined by the amount required to elicit a reproducible avoidance response to the predatory cue in the control group of tadpoles. Once a reproducible avoidance was achieved, experimental results that differed from this reproducible control group could then be observed and validated. At the University of Lethbridge, an amino acid mixture of 0.022 M each of L-alanine, L-serine, and glycine was used. For the Waterloo effluent exposure experiment, the amino acid mixture was

comprised of 0.1 M of each amino acid. We have established that tadpoles sense these cues through the nares as tadpoles rendered anosmic do not respond to chemosensory cues (Heerema et al., manuscript in preparation).

Upon completion of behavioral tests, tadpoles were euthanized in buffered tricaine methanesulfonate (1000 mg/L; TMS, Aqua Life, Syndel Laboratories, Nanaimo, BC, Canada). The rostrum was removed with a sharp razor blade and then cut in half at the midline before preservation in RNAlater solution (Ambion, Foster City, CA, USA) as per manufacturer's instructions. The properly preserved tissues were stored at -20ºC and shipped to the University of Victoria for further dissection and qPCR analysis.

2.2.4 Total RNA isolation and cDNA preparation

All tissue samples were randomized prior to processing and the extraction of RNA. Rostral halves were sub-dissected to isolate the olfactory sac containing the OE. The epithelia sub-dissected from the two rostral halves from each sample animal were then combined in 300 µL TRIzol and mechanically disrupted in a Retsch MM301 Mixer Mill (Thermo Fisher Scientific, Ottawa, Canada) at 25 Hz for two 3-minute intervals, separated by a 180° rotation of the samples. After pelleting insoluble material with centrifugation at 12,000 xg for 10 minutes at 4 °C, the supernatant was transferred to a new RNAse-free tube. RNA was extracted and washed using chloroform, isopropanol, and ethanol treatments and subsequently dissolved in 30 µL diethyl pyrocarbonate-treated water (Sigma-Aldrich) and stored at -80 °C. One µg of total RNA was taken from each sample and used in the preparation of cDNA via a High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor as per the manufacturer's instructions (Applied Biosystems Foster City, CA, USA).

2.2.5 Quantitative real-time polymerase chain reaction (qPCR)

Four separate mRNA transcripts were analyzed across all experimental sets: TH receptor α (*thra*), TH receptor β (*thrb*), TH-induced basic region leucine zipper-containing transcription factor (*thibz*), and olfactory marker protein (*omp*). *Thra*, *thrb*, and *thibz* are well-characterized TH-responsive genes that exhibit hormone-associated up-regulation (Helbing et al., 1992; Maher et al., 2016; Yaoita and Brown, 1990), while *omp* codes for a protein that is uniquely associated with the mature olfactory receptor neurons in most vertebrate species (Dibattista and Reisert, 2016; Margolis, 1982). Three additional gene transcripts were used in the qPCR assay as input normalizers: ribosomal protein S10 (*rps10*), ribosomal protein L8 (*rpl8*), and eukaryotic translation elongation factor 1 α (*eef1a*). Primers were designed using the parameters and MIQE-compliant quality control measures employed for qPCR assay development as described previously (Bustin et al., 2009; Maher et al., 2016; Mochizuki et al., 2012; Veldhoen et al., 2014a). qPCR reactions included 10 mM Tris–HCl (pH 8.3 at 20 °C), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween-20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Life Technologies Corp., Carlsbad, CA, USA), 83.3 nM ROX (Life Technologies), 5 pmol of each primer, 200 mM dNTPs (Bioline USA Inc., Taunton, MA, USA), 2 μL of 20-fold diluted cDNA, and 1 unit of Immolase DNA polymerase (Bioline USA Inc.).

All cDNA samples were analyzed in quadruplicate on either an MX3005P qPCR system (Agilent Technologies Canada Inc., Mississauga, ON, Canada) or a CFX Connect real-time system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Each gene transcript was run on only one machine to eliminate any machine bias. Parameters for qPCR were set at 95 °C for 9 minutes, followed by 40 cycles of 15 s at 95 °C, 30 s at a variable, gene-dependent temperature, and 45 s at 72 °C. For *rpl8*, *thra*, and *thrb*, this

gene-dependent annealing temperature was 64 °C; for *rps10*, *eef1a*, and *omp*, the temperature was 60 °C; and for *thibz* it was 62 °C. Covariation among the average cycle threshold (C_t) values for the three normalizer genes (*rpl8*, *rps10*, *eef1a*) was analyzed using RefFinder and BestKeeper to confirm precision in generating a geometric mean used to normalize sample input variation in the qPCR data of the four gene transcripts investigated for TH-response (*thra*, *thrb*, *thibz*, *omp*).

2.2.6 Statistical analyses

For behavioral experiments, tadpoles that did not leave the acclimation chamber throughout the test were removed from the analysis, as determined *a priori*. For each treatment, the average time spent in the cue and blank arm was calculated. Parametric assumptions were tested using the Shapiro-Wilk normality test on the paired differences of time spent in the cue and blank arms of the maze. For data that satisfied parametric assumptions, mean time spent in the blank and cue arm were compared with a paired t-test. When transformations were unsuccessful in reclaiming parametric assumptions, a permutation t-test was used to compare mean time (Legendre and Blanchet, 2015). Mean differences were considered significant when $p \leq 0.05$.

For the molecular analyses, relative fold difference data obtained from the qPCR assays were not normally distributed (Shapiro-Wilk) and displayed unequal variances (Levene's). Therefore, nonparametric Kruskal-Wallis and Mann-Whitney U tests were performed using R 3.3.3 in R Studio (R Core Team, 2017). Fold changes were calculated and statistical significance was taken to be $p \leq 0.05$.

2.3 Results

2.3.1 Model chemical exposures

Olfactory-mediated avoidance responses in bullfrog tadpoles were measured in a linear trough-style maze (I-maze). Three separate exposure trial sets were performed; one for each hormone tested. For the T_4 set, tadpoles in the control groups (dechlorinated water and NaOH vehicle) avoided the amino acid mixture, and thereby spent significantly less time in the cue arm than the blank arm (water: $t_{14} = 2.15$, $p = 0.04$; vehicle: $t_{16} = 2.37$, $p = 0.03$; **Figure 2.1**). Similarly, after exposure to physiologically relevant concentrations of T_4, tadpoles avoided the amino acid mixture (0.5 nM T_4: $t_{14} = 2.19$, $p = 0.04$; 5 nM T_4: $t_{13} = 2.39$, $p = 0.03$; 50 nM T_4: $t_{14} = 2.55$, $p = 0.01$; **Figure 2.1**).

For the T_3 set, after exposure to the water and NaOH treatments, tadpoles spent significantly more time in the blank arm than the cue arm of the maze (water: $t_{26} = 2.3$, $p = 0.02$ vehicle: $t_{29} = 1.9$ $p = 0.05$; **Figure 2.2**). Conversely, after exposure to physiologically-relevant concentrations of T_3, tadpoles failed to avoid the stimulus and therefore spent a similar amount of time in the blank and cue arms

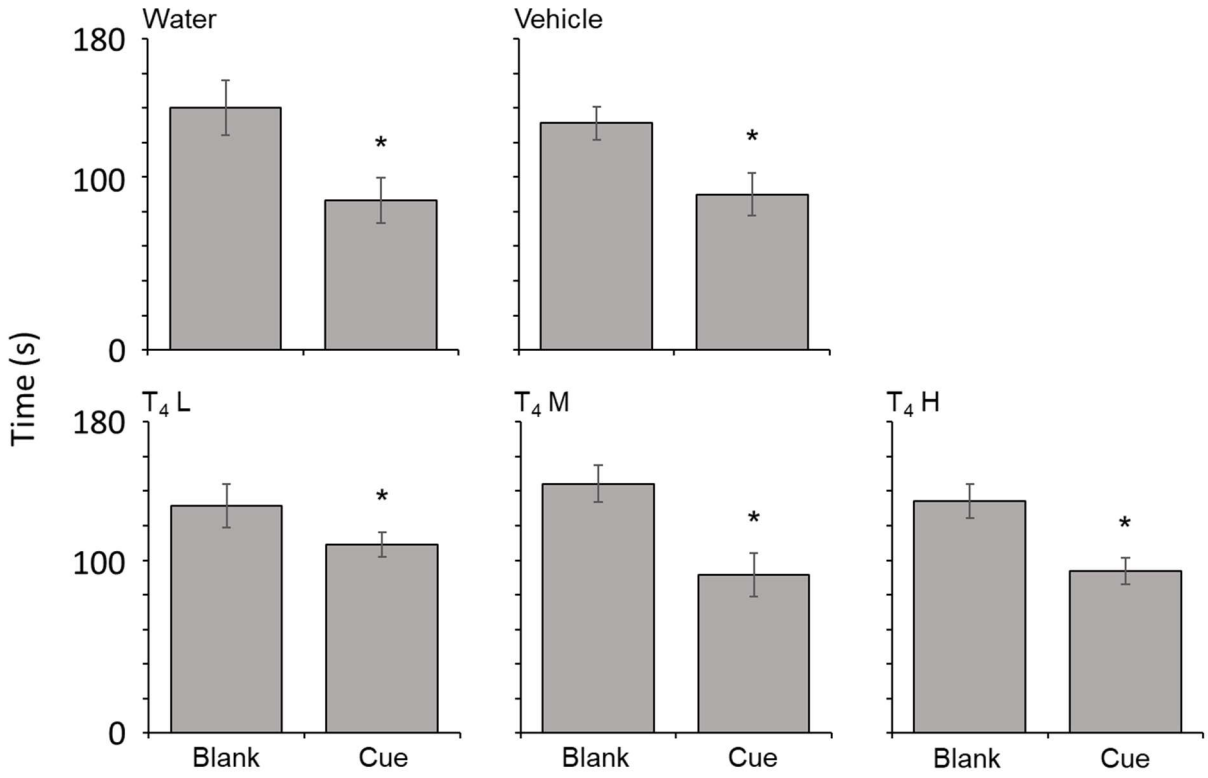

Figure 2.1 Time *R. catesbeiana* tadpoles spent in the blank (water) and cue (amino acid mixture) arms of the I-maze after exposure to water (Water), 800 nM NaOH (vehicle), 0.5 nM T_4(T_4L), 5.0 nM T_4 (T_4M), or 50 nM T_4 (T_4H). An asterisk denotes a significant difference from the blank ($p \leq 0.05$), n = 14 – 16.

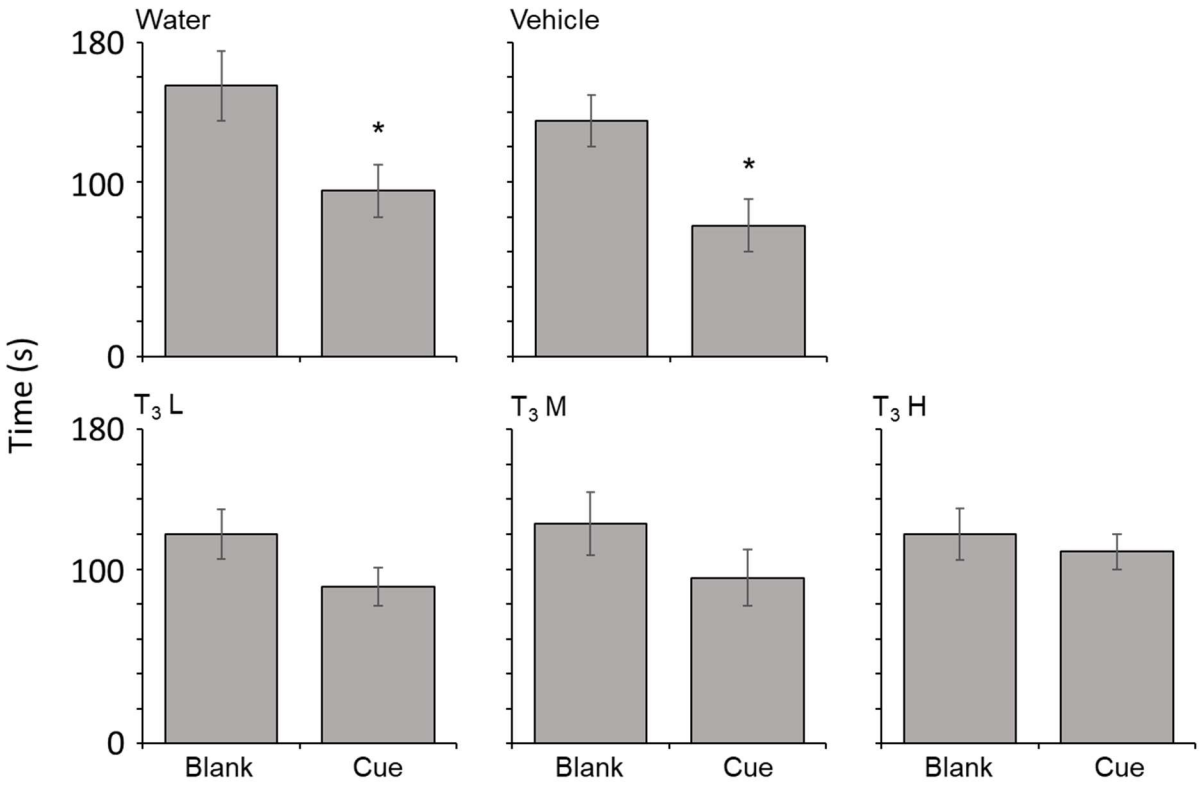

Figure 2.2 Time *R. catesbeiana* tadpoles spent in the blank (water) and cue (amino acid mixture) arms of the I-maze after exposure to water (Water), 800 nM NaOH (vehicle), 0.1 nM T_3(T_3L), 1.0 nM T_3 (T_3M), or 10 nM T_3 (T_3H). An asterisk denotes a significant difference from the blank ($p \leq 0.05$), n = 25 – 31.

of the I-maze (0.1 nM T_3: $t_{29} = 1.3$, $p = 0.18$; 1 nM T_3: $t_{24} = 1.0$, $p = 0.29$; 10 nM T_3: $t_{30} = 0.5$, $p = 0.59$; **Figure 2.2**).

Exposure to E_2 had no effect on avoidance responses to the amino acid mixture. In all treatment groups tadpoles spent significantly more time in the blank arm than in the cue arm of the maze (water: $t_{15} = 2.15$, $p = 0.04$; 0.1 M E_2: $t_{15} = 2.15$, $p = 0.04$; 1.0 M E_2: $t_{15} = 3.83$, $p = 0.001$; 10.0 M E_2: $t_{15} = 4.15$, $p < 0.001$; **Appendix K**).

qPCR was run on olfactory epithelium RNA samples from the vehicle controls, 50 nM T_4- and 10 nM T_3-exposed tadpoles tested in the behavioral experiment. T_4 exposure resulted in significant increases in the abundance of *thra* (2-fold; $p < 0.001$), *thrb* (13-fold; $p < 0.001$), and *thibz* (277-fold; $p < 0.001$) relative to the vehicle control while *omp* transcript abundance significantly decreased (0.8-fold; $p = 0.01$; **Figure 2.3**). T_3 exposure resulted in significant increases in the abundance of *thra* (3-fold; $p < 0.001$), *thrb* (18-fold; $p < 0.001$), and *thibz* (422-fold; $p < 0.001$) relative to the vehicle control while *omp* transcript abundance trended downward (0.8-fold; $p = 0.07$; **Figure 2.4**). E_2 exposure did not affect the abundance of *thra*, *thrb*, or *thibz* transcripts (data not shown). Comparison of the relative responses of *thra* and *thibz* transcripts between the two THs showed a significantly higher response to T_3 compared to T_4 ($p = 0.01$ and 0.02, respectively) while *thrb* and *omp* responses were not ($p = 0.18$ and 0.32, respectively; compare **Figures 2.3** to **2.4**).

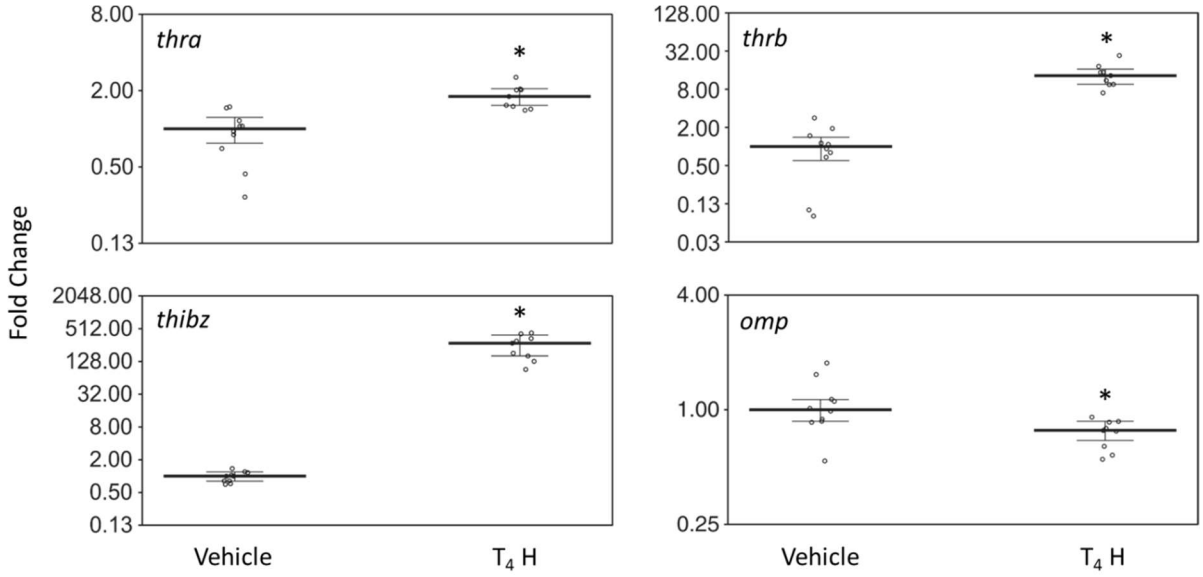

Figure 2.3 Premetamorphic tadpole olfactory epithelium transcript abundance for TH receptor α (*thra*), TH receptor β (*thrb*), TH-induced basic region leucine zipper-containing transcription factor (*thibz*), and olfactory marker protein (*omp*) after 48 h exposure to 800 nM NaOH (vehicle) or 50 nM T_4 (T_4H) as measured by qPCR. The wide bar represents the median, the whiskers represent the median absolute deviation, and the open circles represent the data points of individual animals. An asterisk denotes a significant difference relative to the vehicle ($p \leq 0.05$).

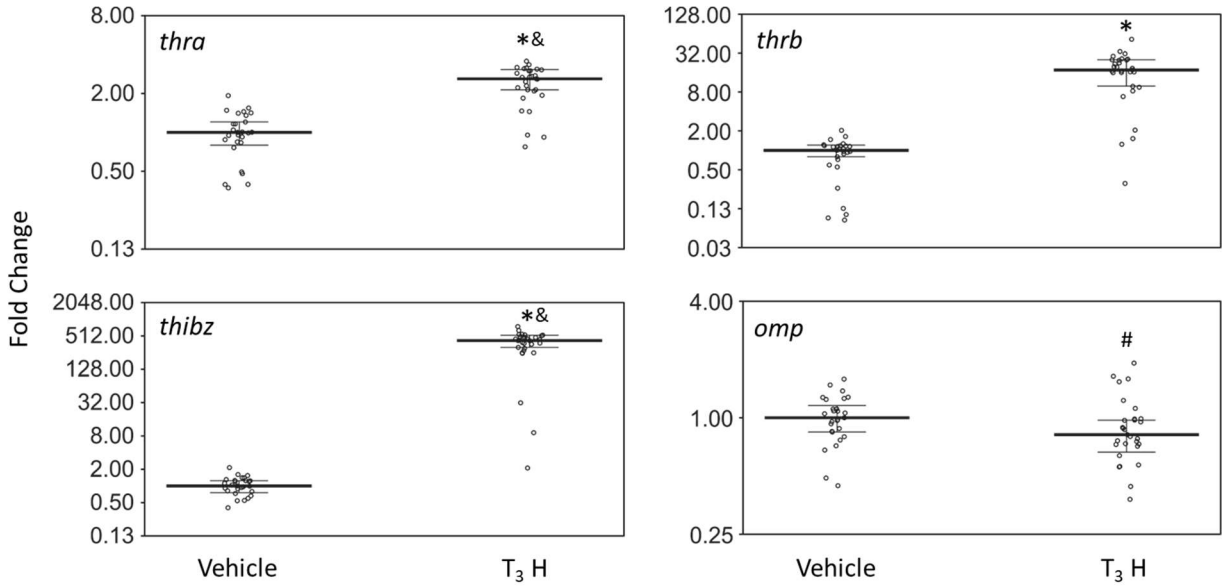

Figure 2.4 Premetamorphic tadpole olfactory epithelium transcript abundance for *thra*, *thrb*, *thibz*, and *omp* after 48 h exposure to 800 nM NaOH (vehicle) or 10 nM T_3 (T_3H) as measured by qPCR. Refer to the Figure 2.3 legend for more details. An asterisk denotes a significant difference relative to the vehicle ($p \leq 0.05$) and '#' denotes $p = 0.07$. An ampersand denotes a significant difference between the T_3H and T_4H groups (compare this figure to Figure 2.3).

2.3.2 Effluent exposures

Manipulation of the influent composition prior to AnMBR treatment resulted in the production of two types of municipal wastewater effluents that were used for tadpole exposures (**Figure 2.5**). Overall, most compounds analyzed were below the detection limit, with some exceptions. Ibuprofen, naproxen, and caffeine were detectable in 15% effluent 1 and effluent 2 treatments. Ibuprofen and naproxen were detected in effluent 1 and were ~4- and 2.5-fold higher, respectively, in effluent 2, as would be expected with the addition of these compounds via the PPCP cocktail to the feed stock. DEHP was consistently present in all water samples from control and effluents. Caffeine levels were reduced by approximately half in effluent 2 as compared to effluent 1, despite the addition of caffeine in effluent 2. All treatments analyzed contained detectable levels of DEHP. Overall, the concentrations of PPCPs observed were consistent through time. All other PPCPs analyzed were not detectable in any treatments.

Avoidance responses to the chemosensory stimulus are presented in **Figure 2.6**. Control tadpoles avoided the chemosensory stimulus and spent significantly more time in the blank arm of the choice maze than the cue arm ($t_{18} = 2.09$, $p = 0.05$). Tadpoles exposed to 7.5% and 15% effluent 1 also spent more time in the blank arm than the cue arm with borderline significance (7.5% effluent 1: $t_{18} = 1.89$, $p = 0.07$; 15% effluent 1: $t_{14} = 1.96$, $p = 0.07$). Tadpoles exposed to 7.5 and 15% effluent 2 spent a similar amount of time in the blank

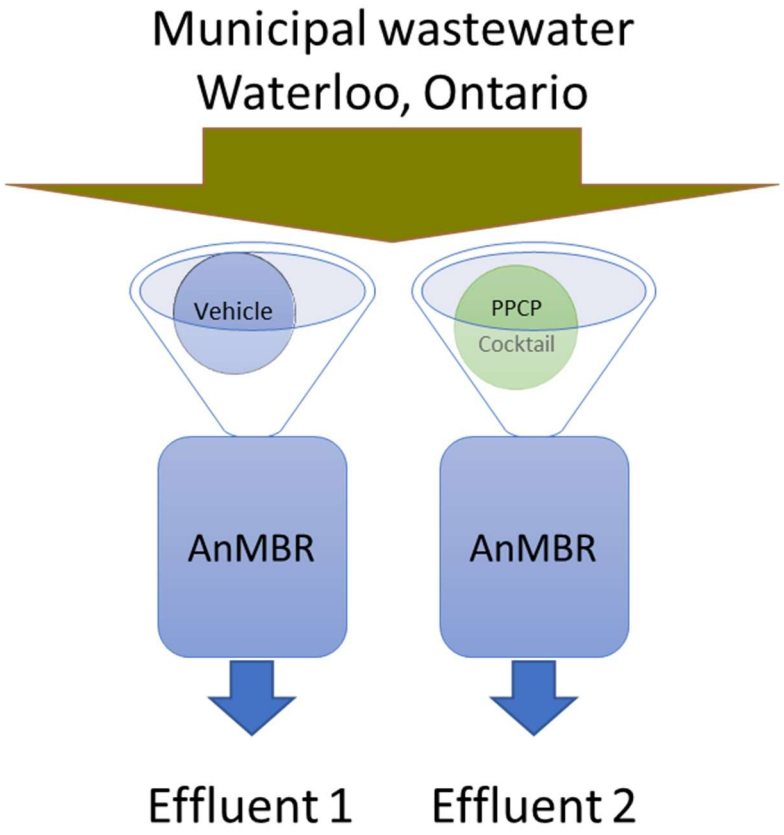

Figure 2.5 Simple schematic of Effluent 1 and 2 production through parallel AnMBRs. Two benchtop treatment set ups were run in parallel, with one processing the vehicle-spiked feed stock to produce Effluent 1 and the other processing the PPCP cocktail-spiked feed stock to produce Effluent 2.

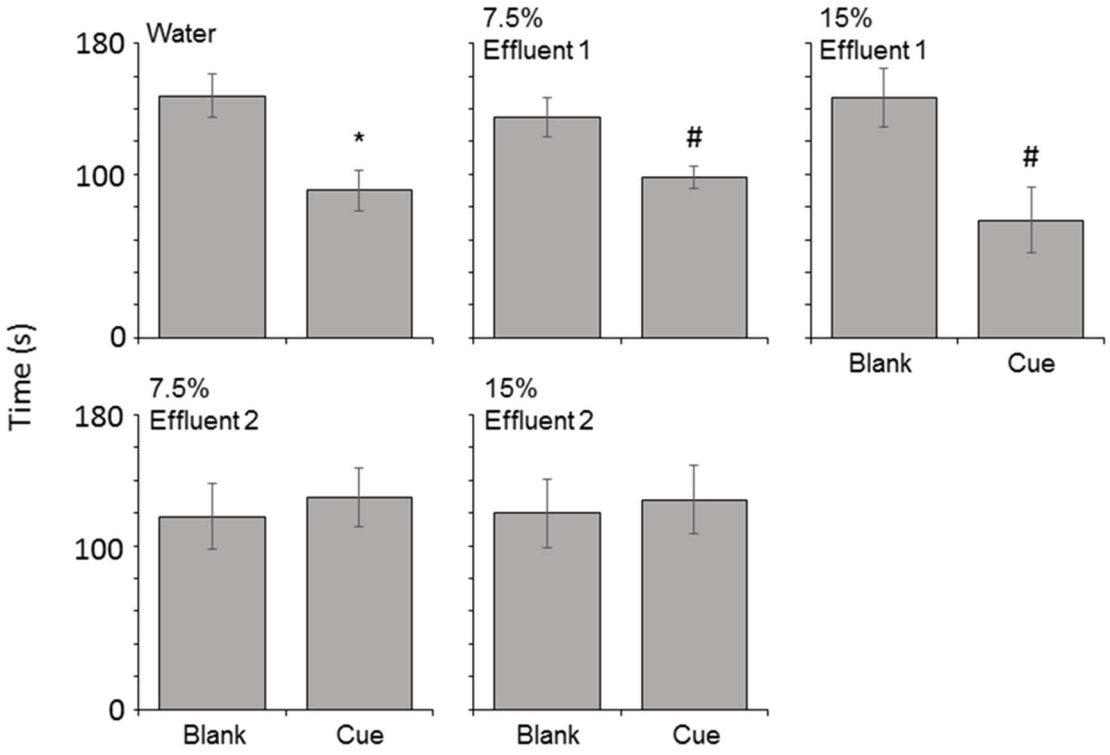

Figure 2.6 Time *R. catesbeiana* tadpoles spent in the blank (water) and cue (amino acid mixture) arms of the linear-style choice I-maze after exposure to water control (Water) or dilutions of University of Waterloo effluents. Error bars represent mean ± standard error. An asterisk denotes a significant difference from the blank ($p \leq 0.05$) and '#' denotes $p = 0.07$, $n = 15 – 20$.

and cue arms of the maze ($t_{19} = -0.37$, $p = 0.71$; $t_{16} = -0.19$, $p = 0.86$, respectively).

Transcript abundances of *thra* and *thrb* in olfactory epithelium isolated from the same animals that were tested for predator cue avoidance showed no significant changes in any of the four treatment conditions (**Appendix L**). However, the levels of *thibz* mRNAs showed a 2-fold significant increase in the higher concentrations of both effluent 1 ($p < 0.001$) and effluent 2 ($p = 0.004$) compared to the control (**Figure 2.7**).

2.4 Discussion

The present study demonstrates for the first time, linkages between TH, behavioral, and gene expression responses in the olfactory epithelium of bullfrog tadpoles. Avoidance responses to an olfactory stimulus were significantly reduced after exposure of premetamorphic *R. catesbeiana* tadpoles to T_3 but not to T_4, suggesting that T_3 modulates changes in sensory perception in the OE, while T_4 does not. While both hormones elicited changes in the expression levels of some classic TH-responsive transcripts, T_3 had a significantly stronger impact on these transcripts.

Tadpoles were exposed to T_3 at concentrations comparable to circulating plasma T_3 concentrations in tadpoles at metamorphic climax (White and Nicoll, 1981). Exposure to T_3 may have initiated precocious metamorphic changes on a cellular level in the olfactory epithelium. The observed reduction in olfactory-mediated avoidance responses after T_3 exposure is corroborated by the cellular-level developmental changes that occur in the olfactory epithelium during the metamorphic climax. For example, Manzini and Schild (2004) reported a change in olfactory acuity to amino acids (including L-alanine, L-serine, and glycine) between premetamorphic and prometamorphic *X. laevis* tadpoles, as measured by calcium imaging. More specifically OSNs from tadpoles NF 51 – 53

(Nieuwkoop and Faber, 1956) (comparable to TK IV – VI) responded to more amino acids than OSNs from tadpoles NF 54 - 56 (comparable to TK VII - XIV; Manzini and Schild 2004). Furthermore Dittrich et al. (2016) demonstrated that OSN apoptosis increased with increasing developmental stage in *X. laevis* tadpoles and peaked at the metamorphic climax, when TH concentrations are highest (Leloup and Buscaglia, 1977).

The discrepancy between the effects of T_3 and T_4 on olfactory-mediated responses observed in the present study was unexpected. The concentrations used for both T_3 and T_4 (5x higher) were expected to simulate levels measured during metamorphic climax, and induce the same changes. Traditionally, T_4 is considered a precursor for the more biologically active T_3. Deiodinase enzymes (D1 and D2) convert T_4 to T_3 locally at target tissues (Grimaldi et al., 2013). Although premetamorphic tadpoles have no circulating endogenous THs, many tissues are enriched with deiodinase enzymes and TRs and are able to respond to exogenous THs (Tata, 2006). However, one form of deiodinase enzyme, D3, converts both T_4 and T_3 to TH derivatives (rT_3 and T_2, respectively), which renders them biologically inactive. A recent analysis has even found that T_2 has ability to differentially regulate different isoforms of TH receptors, painting an even more complex system of modulation than previously stipulated (Mendoza et al., 2013). Previous studies have hypothesized that ratios of D1, D2, and D3 in tissues contribute to the regulation of coordinated metamorphosis (Becker et al., 1997). For example, tissues that change at the end of metamorphosis, such as the retina and tail fin, are rich in D3, and are depleted in D1 and D2 during premetamorphosis to keep THs from binding too early and maintain metamorphic timing (Forrest et al., 2002; Maher et al., 2016).

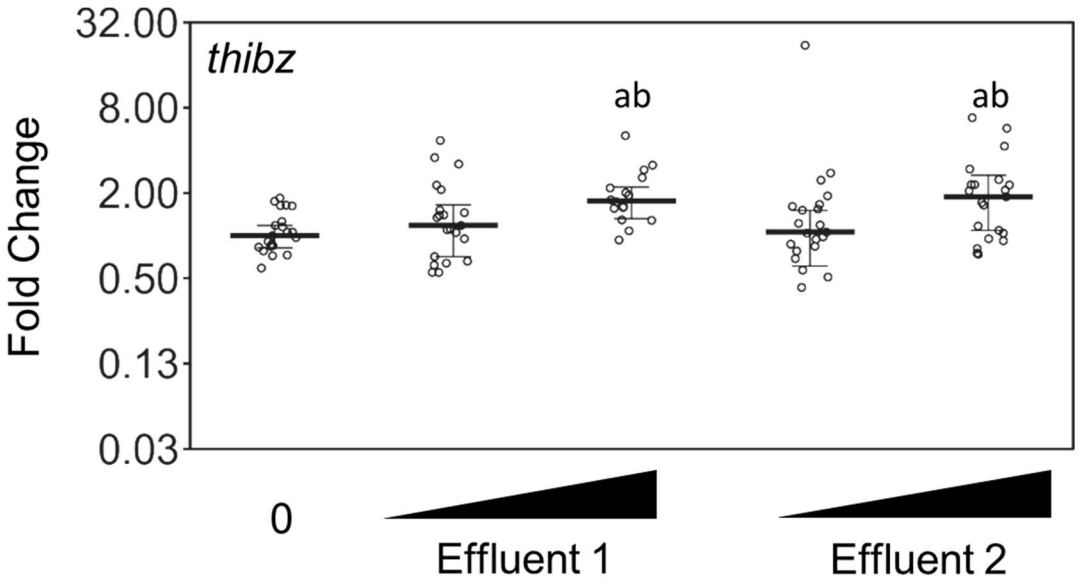

Figure 2.7 Premetamorphic tadpole olfactory epithelium transcript abundance for *thibz* as measured by qPCR. Animal groups were exposed to well water control ("0") or 7.5% or 15% Effluent represented by the bevel for each effluent type for 48 h. Refer to the **Figure 2.3** legend for more details. 'a' denotes significance between treatment and the control. 'b' denotes significance between the two concentrations within an effluent type.

Generally, tissue gene expression programs are altered via TH binding through their nuclear receptors. T_4 binds with ~5-fold lower affinity to these receptors compared to T_3 (Frieden, 1968; White and Nicoll, 1981; Zhang et al., 2006). If T_4 acts directly on a tissue, then a 5-fold higher concentration of T_4 would elicit a similar gene expression response compared to T_3 (e.g. compare 0.1 nM T_3 to 0.5 nM T_4 response; 1.0 nM T_3 versus 5.0 nM T_4; 10 nM T_3 versus 50 nM T_4 (Maher et al., 2016)). If T_4 is converted to T_3 through deiodinase activity within the OE, then a stronger transcript response is expected from T_4 compared to T_3 under non-saturating conditions. Both of these scenarios have been previously observed in other tissues in premetamorphic *R. catesbeiana* tadpoles (Maher et al., 2016). However, we observed a significantly higher response in olfactory epithelium from tadpoles treated with T_3 compared to T_4 for two classical direct TH-response gene transcripts (*thra* and *thibz*). These findings, along with the marked difference in disruption of behavior upon T_3 exposure, suggest that there are differential preferences for either T_4 or T_3 in the metamorphic program executed in the olfactory epithelium. To discern the apparent differences in mechanisms of action between these two THs, a systems-based analysis is warranted using RNA-seq approaches. Ultimately, these analyses will inform adverse outcome pathways to determine deleterious exposure effects.

Estrogenic compounds are commonly measured in wastewater effluent (Boyd et al., 2003; Ryu et al., 2011), but few studies have investigated their effects on the thyroid system in tadpoles. There is some evidence to suggest estrogenic compounds may behave antagonistically in the thyroid system. For example, *X. laevis* and *R. pipiens* tadpoles exposed to high levels of E_2 or 17α-ethynylestradiol (EE_2), respectively, experienced

delayed metamorphosis (Gray and Janssens, 1990; Hogan et al., 2008; Vasudevan et al., 2002). It has been demonstrated that estrogen receptors are significantly increased when the tadpole has reached metamorphic climax (Hogan et al., 2007), and that rising levels of TH can modulate the expression of estrogen pathways (Duarte-Guterman and Trudeau, 2010). An absence of an effect by E_2 exposure in the present study may be a factor of the lack of circulating ERs that are able to modulate such an effect. With the tadpoles in the present study being premetamorphic, and not being exposed to exogenous TH, as were those in the E_2 exposure groups, minimal effect may then be expected. It is possible that if exposed to E_2 later in metamorphosis, there could be a very different transcriptomic result. A number of studies have demonstrated an effect of estrogenic compounds on adult olfactory acuity (Gilbert, 2000; Nakazawa et al., 2009), however, this is the first analysis to our knowledge of the effect of estrogens on gene expression in the tadpole olfactory system. It appears that the avoidance cue behavior is refractory to this hormone suggesting that responsiveness to estrogens requires further maturation of the olfactory system.

Exposure to the treated wastewater effluent resulted in reduced olfactory-mediated avoidance responses to the chemosensory stimulus. Although tadpoles exposed to effluent 1 did not significantly avoid the chemosensory stimulus, it is evident these tests were trending towards significance ($p=0.07$), as tadpoles tended to spend more time in the blank arm than the cue arm. Tadpoles exposed to effluent 2, however, spent a similar amount of time in each arm. Though previous studies have reported effects of EDCs on behavioral endpoints including activity, startle response, and survivorship (Fraker and Smith, 2004; Smith and Burgett, 2005), very few studies have investigated the effects of

EDCs on olfactory disruption in anurans. However, a study completed by Troyer and Turner (2015) reported that *Hyla versicolor* tadpoles exposed to treated effluent and presented with a predator chemosensory stimulus were more active than control groups. The authors concluded that poor water quality in the wastewater effluent interfered with the chemosensory stimulus which resulted in reduced responses to the chemosensory stimulus (Troyer and Turner, 2015). In the present study however, it is unlikely poor water quality contributed to the differential avoidance responses to effluents 1 and 2 given that the water quality was comparable before and after dilution. Therefore, the reduced olfactory acuity observed in the present study cannot be attributed to contaminant and stimulus interactions.

Other studies have reported reduced behavioral or electrophysiological responses to chemosensory stimuli after exposure to pesticides in fish (Moore and Waring, 2001) and tadpoles (Ehrsam et al., 2016). For example, Moore and Waring (2001) suggested olfactory responses were reduced in Atlantic salmon after exposure to the pesticide cypermethrin as a result of disrupted sodium channels in the olfactory epithelium, as measured by a neurophysiology technique. This experiment and those previously mentioned were completed with constant exposure to pesticides. Furthermore, Rohr et al. (2009) demonstrated that deleterious effects of pesticide exposure on olfactory acuity in *Bufo americanus* tadpoles were reversed after 30 minutes in clean water (Rohr et al., 2009). In the present study, tadpoles were subjected to a 20-minute acclimation period in clean water in the behavioral mazes prior to the start of each test. Therefore, it is highly unlikely the reduced avoidance responses to the chemosensory stimulus in the current study were a result of fatigued olfactory neurons.

Reduced olfactory-mediated avoidance responses after exposure to treated effluent observed in the present study may have been a consequence of TH disruption since an increase in *thibz* transcript abundance, a sensitive TH-induced responder, was observed. However, this significant response was observed in effluent 1 as well. This suggests that there may be other as-yet-undiscovered distinguishing molecular bioindicators that may better discriminate this observation. Further demonstration of these possible TH agonistic effects are found in observations of the early onset of metamorphosis after exposure to EDCs (Crump et al., 2002; Veldhoen et al., 2006). Therefore, the mechanism driving the reduced avoidance responses in the present study could be partly developmental.

However, behavior is not influenced by olfaction alone, but also by multiple hormone and sensory systems (Scott and Sloman, 2004). Considering the diverse composition of wastewater effluent, more than just the thyroid system may have been affected in the effluent-exposed tadpoles. For example, Scholz et al. (2000) reported neurotoxic effects of the pesticide diazinon. *Oncorhynchus tshawytscha* exposed to diazinon inhibited neurotransmitters and resulted in reduced olfactory-mediated avoidance responses (Scholz et al., 2000). Given that pesticides are commonly measured in wastewater effluent (Kolpin et al., 2002), it is possible they were also present in the Waterloo effluent. Therefore, the effects of the Waterloo effluent exposure on the olfactory system were likely not resultant solely of TH disruption, but also by disruption of other pathways.

The AnMBR treatment was effective at removing most of the added EDCs from the PPCP-spiked effluent 2, however, some EDCs were detected. Caffeine, DEHP, ibuprofen, and naproxen persisted in both treated effluent 1 and effluent 2. DEHP was

consistently present in all water samples from control and effluents. Of these, there is evidence that DEHP, ibuprofen, and naproxen can affect TH action. With respect to DEHP, there is no current literature on its effects on amphibian or fish behavior, but physiological effects in fish have been reported (Jia et al., 2016). *Danio rerio* (zebrafish) embryos exposed to DEHP experienced an increase in endogenous TH concentrations, which suggests that DEHP can act agonistically on the thyroid system. Non-steroidal anti-inflammatory drugs (NSAIDs), including ibuprofen and naproxen, are commonly found in wastewater effluent (Kolpin et al., 2002) and can be thyroid active, but overall their effects on aquatic organisms are poorly studied. However, we previously demonstrated that ibuprofen can have agonistic effects on THs in *R. catesbeiana* tadpoles, as measured by the mRNA profile in the liver (Veldhoen et al., 2014a). Of these persistent EDCs, it seems most likely that ibuprofen and naproxen may be the strongest correlates with the observed increase in disruption of olfactory-mediated behavior in effluent 2. These compositions are obviously complex and contain many unknown agonistic and antagonistic effects, so these correlations are limited in scope and should be investigated further.

These dynamic interactions among the compounds in the PPCP cocktail are further complicated by the unidentified mixture of compounds that exist in the extracted Waterloo effluent itself, which were not analyzed or tested for. Kolpin et al. (2002) measured 82 different contaminants across 139 effluent-affected streams throughout the USA. Most streams contained seven contaminants, but a maximum of 38 were identified in one stream (Kolpin et al., 2002). The large number of measured contaminants provides a multitude of possible unique mixtures. Mixtures can result in additive, synergistic, or

antagonistic effects, and studies have reported toxicity at lower concentrations in mixtures than when compounds are alone (reviewed in Petrie et al., 2015). For example, exposure to a mixture of NSAIDs inhibited tactile function in *Limnodynastes peronii* tadpoles at lower concentrations than when provided individually (Melvin et al., 2014). Therefore, the individual EDCs measured in the present study may not account for the entirety of the disruption olfactory-mediated behavioral responses. As the present study used wild-caught tadpoles, and treated wastewaters are released directly into the ecosystem, it is clear that exposure to wastewater effluent has the potential to affect important sensory systems necessary for survival.

In summary, we have shown that exposure of premetamorphic *R. catesbeiana* tadpoles to exogenous TH disrupts olfactory-mediated avoidance behavior while estrogen does not, and have established that the olfactory epithelium responds to TH by changes in gene expression. Therefore, olfaction should be considered as a critical system when evaluating the impact of EDCs during this sensitive time of amphibian development.

3 Transcriptomic analysis of the *Rana (Lithobates) catesbeiana* tadpole olfactory epithelium in response to thyroid hormones, estrogen, and treated municipal wastewater effluent exposures

Abstract

Orchestration of metamorphic changes in the olfactory system of frog tadpoles is critical for the successful transition from an aquatic herbivore to a terrestrial carnivorous juvenile frog. Extensive restructuring is induced by the thyroid hormones (THs), L-thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3). The nature of the gene expression programs involved in this process is poorly understood and disruption of its normal function and regulation by anthropogenic endocrine disrupting chemicals (EDCs) may deleteriously affect anuran fitness. Premetamorphic *Rana (Lithobates) catesbeiana* tadpoles were exposed to biologically-relevant concentrations of T_4, T_3, or 17-beta-estradiol (E_2) for 48 h and the gene expression programs were examined in the olfactory epithelium (OE) and olfactory bulb (OB) using a combination of quantitative real-time polymerase chain reaction (qPCR) and RNA-seq analyses. The OE was more sensitive to hormone exposure than the OB and RNA-seq analyses of the OE revealed sensitivity towards THs compared to E_2. T_3 and T_4 exposure resulted in more differentially expressed contigs (38,830 and 31,439, respectively) than exposure to E_2 (267) with few shared contigs among all three hormones. While a substantial portion of contigs were common between the THs (25,245), 13,486 and 6,124 contigs were unique to T_3 and T_4, respectively. Classical and new bioindicators queried TH-disrupting activity in tadpoles exposed to treated municipal wastewater effluent from two different treatment systems: anaerobic membrane bioreactor (AnMBR) and membrane enhanced biological phosphorous removal (MEBPR). While substantial removal of known and suspected

EDCs was observed, evidence for TH disruption remained in tadpole OE exposed to effluent.

3.1 Introduction

Olfaction is an essential sensory function for the survival and fitness of frogs throughout their life. Olfactory requirements develop and change as the animal transitions from the herbivorous aquatic lifestyle of the larval tadpole into the carnivorous, terrestrial lifestyle of the juvenile frog. While the fundamentals of the olfactory system remain the same - receptors on the surface of the olfactory epithelium (OE) bind odorants, propagate a signal to the olfactory bulb (OB) of the brain which elicits a behavioral response to the odorant (Ache and Young, 2005; Gascuel and Amano, 2013) - what constitutes an appropriate response to different odorants differs depending upon life phase and is essential for survival both in terms of food location and predator evasion.

This metamorphic restructuring of the olfactory tissues is a process initiated by the thyroid system and its two primary thyroid hormones (THs), thyroxine (T_4) and 3,5,'3-triiodothyronine (T_3). Premetamorphic tadpoles have no endogenously circulating THs, and exogenous exposure can induce the onset of metamorphosis (Brown and Cai, 2007; Maher et al., 2016; Tata, 2006). The receptors for these hormones, TRs α and β, are members of the steroid and thyroid hormone receptor superfamily (Evans, 1988), a group of heterodimeric receptors whose structural similarities have exhibited the possibility of cross-regulation or cross-talk with receptors of steroid hormone regulation systems (Zhang et al., 1996). Estradiol (E_2) is a well-studied steroid sex hormone whose response to ecological endocrine perturbations in many wild animal species has been observed and characterized (Colborn et al., 1996; Pinto et al., 2014; Shanle and Xu, 2011).

Despite the clear importance of the olfactory system, very little is known about the gene expression programs affected by these hormones. We have previously demonstrated that THs, but not E_2 exposure, induce the accumulation of classic TH-response gene transcripts (*thra*, *thrb*, and *thibz*) in the OE. Yet only T_3, and not T_4, disrupts the tadpole avoidance response to a predator cue, suggesting that other components in the gene expression program may be linked to the observed behavioral outcomes (Heerema et al., 2017). Using novel transcriptomic resources (Hammond et al., 2017), the present study defines transcriptomic profiles of the olfactory system of premetamorphic *Rana [Lithobates] catesbeiana* tadpoles when exposed to T_4, T_3, or E_2. These analyses provide a framework for the identification of additional hormone-responsive transcripts and the generation of specific, validated quantitative real-time polymerase chain reaction (qPCR) tools for use as bioindicators of endocrine disruption in aquatic systems. The results of the present study indicate that the OE is extremely sensitive to THs compared to estrogen.

We applied this knowledge to evaluate the performance of two different municipal wastewater treatment technologies, membrane enhanced biological phosphorous removal (MEBPR) and anaerobic membrane bioreactor (AnMBR), in removing TH-disrupting compounds. MEBPR is a common secondary treatment technology (Monti et al., 2006), while AnMBR is an embryonic secondary treatment technology that is more sustainable from an energy perspective (Chang, 2014; Liao et al., 2006). Each treatment type utilized municipal wastewater feed stock which was then split into two parallel treatment trains. The feed stock to one treatment train was spiked with a pharmaceutical and personal care product (PPCP) cocktail of established endocrine disrupting chemicals (Heerema et al.,

2017) common in municipal wastewater (effluent 2) and the feed stock flowing into the parallel system received vehicle alone (effluent 1). We then compared the removal of PPCPs, as detected via analytical chemistry methods, with the responses of TH-regulated transcripts present within tadpole OE in order to determine the efficacy of both treatment technologies in removing endocrine disrupting effects from wastewater.

3.2 Materials and methods

3.2.1 Experimental animals

Rana catesbeiana tadpoles of mixed sex were caught locally near Victoria (BC, Canada) by Westwind Sealab Supplies and Taylor and Kollros (TK) staging was used to select premetamorphic tadpoles (Taylor and Kollros, 1946). Tadpoles were fed *Spirulina* (Aquatic ELO-systems, Inc., FL, USA) daily and kept at the University of Victoria Outdoor Aquatics Unit in 378.5 L (100 gallon) covered fiberglass tanks containing recirculated dechlorinated municipal water at 15 ± 1 °C, pH 6.8 and 96-98% dissolved oxygen (DO). Animal husbandry was performed according to the guidelines established by the Canadian Council on Animal Care and the Animal Care Committee of the University of Victoria under permit #2011-030.

Tadpoles were then sent to the Pacific Environmental Science Centre (PESC) and housed on-site in North Vancouver (BC, Canada) in a covered outdoor facility. Tadpoles were brought indoors 96 h prior to the start of the experiment and kept at 20 °C under a light: dark 16: 8 h photoperiod with daily feeding of *Spirulina* flakes *ad libitum*. Tadpoles were fed Nutrafin Max spirulina meal tablets (A6762C) at a ratio of ½ spirulina tablet per animal (9 tablets/tank) on the day that they were moved inside.

3.2.2 Experimental exposures

3.2.2.1 Hormone exposures

Premetamorphic tadpoles (TK stages I-VI) were exposed at 21 °C for 48 h to concentrations of T_3 (Sigma-Aldrich, Oakville, ON; Catalog #T2752, CAS 55-06-1), T_4 (Sigma, Catalog #T2501, CAS 6106-07-6), or E_2 (Sigma, Catalog #E4389, PubChem Substance ID: 329799056). Tadpoles were exposed to one concentration of T_3 (0.1, 1, 10 nM), T_4 (0.5, 5, 50 nM), E_2 (0.1, 1, 10 nM), or 800 nM NaOH vehicle control for the TH exposures or dechlorinated water (E_2 control). The concentrations chosen were based observed physiological and environmental relevance (Maher et al., 2016). All exposures were conducted in aerated 20 L aquaria at a ratio of one tadpole per 10 L (2 tadpoles per aquarium; 12 per treatment condition). Tadpole morphology for each exposure group and water quality measures are reported in **Appendices M and N**, respectively. The same hormone stock solutions were used as in Heerema et al. (2017). Since the actual measured hormone concentrations were very close to nominal (Heerema et al., 2017), the nominal concentrations will be used in the present manuscript.

3.2.2.2 Natural metamorphosis

Tadpoles were grouped into premetamorphic (TK I-VI; n=6), early prometamorphic (TK XV-XVI; n = 7), and late prometamorphic (TK XVII-XVIII; n=7) stages and held at 20-21 ºC for 2 d before tissue collection. Tadpole morphology details and water quality parameters for three TK stages examined are found in **Appendices O and P**, respectively.

3.2.2.3 Municipal wastewater effluents

Two separate municipal wastewater exposure experiments were conducted. The first experiment was conducted using an AnMBR system to treat municipal wastewater from

Waterloo, Ontario at the University of Waterloo as described in Heerema et al., (2017). The second experiment was conducted using an MEBPR activated sludge system (Monti et al., 2006) to treat municipal wastewater from Vancouver, British Columbia at the University of British Columbia. Detailed information regarding the AnMBR set-up and exposure conditions have been described in Heerema et al., (2017), and the MEBPR system in **Appendix Q**. Design specifications of the biological zones of the MEBPR wastewater treatment pilot plant are given in **Appendix R**.

At both sites, two separate benchtop treatment plants were run in parallel to produce two separate treated effluents. Raw sewage was collected every other day from nearby sanitary sewers and spiked with either a PPCP cocktail or a vehicle control (0.0017% methanol, 0.0080% ethanol; Heerema et al., 2017). The PPCP cocktail was composed of fifteen endocrine disrupting chemicals that are commonly found in municipal wastewater and the details of its composition can be found in Heerema et al., (2017). This cocktail was prepared at the University of Victoria, aliquoted, and shipped to both locations. The post-treatment product of the vehicle-spiked wastewater will be referred to throughout the present work as effluent 1, and the post-treatment product of the PPCP cocktail-spiked wastewater as effluent 2.

The reactors at both sites were monitored over a two-month period with consistent addition of spiked reagents to the wastewater to ensure consistent performance. After this period, effluent was collected over the course of 4 d, stored at 4 °C, and shipped in coolers overnight to PESC. Operational performance characteristics have been summarized previously for the AnMBR system (Heerema et al., 2017) and are presented for the MEBPR system in **Appendix S**. A primary difference between the two

technologies in effluent quality was the amount of ammonia (~40 versus 0.1 mg/L, respectively).

The dilutions of treated effluent selected for use in exposures were determined with a mortality range-finder test. Premetamorphic tadpoles were exposed to a geometric dilution series of effluent for 48 h to determine the highest concentration of effluent at which no mortality of exposed tadpoles occurred. For the Waterloo AnMBR effluent, this was 15% and for the Vancouver MEBPR effluent, this was 100%. Two separate exposures in which premetamorphic tadpoles were exposed for 48 h at 16-19 °C at a density of one tadpole per 3.5 L in aerated aquaria (3-4 tadpoles/aquarium) were performed: the first with Waterloo AnMBR effluent at 7.5% and 15% strengths and the second with Vancouver MEBPR effluent with 50% and 100% strengths. Each exposure set had its own well water control, and tadpole morphology measurements for each exposure group were tabulated (see Heerema et al., 2017 for AnMBR set and **Appendix T** for the MEBPR set).

3.2.3 Total RNA isolation and cDNA preparation

Upon completion of the experimental exposures, tadpoles were euthanized in buffered tricaine methanesulfonate (1000 mg/L; TMS, Aqua Life, Syndel Laboratories, Nanaimo, BC, Canada) and the olfactory bulb (OB) and rostrum of each were dissected. These tissues were preserved using RNAlater solution (Ambion, Foster City, CA, USA) as per the manufacturer's instructions. These tissues were stored at -20 °C and shipped on blue ice to the University of Victoria where they were stored at -20 °C until RNA isolation and molecular analysis.

Prior to RNA extraction, all tissue samples were assigned randomized processing numbers to blind the operator until data analysis. The olfactory epithelium (OE) was dissected from the rostrum before RNA extraction as previously described (Heerema et al., 2017). The OB was processed using all the same steps, except that it was mechanically disrupted in the mixer mill at 20 Hz rather than 25. The extracted RNA was stored at -80 °C for RNA-seq, and a portion was converted into cDNA for qPCR.

3.2.4 RNA sequencing (RNA-seq) and transcriptome assembly

Isolated RNA was analyzed for quality and concentration using a Bioanalyzer 2100 (Agilent, Mississauga, Ontario, Canada) and five high-quality (RNA integrity number (RIN) >8) biological replicates were selected for each treatment condition. RNA was shipped at -20 °C to the Michael Smith Genome Sciences Centre (Vancouver, BC, Canada) for strand specific mRNA library construction and sequencing using the HiSeq 2500 (Illumina, San Diego, California, USA) paired-end platform to generate 2x75 base pair reads in the same manner as has been described previously (Hammond et al., 2017). All raw reads can be found under National Center for Biotechnology Information (NCBI) BioProject PRJNA417510.

Read counts were determined by alignment to a >1.9 million contig *R. catesbeiana* reference transcriptome (Bullfrog Annotation Resource for the Transcriptome (BART)) compiled from 132 tadpole samples and six tissue types as outlined in Hammond et al., (2017). The raw sequencing reads of the 30 experimental OE samples (T_4 n=10; T_3 n=10; E_2 n=10) in the present study were then aligned to BART to generate read counts. The total number of aligned contigs were 59,428, 59,111, and 52,699 for T_4, T_3 and E_2, respectively (**Appendix U**). Contigs were annotated using UniProt/SwissProt

(The UniProt Consortium, 2017) and the GenBank Transcriptome Shotgun Assembly (TSA) database to assist with functional annotation. Uniprot IDs were used to run GO analyses (www.geneontology.org) and ReviGO (www.revigo.irb.hr) analyses (Ashburner et al., 2000; Supek et al., 2011).

3.2.5 Quantitative real-time polymerase chain reaction (qPCR)

qPCR primer sets were designed according to a rigorous, multi-tiered quality control regime as outlined in Veldhoen et al., (2014a). Transcript abundances of TH receptor α (*thra*), TH receptor β (*thrb*), TH-induced basic region leucine zipper-containing transcription factor (*thibz*), and iodothyronine deiodinase 2 (*dio2*), were determined using methods described previously (Heerema et al., 2017). Following minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009), three constitutively expressed transcripts were used for normalization: ribosomal protein S10 (*rps10*), ribosomal protein L8 (*rpl8*), and eukaryotic translation elongation factor 1 α (*eef1a*). These normalizers were analyzed for covariation using BestKeeper (Pfaffl et al., 2004), and for Cronbach's Alpha provided by reliabilitycalculator2 by Del Siegle (https://researchbasics.education.uconn.edu/instrument_reliability/). A representative example of the results of these analyses is provided for the T_3 exposure set in **Appendix V**. Details regarding primer sequences and amplification conditions can be found in Heerema et al. (2017).

A thorough analysis of differential expression using DEseq2 (Love et al., 2014) on the RNA-seq data was performed to locate the strongest possible qPCR targets for sensitive bioindicators of T_3 and T_4 exposure for future studies. These targets were chosen based

on a combination of TH-induced response strength, confidence values based on the separation of TH-treated and control sample groups, biological relevance to olfaction and sensory systems, and biological relevance to development and growth. After rigorous qPCR primer design and validation, transcripts encoding stromelysin 3 *(st3*; *mmp11*), transient receptor potential cation channel subfamily V member 1 (*trpv1*), and two unannotated transcripts were examined.

One of the unannotated transcripts was comprised of a set of similar contigs (sequences assembled *in silico* from raw RNA sequencing reads that may or may not be complete mRNA transcripts) that were related, but did not have any functional annotation except for confirmation of their existence from prior sequencing in the TSA database. Upon assessing recently available extensive genomics data (Hammond et al., 2017), and a combination of CAP3 (Huang and Madan, 1999) and BLAST (Altschul et al., 1990) analyses, we discovered that this group of contigs were actually part of the *thibz* gene. We found three distinct *thibz*-related contigs that are derived from the same gene based upon alignment to the bullfrog genome using GMAP (Wu and Watanabe, 2005) (data not shown; Hammond et al., 2017). Two contigs encode the thibz protein and the third does not have notable coding potential and may possibly be a non-coding RNA (ncRNA). The primer set used in the present study that detects the two splice variants with equal coding potential is subsequently referred to as *thibz*. This primer set has been used in previous studies (Heerema et al., 2017; Maher et al., 2016). The second primer set detects these two splice variants plus the third putative non-coding variant and hybridizes with the 3' UTR. This primer set is referred to as *thibz*-all. An alignment

of the *thibz* variant sequences, with corresponding primer sets and located functional ORF is shown in **Figure 3.1**.

The second unannotated transcript did not have a substantial putative ORF. The longest ORF produced a 121-amino acid theoretical protein that aligned only with 15% coverage and 44% identity with a neuronal regeneration-related protein (NREP) upon NCBI blastx analysis. NREP has recently been identified as a biomarker of liver and kidney toxicity for drug compound screening in rats and human cell lines (Kim et al., 2015). Analysis of the *R. catesbeiana* genome (data not shown) confirms the existence of the corresponding gene sequence. Until further investigation and characterization, it will from this point forward be referenced as a bioindicator of TH-action, and be named *heket*, after the Egyptian frog goddess. Sequence accession numbers for *thibz* and *heket* can be found under NCBI accession numbers MG459288and MG459291, respectively. Information regarding the primer sets designed for the above transcripts is in **Appendix W**. Amplification cycles for all four targets were run with an annealing temperature of 60 °C. All qPCR was performed using SYBR Green on a CFX Connect real-time system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada).

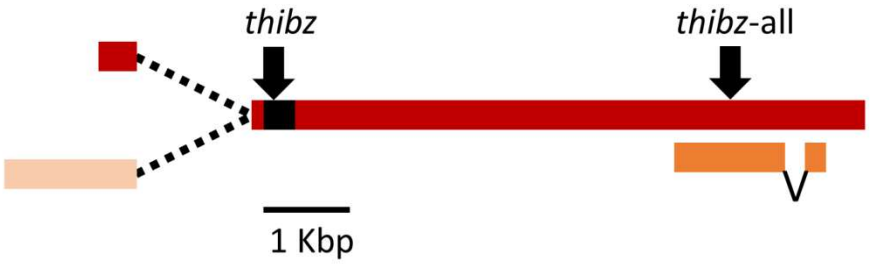

Figure 3.1 Schematic diagram of putative splice variant alignment of the *R. catesbeiana thibz* gene. Two variants have different presumed first exons (red and light red) spliced to a common exon 2. The third putative variant (orange) is exclusive to the 3' UTR with a 320 bp deletion. The ORF is indicated in black. The position of the *thibz* and *thibz*-all primer sets is indicated by the arrows.

3.2.6 Statistical Analyses

Differential expression analyses were conducted using DEseq2 (Love et al., 2014) with read counts generated as described in Materials and Methods 3.2.4. Relative fold difference data obtained from the qPCR assays were not normally distributed (Shapiro-Wilk) and displayed unequal variances (Levene's). Therefore, nonparametric Kruskal-Wallis and Mann-Whitney U tests were performed. Analyses of fold changes were performed using R 3.3.1 (R Core Team, 2017) and statistical significance determined using median and median absolute deviation was defined at $p \leq 0.05$.

3.3 Results

3.3.1 Evaluation of classic TH-responsive gene transcripts

We ran qPCR on the classic TH-response gene transcripts, *thra*, *thrb*, and *thibz*, on the OE of all tadpoles exposed to hormones or their respective controls (n = 12 per treatment group). OE responses to T_4, T_3, and E_2 in the present study produced similar fold changes as those reported in Chapter 2 (**Figure 3.2**). We also ran qPCR on the RNA from the OB of the same animals to compare fold change sensitivity and magnitude with that of the OE. Exposure to E_2 resulted in no significant responses to any of the transcripts tested at any of the concentrations tested in either OB or OE (**Appendix X**). Regarding *thra*, *thrb*, and *thibz*, the OE was markedly more sensitive in response to both T_3 (1.3-, 2.8, and 14-fold change at low concentration for *thra*, *thrb,* and *thibz*, respectively) and T_4 (1.3-, 2.8-, and 26.1-fold for *thra*, *thrb,* and *thibz*, respectively) than the OB (between 0.7 and 1.1-fold; **Figure 3.2**). *Thra*, *thrb*, and *thibz* all showed significant responses at all three concentrations of exposure, to both T_3 and T_4 in the OE (**Figure 3.2**). This response was not seen in the OB, as fewer significant differences relative to the control were observed in both T_3 and T_4 treatments (**Figure 3.2**). OE also exhibited a stronger fold change at

every concentration and in every transcript to both T_3 and T_4 (for example 6.7-fold (OE) versus 1.3-fold (OB) for *thrb* and *thibz* at 192.6 compared to 1.3, respectively, upon treatment with 1 nM T_3; **Figure 3.2**). OB exhibited significantly stronger responses to T_4 than to T_3 in the medium and higher concentrations of *thrb*, *thibz*, and *dio2* (**Figure 3.2**). This phenomenon was not observed in the OE (**Figure 3.2**). Progression through metamorphic stages revealed transcript abundance patterns that mimic those of the precociously-induced exposures (**Appendix Y**). While the pattern is the same, and the changes significant, the magnitude of fold change differences is less when compared to exogenously TH-exposed animals.

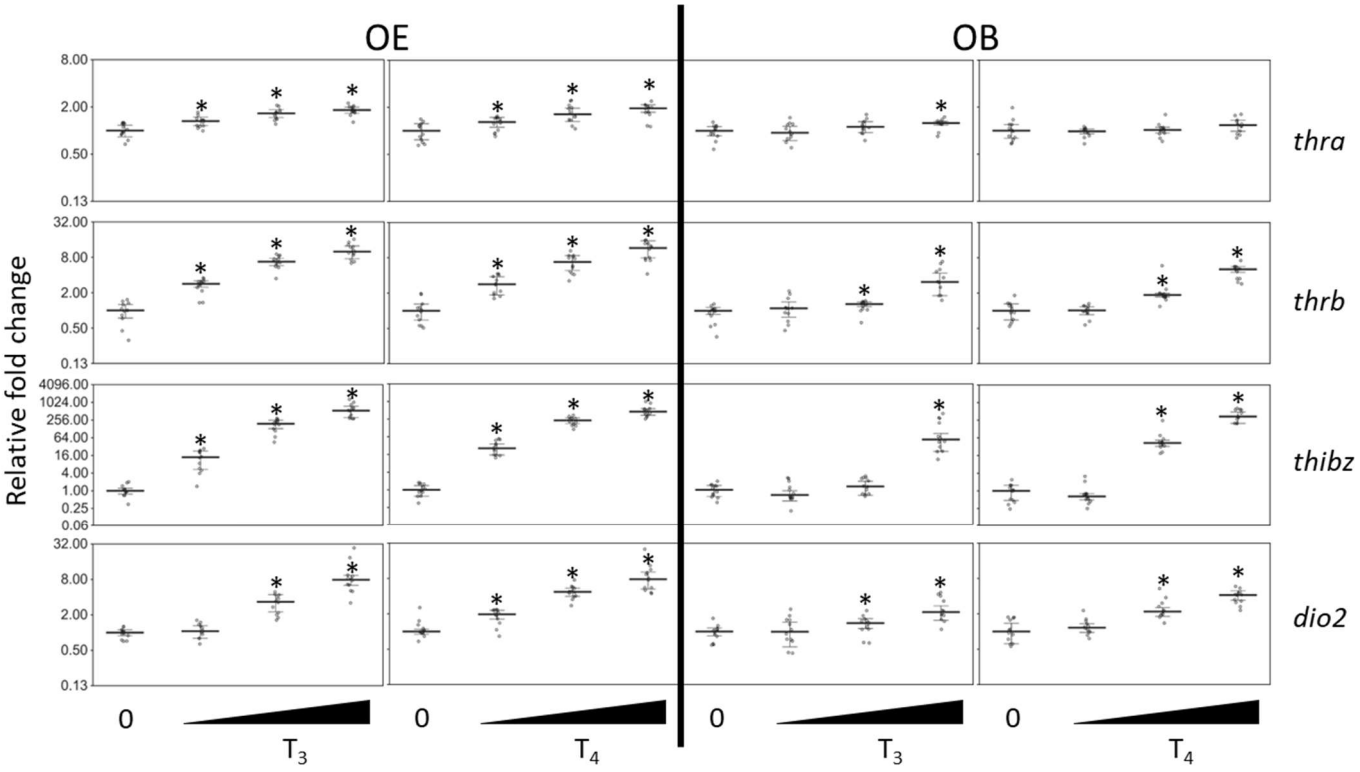

Figure 3.2 qPCR plots of four classical TH-responsive gene transcripts: TH receptor α (*thra*), TH receptor β (*thrb*), TH-induced basic region leucine zipper-containing transcription factor (*thibz*), and iodothyronine deiodinase 2 (*dio2*), from olfactory epithelium (OE) or olfactory bulb (OB) after premetamorphic *R. catesbeiana* tadpole exposure to THs (n=12 per treatment group) for 48 h. The bevel represents increasing concentrations of T_3 (0.1, 1, or 10 nM) or T_4 (0.5, 5.0, or 50 nM). The thick horizontal bar represents the median, the whiskers represent the median absolute deviation, and the circles represent individual animals. An asterisk indicates statistical significance ($p < 0.05$). '0', vehicle control.

3.3.2 High throughput RNA sequencing (RNA-seq)

Due to the marked difference in TH-sensitivity in the OE as compared to the OB, the OE transcriptomes were further evaluated by RNA-seq. Five biological replicates from each of the vehicle and high concentration conditions for each of the E_2, T_4, and T_3 exposure sets were selected for a total of 30 samples. Raw reads generated were similar across all samples (mean = 1.0×10^8, range = $8.24 \times 10^7 – 1.20 \times 10^8$; **Appendix Z**). After querying against BART, 99.6% of these raw reads were successfully aligned to assembled contigs.

Once aligned, contig read counts were processed through a DEseq2 pipeline. The number of differentially expressed (DE) contigs differed dramatically between treatments with 38,830 DE contigs upon T_3 treatment and 31,349 DE contigs upon T_4 treatment. In comparison, there were markedly fewer DE contigs (267) responsive to E_2. Principle component analysis demonstrated a strong separation on the first component between control and hormone treatments (70-75% of the variation), and the clear separation of treatment groups along the x-axis lends strength to the interpretation of the differentially expressed results being from the hormone exposures (**Figure 3.3**). Volcano plots indicate a substantially greater number of T_3-responsive contigs in comparison to T_4 (**Figure 3.4)**. The propensity for TH exposure to elicit greater differential transcript responses as compared to E_2 is visible in the ratios of grey (insignificant) contigs to red (significant; **Figure 3.4**). The response magnitudes in E_2 were comparatively weak overall, but a few strong responders of >20-fold were evident, and included contigs annotated for such genes as zinc finger protein 567 (ZNF567) and sentrin-specific peptidase 5 (SENP5). **Figure 3.5** revealed that approximately half of the significant contigs are increased and half are decreased, regardless of which hormone treatment was

administered and the majority of significantly differentially expressed contigs were shared between T_4 and T_3 treatments (**Figure 3.5**; 25,245). A number of contigs were specifically induced by T_3 or T_4, with twice as many in the T_3 group compared to the T_4 group (13,486 and 6,124, respectively). Approximately half of the E_2-responsive contigs (112) were shared with the THs (**Figure 3.6**). As confirmation of the validity of the response to E_2 treatment observed in this tissue, vitellogenin (VTG)-encoding contigs were analyzed in RNA-seq data from the liver of animals from the same exposure set (Partovi et al., 2017). As expected for a response to estrogen, *vtg* transcripts exhibited abundance increases of two to three orders of magnitude relative to the control (data not shown).

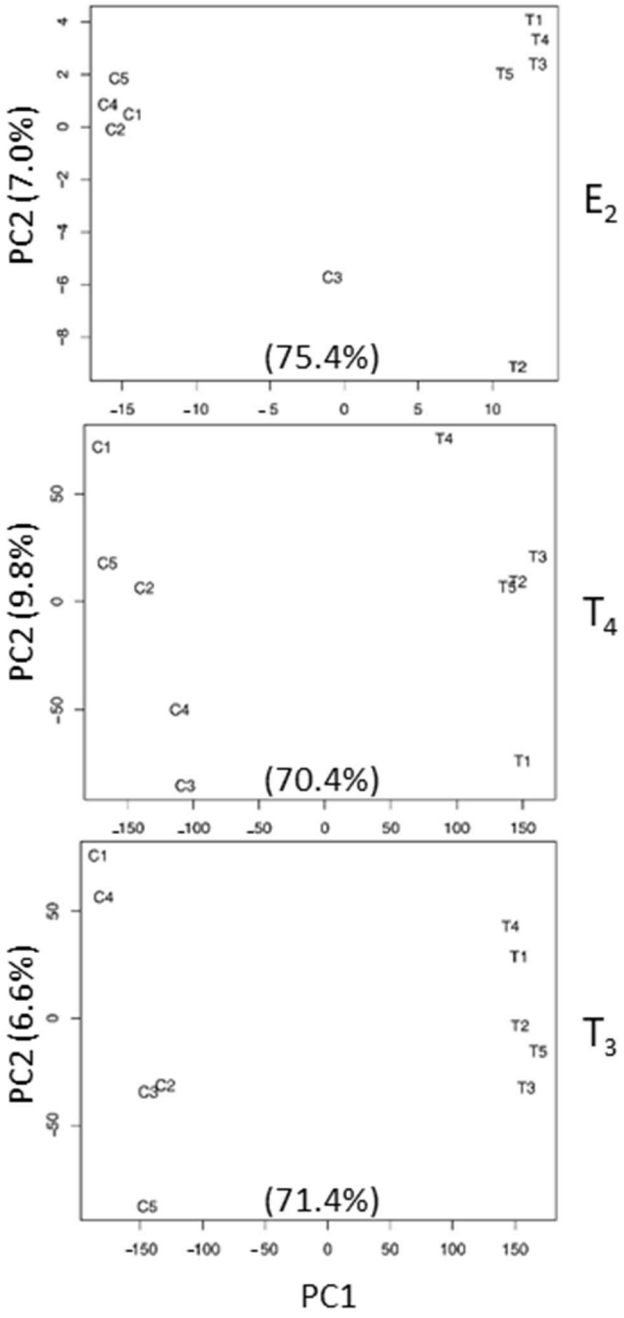

Figure 3.3 PCA plots of premetamorphic tadpole olfactory epithelium differentially expressed contigs from control, E_2, T_4, and T_3 treatments. The x-axis represents principle component 1 (PC1) and the y-axis represents principle component 2 (PC2). C1-C5 are untreated control biological replicates, T1-T5 are biological replicates exposed to 10 nM E_2, 50 nM T_4, or 10 nM T_3. The percent variation of PC1 and PC2 are indicated in brackets.

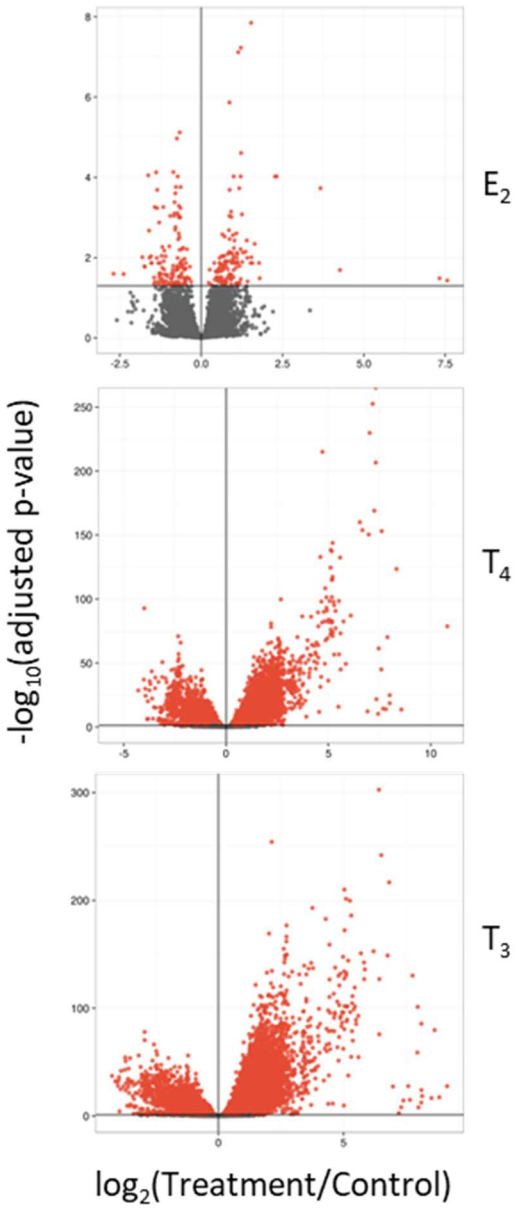

Figure 3.4 Volcano plots of premetamorphic tadpole olfactory epithelium differentially expressed contigs from control, 10 nM E_2, 50 nM T_4, or 10 nM T_3 treatments. The x-axis represents log2 fold change down (left of '0') or up (right of '0') and the y-axis represents log10 adjusted p-values. Note that the y-axes are not equivalent across plots. Each dot on the plot represents an individual contig. Red dots are statistically significant as determined by DESeq2 analysis and grey dots are not significant (adjusted p-value<0.05).

Functional annotation using the UniProt/SwissProt database (The UniProt Consortium, 2017) allowed for an analysis using gene ontology (GO) terms. Annotation across all three treatment groups averaged 72.6% of contigs annotated with UniProt IDs (E_2 = 70%; T_4 = 72%; T_3 = 75%). Qualitative examination of GO component trees identified that the top five major groups for T_3 and T_4 were shared: RNA processing, negative regulation of biological process, intracellular transport, cellular component biogenesis, and cellular metabolism (**Figure 3.7**). There was, however, a notable difference in the sixth-most dominant group, wherein for T_3 it was detection of chemical stimulus involved in sensory perception and for T_4 it was cellular response to stress. In contrast, the top three major groups for E_2 were vesicle-mediated transport, antigen processing and presentation of exogenous peptide antigen via MHC class I, and nucleoside diphosphate phosphorylation.

3.3.3 Bioindicator toolbox

Four of the most robust responders as identified by RNA-seq were selected for qPCR assay development. *St3*, *thibz-all*, and *heket* showed significant increases in all three concentrations upon exposure to both T_3 (*st3* 245-fold; *thibz-all* 204-fold; *heket* 63-fold) and T_4 (*st3* 172-fold; *thibz-all* 158-fold; *heket* 54-fold; **Figure 3.8**). None of these transcripts were affected by E_2 treatment (**Figure 3.9**). These responses follow a similar pattern to those observed in the classical tools tested. *Trpv1* showed a significant decrease in the highest concentration of T_3 (3-fold) and both the medium and high concentrations of T_4 (2.4- and 4-fold, respectively; **Figure 3.9**). The sensitivities (response at low hormone concentration) and strengths of response (response at high hormone concentration) for these potential bioindicator tools as compared to the classical qPCR

tools are shown in **Figure 3.10**. The bioindicators selected are within the top four TH-responsive transcripts for both strength of response and sensitivity. Transcript counts of these newly examined genes from unexposed tadpoles progressing through metamorphosis are presented in the same manner as the classical genes noted in section 3.3.1 and are shown in **Appendix AA**.

3.3.4 Effluent exposures

PPCP concentrations and water quality analyses were performed at PESC on water samples that were collected on the first and last experimental days from the exposure aquaria (see Heerema et al., 2017 for AnMBR set and **Appendices BB and CC**, respectively, for the MEBPR set)**.**

Thra and *thrb* showed no significant response when the animals were exposed to effluent from either the AnMBR or MEBPR systems (**Appendix DD**). *Thibz* showed a significant increase in transcript levels in the exposures at the highest concentrations of AnMBR effluent 1 and effluent 2 (Chapter 2, Heerema et al., 2017). In contrast, there was a modest, but significant decrease (1.3-fold) in *thibz* transcript levels after exposure to the 50% dilution of MEBPR effluent 1 but not 2 (**Figure 3.11**). While AnMBR effluent had no effect on *dio2* transcript levels (Chapter 2, Heerema et al., 2017), exposure of the animals to 100% MEBPR effluents 1 and 2 resulted in a significant decrease (1.5- and 1.6-fold respectively; **Figure 3.11**).

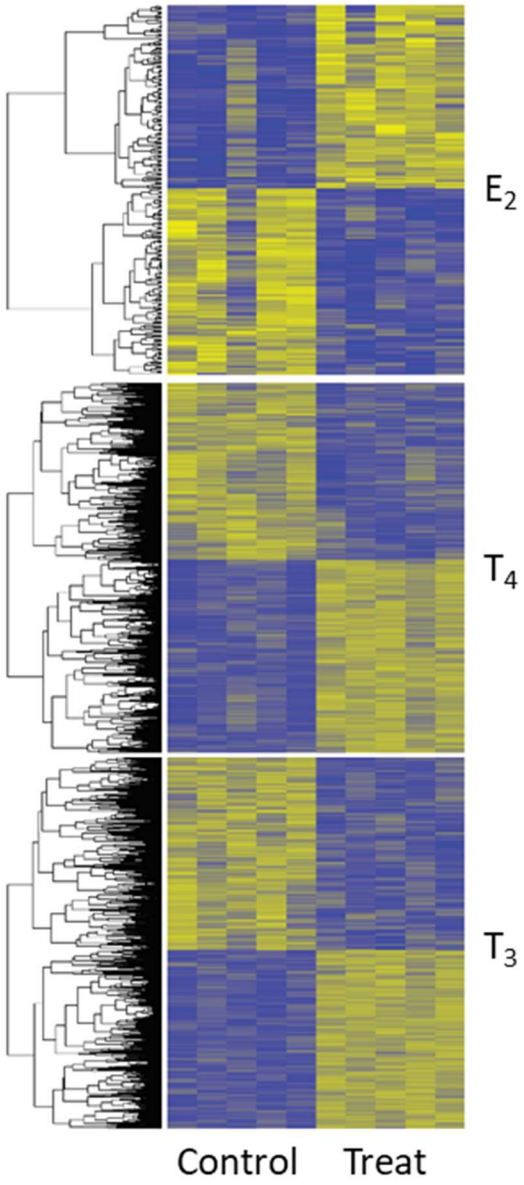

Figure 3.5 Heat maps of premetamorphic tadpole olfactory epithelium differentially expressed contigs from control, E_2, T_4, and T_3 treatments. The x-axis represents experimental samples where each column represents a biological replicate (n=5 per control or treatment ('Treat') condition). The treatments are either 10 nM E_2, 50 nM T_4, and 10 nM T_3. The y-axis represents a z-score (blue: -2; yellow: +2) mediated cluster analysis of statistically significant differentially expressed contigs (adjusted p-value<0.05).

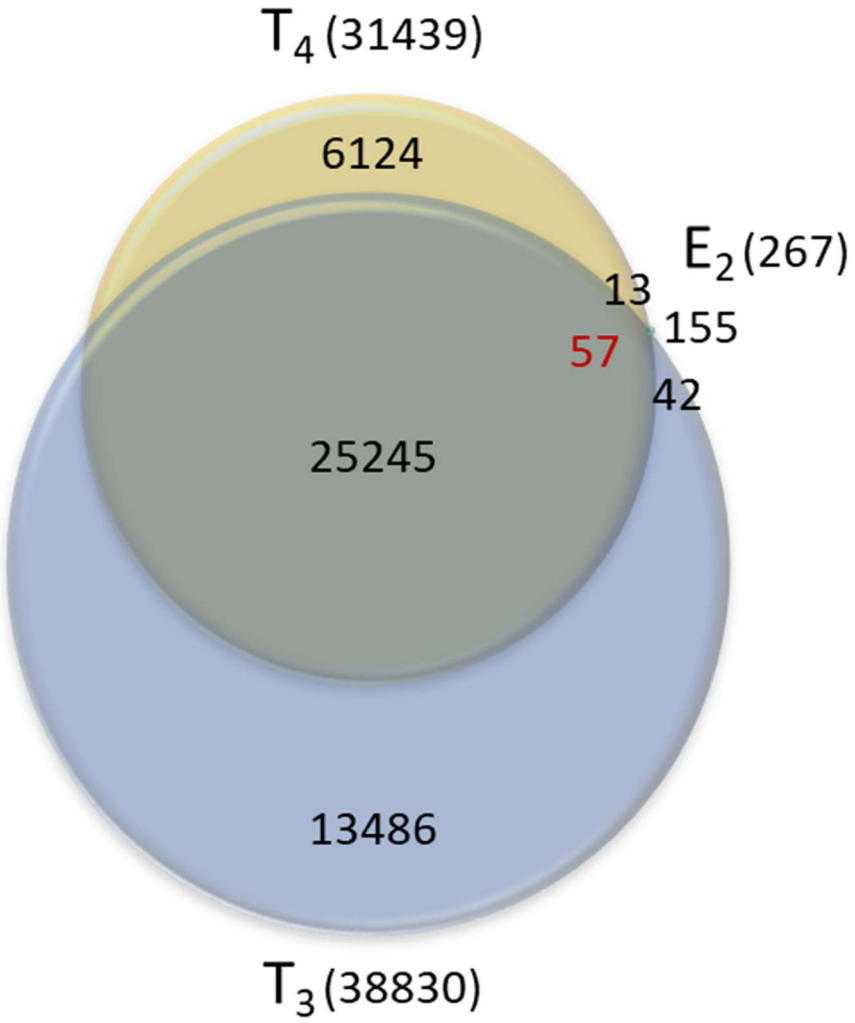

Figure 3.6 Venn diagram comparison of statistically significant (adjusted p<0.05) differentially expressed contigs identified in 10 nM E_2, 50 nM T_4, and 10 nM T_3 treatments in the olfactory epithelium of premetamorphic *R. catesbeiana* tadpoles following RNA-seq. Red numbers indicate significance in all three treatments. Circles were scaled to indicate the difference in total contigs represented in each hormone exposure group.

Of the newly designed tools, *thibz-all* primers exhibited a similar exposure response profile as *thibz* in the AnMBR set, with significance at the high concentrations of both effluents 1 and 2 (1.9- and 1.4- fold, respectively). This effect was not present in the MEBPR data set (**Figure 3.11**). *Heket* transcript levels displayed a significant increase upon exposure to the low concentration of effluent 1 from the AnMBR set (1.9-fold change), but showed no significant change under any other conditions of either treatment set (**Figure 3.11**). Despite a high-magnitude response at high concentrations of T_3 and T_4 (**Figure 3.10**), *st3* showed no significant changes in either treated effluent set (**Appendix DD**). *Trpv1* had the lowest strength of response of the new tools tested (**Figure 3.10**), and no significant changes in either treated effluent set were detected (**Appendix DD**).

3.4 Discussion

The present study is the first in-depth molecular analysis of TH-responsiveness in the olfactory epithelium (OE) of *R. catesbeiana*. Heerema et al. (2017) performed an initial investigation into TH-responsiveness in OE using established qPCR tools, but not at this depth of RNA sequencing, transcriptomic analyses, or novel qPCR tool design. Prior studies have demonstrated TH-response of classical responsive qPCR tools on the brain (Maher et al., 2016), but not specifically on the isolated olfactory bulb (OB) as presented herein. In this instance, the qPCR data for the olfactory bulb nearly replicated what was observed in the *R. catesbeiana* brain tissue in Maher et al., (2016).

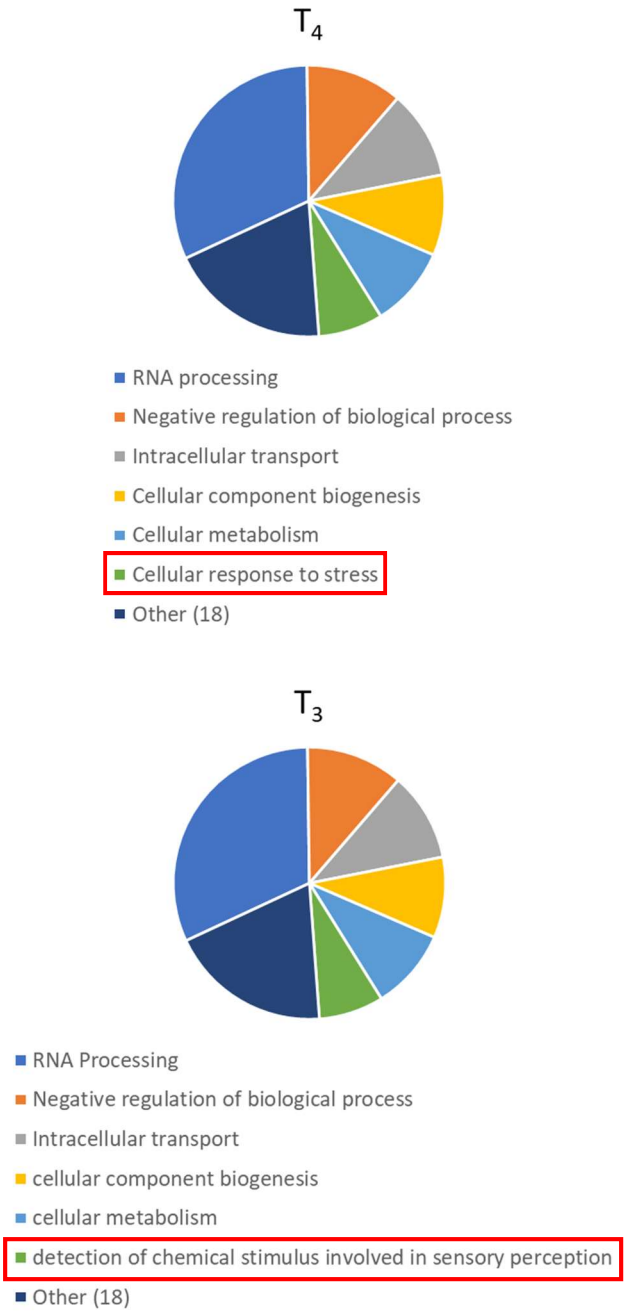

Figure 3.7 Pie charts representing the top six biological process categories from REVIGO gene ontologies of T_4-treated and T_3-treated differentially expressed olfactory epithelium contigs. Groups outlined with thick red lines represent where T_4 and T_3-treated animals exhibited a difference. There was no overlap in the top categories between TH and E_2 categories. See text for more details.

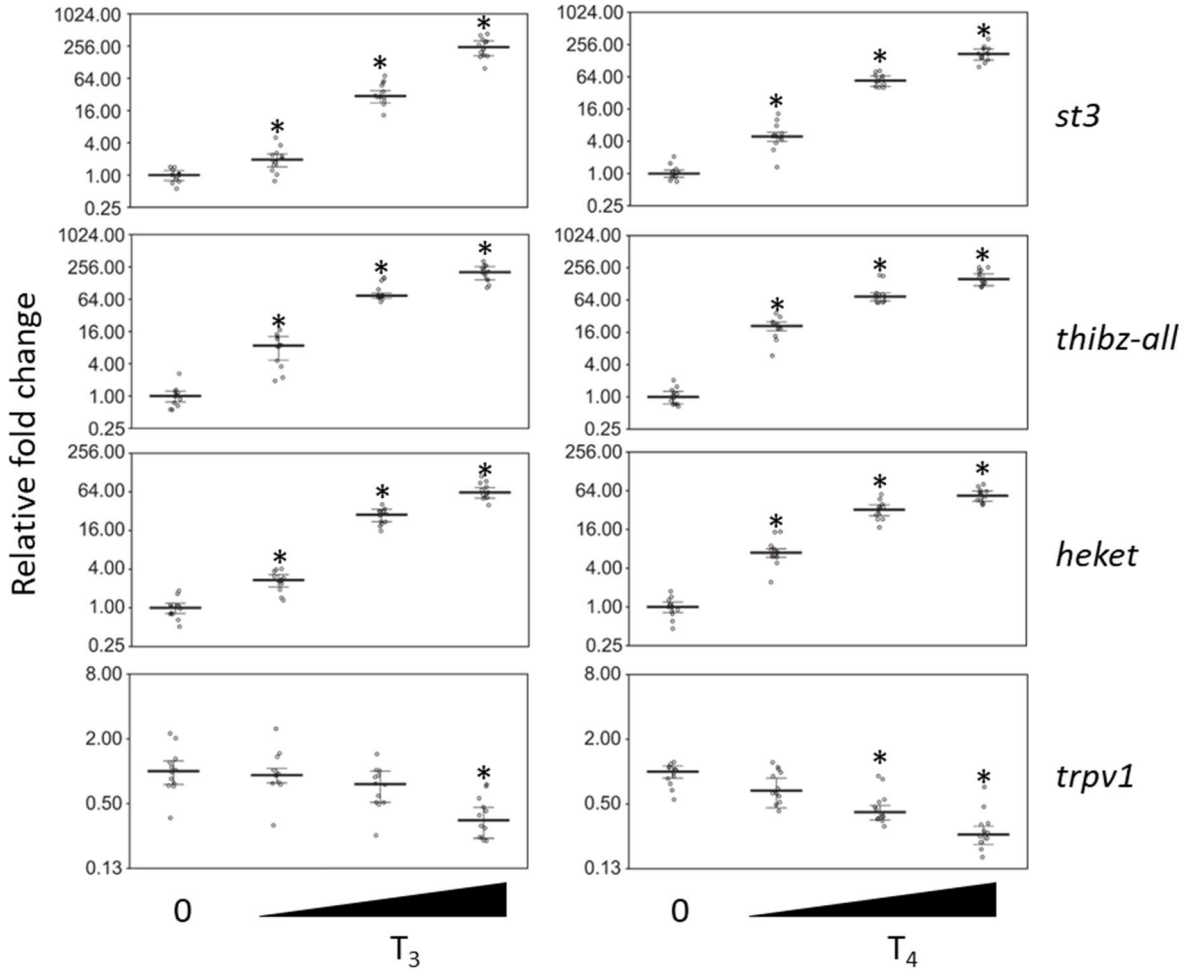

Figure 3.8 qPCR plots of four additional TH-responsive gene transcripts identified by RNA-seq: stromelysin 3 (*st3; mmp11*), an isoform of TH-induced basic region leucine zipper-containing transcription factor (*thibz-all*), an unannotated transcript (*heket*), and transient receptor potential cation channel subfamily V member 1 (*trpv1*) from olfactory epithelium after premetamorphic *R. catesbeiana* tadpole exposure to T_3 or T_4 (n=12 per treatment group) for 48 h. An asterisk indicates statistical significance $p < 0.05$). '0', vehicle control. See Figure 1 legend for additional details

It is evident that the OE is more sensitive to TH than the OB at low concentrations and has a higher magnitude response at the higher concentrations of exposure as well. This demonstrates the utility of the OE as being an excellent tissue for use in TH-responsive transcriptomic analysis. Of the two olfactory system tissues examined with this exploratory qPCR, these results made OE the desired target for an in-depth RNA-seq experiment.

In a study investigating the roles of T_3 and T_4 in different tissues as direct-acting hormones in relation with the presence of 5' deiodinases, Maher et al. found that the brain exhibited a much stronger fold change response to T_4 exposure than it did to T_3 (Maher et al., 2016). It was postulated that this effect was due to the presence of the 5'-deiodinase, dio2, that converts T_4 into T_3, the more biologically active form. Due to the 5X compensatory dosage of T_4 to T_3 (0.5, 5, 50 to 0.1, 1, 10 nM, respectively), T_4 would give rise to nearly 5X concentration of T_3, directly leading to a stronger response in TH-activated transcripts. This effect indeed appears to have been demonstrated in the OB tissue of the present study. We extend these results further by examining a new tissue, the OE. The OE is more sensitive to both T_4 and T_3 than the OB. However, although the OE expresses *dio2*, the expected *dio2*-mediated difference between T_4 and T_3 response magnitude was not observed. The responses across increasing concentrations of T_3 and T_4 in the classical responders of the OE are nearly superimposable, an unexpected result in a dio2-rich tissue.

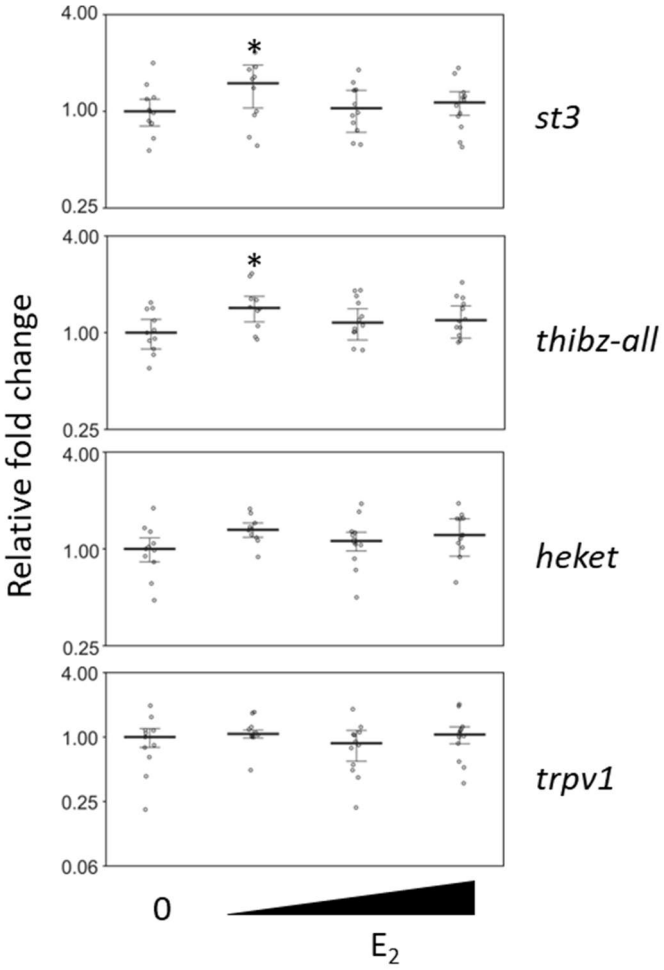

Figure 3.9 qPCR plots of four additional TH-responsive gene transcripts identified by RNA-seq: stromelysin 3 (*st3; mmp11*), an isoform of TH-induced basic region leucine zipper-containing transcription factor (*thibz-all*), an unannotated transcript (*heket*), and transient receptor potential cation channel subfamily V member 1 (*trpv1*) from olfactory epithelium after premetamorphic *R. catesbeiana* tadpole exposure to E_2 (n=12 per treatment group) for 48 h. The bevel represents increasing concentrations (0.1 nM, 1 nM, 10 nM). An asterisk indicates statistical significance ($p < 0.05$). '0', vehicle control. See Figure 1 legend for additional details.

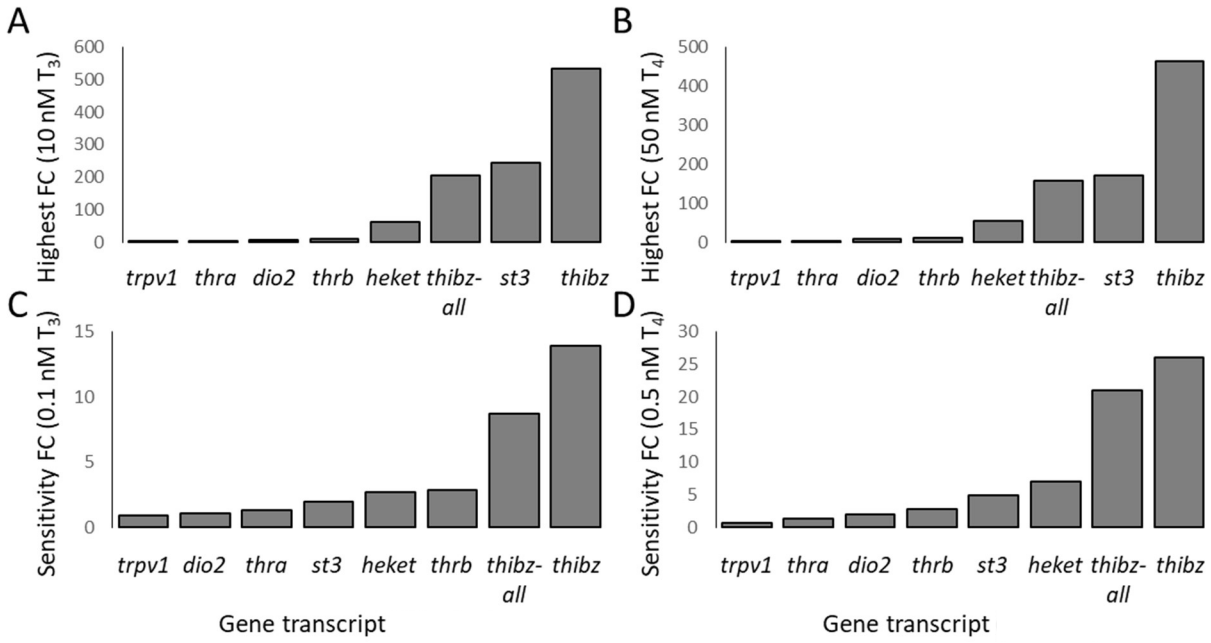

Figure 3.10 Relative responses and sensitivities of TH-responsive transcripts in the premetamorphic *R. catesbeiana* olfactory epithelium. Fold changes observed for each indicated transcript were ordered according to magnitude after A) 10 nM T_3 or B) 50 nM T_4 exposure to evaluate the strongest responses or after exposure to C) 0.1 nM T_3 or D) 0.5 nM T_4 to identify the most sensitive transcript responses.

When comparing these results to the back skin of the tadpole (Maher et al., 2016), another epithelial tissue, the same superimposable response curves are seen. Back skin contains reliably detectable amounts of *dio2* transcript, yet it does not significantly respond at any concentration of T_3 or T_4 (Maher et al., 2016), in contrast to OE, which responds quite strongly. It is possible that the *dio2* transcripts may not yield functional protein at this developmental stage, and further investigation into this phenomenon is warranted.

This phenomenon becomes more interesting after transcriptomic analysis as the results suggest a strong sensitivity to T_3 in the OE. The number of total contigs represented in the T_3 samples is 24% greater than that of T_4. This effect is exaggerated in the amount of contigs that were found solely in the T_3 samples (13,486) and not in any of the other treatment conditions, which was more than double that of the T_4-only (6,124) contig set. This phenomenon is intriguing when taken into consideration with the behavioral effects that were seen in the Heerema et al., (2017) study, which saw a significant effect on olfaction-mediated behavior in tadpoles that had been exposed to T_3, but no significant effect in those exposed to T_4. While there were several similarities in GO groups identified between the two THs, a notable difference in the top groups was in the "chemical stimulus involved in sensory perception" category upon T_3 treatment that was not prominent in the T_4-exposed OE. This new transcriptomic information indicates that there are a substantial number of transcripts in this tissue that are only showing differential expression under the influence of T_3, and not to a T_4 molecule that would be expected to be converted to T_3, and then exhibit a similar response. Also implied is that T_3 and T_4 signaling, though overlapping to some extent, can have distinctive effects on

gene expression. The present analysis also implies that the OE shows dramatic preference for THs as compared to E_2, and that there is relatively minimal, if any, cross-talk occurring when exposed to exogenous estrogen.

Of the several TH-responsive gene targets identified, we selected four representative bioindicator targets for further development. One of these targets revealed putative splice variants of the *thibz* gene including a putative non-coding RNA and another represented a completely new transcript, *heket*, with little coding potential, both of whose assembly integrity was independently confirmed through alignment to a high-quality draft of the bullfrog genome (Hammond et al., 2017).

The third bioindicator, *st3*, has been established as a TH-induced agent of cell fate and tissue morphogenesis in the intestine of *Xenopus laevis* (Fu et al., 2005), and its extremely strong response to TH-induction (244-fold) in the present study suggests it plays a similar role in the transformation of the OE tissue. This process could have obviously critical effects if disrupted, and these traits make it a relevant bioindicator candidate as identified by this analysis.

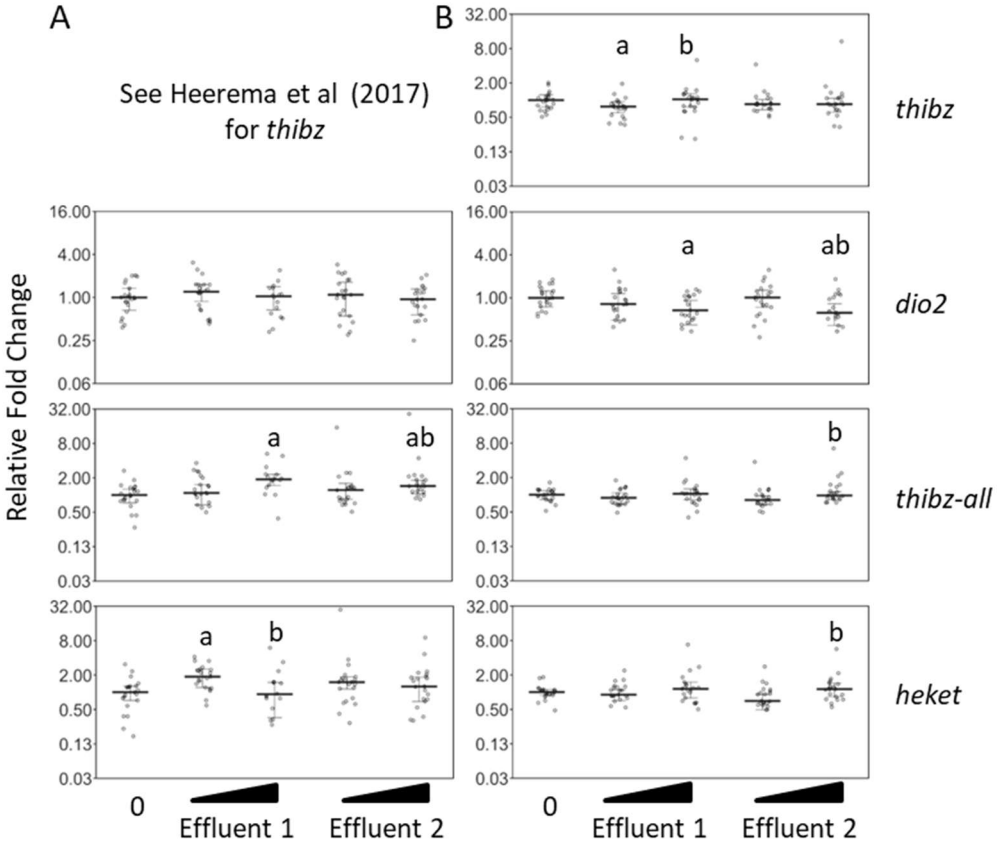

Figure 3.11 qPCR plots of TH-responsive gene transcripts that showed a significant effect when exposed to treated wastewater effluents from the A) AnMBR and B) MEBPR systems. This includes TH-induced basic region leucine zipper-containing transcription factor (*thibz*), iodothyronine deiodinase 2 (*dio2*), multiple splice variants of TH-induced basic region leucine zipper-containing transcription factor (*thibz-all*), and an unannotated transcript (*heket*). The bevel represents increasing concentrations of Effluent 1 or Effluent 2 (AnMBR: 7.5%, 15%; MEBPR: 50%, 100%). The thick bar represents the median of the treatment set, the whiskers represent the median absolute deviation, and the circles represent individual animals. '0', vehicle control; 'a', $p<0.05$ between treatment and control; 'b', $p<0.05$ between the two concentrations within an effluent type.

The last target, *trpv1*, a member of the transient receptor potential cation channel family, is a non-selective cation channel, and has been shown to be involved in sensory reception and activation by chemical agonists (Caterina et al., 1997; Tsuji and Aono, 2012). *Trpv4*, a relative of note, is involved in temperature-dependent sex determination in the American alligator (Yatsu et al., 2016). *Trpv1* transcripts are induced by retinoids, compounds whose effects are mediated by the steroid-TR superfamily (Yin et al., 2013). TH receptors are members of this superfamily, and the present study indeed shows modulation of *trpv1* transcript levels by TH. Disruption to TH systems in this family of ion channels could have far-reaching implications regarding behavior and fitness, and *trpv1* could be a helpful bioindicator of disruption of specific biological functions in aquatic species.

With TH-specific bioindicators in hand, we then applied them to the investigation of EDC activity in municipal wastewater effluents. Of the classical TH-responsive genes (*thra*, *thrb*, *thibz*), only *thibz* presented a significant response to the wastewater effluents of either treatment technology, suggesting the possibility of TH-signaling perturbation (Heerema et al., 2017). The response was exhibited in effluents 1 and 2, suggesting no differential effect when the PPCP cocktail was added to the wastewater, and thus it would seem likely that the cause of the endocrine disruption was already in the effluent stream. This response was replicated with the *thibz*-all primers, showing consistency with the finding that these primers detect variants of the same gene. Though *thibz-all* and *heket* showed significant responses in the AnMBR treated wastewater, no significance was observed in the MEBPR treated sets. These observations are consistent with the more effective general removal of the fifteen measured PPCPs by the MEBPR

system, compared to the AnMBR system, as measured by analytical chemistry. Of note, however, was that *dio2* transcript levels were significantly reduced in the high concentrations of both MEBPR effluents, despite the system being more capable of removing measured PPCPs. In contrast, the exposure to the AnMBR effluents showed no significant effects on *dio2* expression. This suggests not only the ability of the MEBPR-treated wastewater to modulate *dio2* expression, but that it is in a direction opposite and possibly antagonistic to that of normal TH-modulated *dio2* expression. These results exemplify how two different treatment types may selectively eliminate different endocrine disruptors from wastewater. The present study demonstrates that although many PPCP components may be removed through treatment, other unidentified compounds remain that can affect components of the thyroid axis. It is also possible that the compounds being observed do in fact remain in the effluent at a level below the limit of detection of the standardized measuring instrumentation, yet are still inducing a biologically-relevant transcriptional effect.

An example of an important quality of a potential bioindicator is seen in the comparison of *thibz*-all and *st3*. Though *st3* has a slightly stronger magnitude of response to high levels of TH than *thibz*-all, *thibz*-all is nearly four times more sensitive at the low concentrations. This observation was consistent with what was observed in the effluent exposure experiment in which *thibz*-all responded significantly, but *st3* showed no significant response to either effluent at either treatment site.

In summary, we have confirmed the sensitive TH-responsiveness to T_3 and T_4 of the OE (Heerema et al., 2017), and have shown that it is more sensitive to these hormones than the OB. We have shown that, despite expressing *dio2*, the OE does not exhibit the

classical T_3-T_4 response as was seen in other tissues in *R. catesbeiana* (Maher et al., 2016). Transcriptomic examination shows a strong preference for T_3 over T_4 even when corrected for biological activity. While the general gene ontologies overlap considerably, T_3 and T_4 responses notably differ in detection of chemical stimulus involved in sensory perception and cellular response to stress, respectively. This T_3 preference correlates with the observed olfaction-mediated behavioral responses in tadpoles, wherein exposure to T_3 caused a significant behavioral change and exposure to T_4 did not (Heerema et al., 2017). We have shown that the transcriptomic results can be scrutinized to select TH-responsive bioindicators that may be able to detect sensitive perturbations in aquatic systems that may contain xenobiotics that are below limits of detection of contemporary tools. Lastly, we have shown that EDCs persistent in the effluents produced by either MEBPR or AnMBR wastewater treatment technologies can be of biological relevance, affecting some TH-responsive genes, and not others. Studying the olfactory epithelium and its inherent differential responsiveness to T_3 and T_4 can provide important insights into the impact of EDCs on olfaction.

4 Conclusions and future directions

4.1 Conclusions

In addressing the stated thesis objectives, putative bioindicators selected from RNA-sequencing-based transcriptomic profiling did in fact show more sensitive responses than the classical TH-monitoring molecular tools. Though these new bioindicators were not able to show selectivity between T_3- and T_4-mediated modulation of expression, they were able to provide a more discerning response profile of TH activity in treated wastewater effluents. Completing a reliable panel of bioindicators that can exhibit T_3 and T_4 specificity can be completed through further investigation into the transcriptomic results presented herein.

The first objective of this thesis was to evaluate whether molecular responses of multiple classical thyroid hormone-responsive transcripts could be correlated to olfactory-mediated behavioral responses to a predatory cue. Although a differential behavioral response was displayed between T_3 and T_4 treated tadpoles, the qPCR results of the classical transcripts *thra*, *thrb*, and *thibz* indicated that there was not a concretely discernable molecular correlation with the differential behavior.

In addition, the observation that treated municipal wastewater could both induce a significant elevation of TH-modulated transcripts (*thibz*), and also significantly affect the tadpoles' ability to detect and avoid the predatory cue first suggests presence of TH disruption in the treated wastewater, and second that the route of TH disruption may be a component factor contributing to wild tadpoles' ecological fitness in an environment that is exposed to discharge of these treated municipal wastewaters. E_2-exposed tadpoles showing no significant changes in behavioral response suggests that E_2 does not play a

significant role in disruption of olfactory-mediated behavior. It was suggested at the end of this data analysis that there was indeed an effect occurring in T_3 and treated wastewater, that the classical responders were not enough of a tool to link a molecular response to a behavioral one, and that new bioindicator candidates may provide insight.

The second objective of this thesis was to investigate the transcriptome-wide response of the olfactory epithelium to thyroid hormones to identify new hormone-responsive transcripts that could be utilized as bioindicators of endocrine activity in environmental discharge of treated municipal wastewaters. Aside from reaffirming that the OE was consistently sensitive to TH, and that it was significantly more sensitive than the OB, we found a strong preference for T_3 over T_4 in the overall TH-responsive transcriptome. Four new qPCR tools were successfully located in the transcriptomic analysis and used on exposed animal tissues, rendering significant responses to both the individual hormone exposures and to the treated wastewater effluents.

Multiple lines of evidence presented herein suggest that there is indeed a real difference between T_4 and T_3 responses, despite the many similarities. The significantly higher response of classical TH-responsive transcripts, drastic differences in the total differentially expressed contig counts, and the observed difference in GO analysis suggest that there is indeed a correlation between these T_3-responsive transcripts and associated behavioral responses as compared to those of the T_4-exposed tadpoles. Further in-depth investigation of the RNA-seq data is required to identify the linkage between T_3-specific transcripts and the effect of this hormone on behaviour.

Outside of the two main objectives of this thesis, the data show clear evidence that modulation of TH-responsive transcripts does in fact occur in the treated effluents of two

different types of wastewater processing facilities, one in current use across Canada, and the other in the research and development stage for future implementation. The data suggest that these treated wastewaters can significantly affect some TH-responsive genes, while leaving others unaffected. Also evident is that neither treatment system was able to fully prevent disruption, even when all endocrine disrupting chemicals that were tested for were below the limits of detection.

4.2 Future directions

The experiments and results of this thesis were both novel and foundational, in that these are the first data representing the transcriptome-wide and individual gene TH-responsiveness to *R. catesbeiana* olfactory epithelium, the analysis of their use as bioindicators of TH-disruption in unknown mixtures of treated municipal wastewaters, and in observing a correlated behavioral effect. Therefore, this thesis provides a foundation for further experiments and investigations embodying an ultimate goal of maintenance of ecological health and sustainable human practices.

The category of differentially expressed T_3-induced contigs that only appeared in the T_3-exposed experimental group and none of the other groups, as shown in Figure 3.5 of this thesis, would be a prime location to investigate further for genes that may correlate with the discrepancy in T_3 and T_4-exposed behavioral responses. If a qPCR tool could be successfully designed to distinguish between a T_3 and T_4-mimicking disruption, then it would be a very useful and specific tool for interpreting and predicting disruption-associated behavior.

Topologically associating domains (TADs) are fascinating phenomenon that have only recently begun to be characterized. These consist of large sections of chromatin that can operate as discrete regulatory units in which the majority of the genes in the domain are transcriptionally activated or repressed, and that this effect has been observed upon a transient hormone exposure (Le Dily et al., 2014; Le Dily and Beato, 2015). Borders of these TADs exhibit regulatory control regions that when activated, may allow for transcription of the genes contained within as a group. These studies used steroid hormones, the shared superfamily relative of the THs. With the newly curated *R. catesbeiana* genome (Hammond et al., 2017) and the corresponding BART tool, the olfactory epithelium is a ripe candidate for a novel investigation of these TADs and the role they play in TH response, post-larval development, and mechanisms of environmental endocrine disruption. If disruption to a gene within a characterized TAD is occurring, then by elucidating the genes that reside within the shared TAD, inferences can be made as to what other processes would be disrupted and which ones would not.

The complex and vastly uncharacterized interplay of chemical mixtures in wastewater effluents make it difficult to predict when and where in the body endocrine disruption may occur. Armed with sensitive new TH-responsive bioindicators, an experiment can be laid out wherein multiple known and suspected endocrine disruptors are each individually exposed to tadpoles, and then a mixture group is used for exposure of another tadpole group. Additive, synergistic, or antagonistic effects may be observed and classified. An attempt at performing an experiment such as this for the entirety of known and suspected endocrine disruptors would obviously not be possible, but if companioned with

wastewater treatment analysis for specific regions (Sun et al., 2014), it could lend insights into the what the most prominent and relevant EDCs exist to be studied for a region.

With regards to constructing an AOP for TH-disruptive activity in aquatic systems affected by treated wastewater discharge, the present thesis has provided several new gene transcript candidates that could be linked to an adverse outcome of a change in organismal response to an olfactory cue. Future research into the receptor/ligand interaction to the organ response or even to the full-scale population responses will fill in the gaps and paint a more complete picture of the full systematic effects of disruption of TH action. Once a thorough AOP is established, molecular bioindicators can provide a very cost-efficient and relatively quick analysis of water quality from multiple affected regions and organismal and population effects can be dependably extrapolated.

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Appendix

Appendix A Body morphology of tadpoles used in 3,5',3-triiodothyronine (T_3), thyroxine (T_4), and 17β-estradiol (E_2) exposures

Exposure Set		n	Total Length (mm)[a]	Mass (g)[a]	Stage (TK)[b]
T_4	Water	16	85.0 ± 4.59	6.6 ± 0.70	VI, I-XII
	Vehicle	16	83.9 ± 4.56	6.2 ± 0.70	VII, I-XII
	0.5 nM	16	89.9 ± 4.09	7.7 ± 1.03	VII, I-XII
	5 nM	16	92.7 ± 2.74	7.9 ± 0.61	IX, I-XII
	50 nM	16	88.5 ± 3.17	6.6 ± 0.74	VIII, I-XII
T_3	Water	38	73.9 ± 1.75	4.3 ± 0.25	III, I-XI
	Vehicle	38	75.1 ± 1.33	5.0 ± 0.43	IV, I-XI
	0.1 nM	38	89.4 ± 2.15	4.9 ± 0.42	IV, I-XI
	1 nM	38	75.5 ± 1.26	4.9 ± 0.31	IV, I-XI
	10 nM	38	89.0 ± 1.77	4.7 ± 0.42	V, I-XII
E_2	Water	16	86.1 ± 2.90	5.8 ± 0.62	VI, I-XI
	0.1 nM	16	85.4 ± 2.84	5.9 ± 0.55	V, I-XIII
	1 nM	16	95.0 ± 2.94	8.4 ± 0.87	VII, I-XIII
	10 nM	16	92.3 ± 3.27	7.4 ± 0.84	VII, I-XII

[a]Mean ± SEM

[b]Median, range

Appendix B Water quality measurements for 3,5',3-triiodothyronine (T_3), thyroxine (T_4), and 17β-estradiol (E_2) exposures

Parameter	Exposure Set		
	T_3	T_4	E_2
Temperature (°C)[a]	23.7 ± 0.1 (116)	23.5 ± 0.1 (59)	24.5 ± 0.1 (45)
Dissolved oxygen (mg/L)[a]	7.2 ± 0.04 (116)	7.1 ± 0.1 (59)	6.7 ± 0.1 (41)
Ammonia[a]	0.3 ± 0.0 (86)	0.3 ± 0.0 (31)	0.0 ± 0.0 (13)
pH[b]	8.5, 7.8 - 8.7 (16)	8.4, 8.1 - 8.7 (47)	8.3, 8.1-8.5 (28)
Hardness (mg/L as $CaCO_3$)[a]	178 ± 1 (30)	172 ± 1 (35)	170 ± 1 (35)
Alkalinity (mg/L as $CaCO_3$)[a]	136 ± 2 (30)	141 ± 1 (35)	142 ± 2 (35)

[a]Mean ± SEM (sample size)

[b]Median, range (sample size)

Appendix C Actual hormone measurements during 3,5',3-triiodothyronine (T_3), thyroxine (T_4), and 17β-estradiol (E_2) exposures, Mean ± SEM

		nM	
Exposure Set		0h	48h
T_4[a]	Water	<0.1[c]	<0.1[c]
	Vehicle	<0.1[c]	<0.1[c]
	0.5 nM	0.5 ± 0.0	0.4 ± 0.0
	5 nM	5.5 ± 0.0	4.6 ± 0.0
	50 nM	57.4 ± 0.0	52.9 ± 0.2
T_3[a]	Water	<0.1[c]	<0.1[c]
	Vehicle	<0.1[c]	<0.1[c]
	0.1 nM	0.1 ± 0.0	0.1 ± 0.0
	1 nM	0.8 ± 0.0	0.8 ± 0.0
	10 nM	9.6 ± 0.3	7.5 ± 0.2
E_2[b]	Water	<0.2[c]	<0.2[c]
	0.1 nM	ND[d]	ND
	1 nM	ND	ND
	10 nM	1.4	1.8

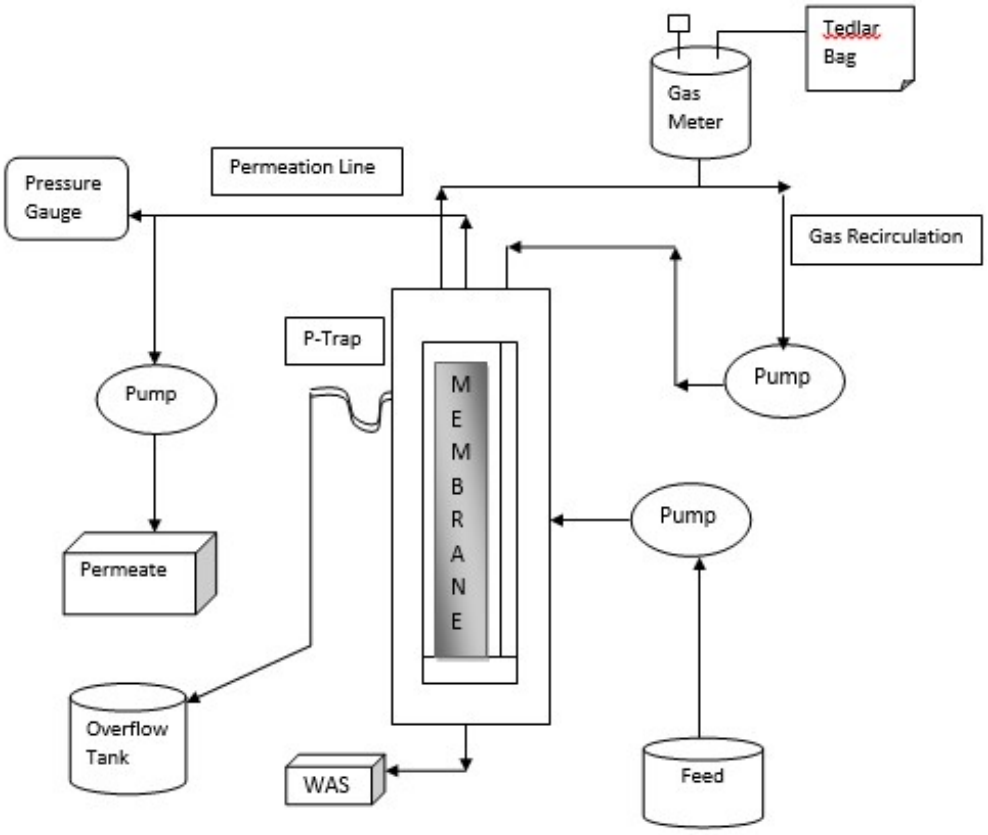
[a]LC-MS/Q-TOF, n=2

[b]LC-MS/MS, n=1

[c]BQL = Below quantitation limit

[d]ND = not determined

Appendix D Schematic of the lab-scale AnMBR



Appendix E Common PPCP constituents found in municipal wastewater and their final concentrations administered to municipal wastewater influent before AnMBR treatment to produce Effluent 2. A stock concentration of this PPCP cocktail was diluted 10,000x into collected municipal wastewater in Waterloo, ON, Canada. A parallel reactor received the same municipal wastewater plus vehicle only to produce Effluent 1.

Compound	Classification	Target concentration added to AnMBR 2 (μg/L)	Reference	CAS
Butylparaben	Antimicrobial	1	(Bergman et al., 2013)	94-26-8
Caffeine	Stimulant	45	(Bergman et al., 2013)	58-08-2
Decamethylcylopentasiloxane (D5)	Emollient	5	(Bergman et al., 2013)	541-02-6
Di (2-ethylhexyl) phthalate (DEHP)	Plasticizer	40	(Kolpin et al., 2002)	117-81-7
Estrone (E1)	Estrogen	0.07	(Bergman et al., 2013)	53-16-7
17α - Ethynlestradiol (EE_2)	Estrogen	0.006	(Gilbert, 2000)	57-63-6
Ibuprofen	Analgesic	27	(Bergman et al., 2013; Kolpin et al., 2002)	15687-27-1
Ketoconazole	Antifungal	0.02	(Kolpin et al., 2002)	65277-42-1
Naproxen	Analgesic	15	(Waye and Trudeau, 2011)	22204-53-1
4-Nonylphenol	Detergent	22	(Bergman et al., 2013)	104-40-5
Octamethylcyclotetrasiloxane (D4)	PPCP constituent	5	(Kolpin et al., 2002)	556-67-2
Thyroxine (T_4)	Thyroid hormone	0.1	(Melvin et al., 2014)	7-6-6106
Tonalide	Musk	1	(Bergman et al., 2013)	21145-77-7
Triclocarban (TCC)	Antimicrobial	3	(Quanrud and Propper, 2010)	101-20-2
Triclosan (TCS)	Antimicrobial	3	(Bergman et al., 2013)	3380-34-5

Appendix F Water quality data for ANMBR permeates, Mean ± SEM (n=7)

Parameter (mg/L)	Feed	Effluent 1	Effluent 2
Orthophosphate	10.1 ± 0.7	9.6 ± 0.4	9.5 ± 0.4
Ammonia - N	43.7 ± 0.5	38.4 ± 0.8	36.3 ± 0.8
BOD	146.2 ± 6.1	23.4 ± 1.4	20.8 ± 1.5

Appendix G Body morphology of tadpoles used in the effluent exposures.

Exposure	n	Total Length (mm)[a]	Mass (g)[a]	Stage (TK)[b]
Well water	21	86.2 ± 3.2	5.14±0.66	III, I-XI
7.5% Effluent 1	21	95.9 ± 2.7	7.01±0.64	II, I-X
15% Effluent 1	16	90.3 ± 3.6	5.72±0.84	II, I-X
7.5% Effluent 2	21	92.6 ± 2.2	6.38±0.55	III, I-IX
15% Effluent 2	21	92.4 ± 2.9	6.37±0.64	II, I-XII

[a]Mean ± SEM

[b]Median, range

Appendix H Water quality measurements for the effluent exposures, Mean ± SEM (n=6)

	Timepoint									
	0 h					48 h				
Parameter	Well Water	7.5% Effluent 1	15% Effluent 1[a]	7.5% Effluent 2	15% Effluent 2	Well Water	7.5% Effluent 1	15% Effluent 1[a]	7.5% Effluent 2	15% Effluent 2
Temperature (°C)	13.3 ± 0.2	13.3 ± 0.2	13.4 ± 0.2	13.4 ± 0.1	13.5 ± 0.1	16.8 ± 0.1	16.8 ± 0.1	16.6 ± 0.1	16.6 ± 0.1	16.7 ± 0.2
Dissolved oxygen (mg/L)	10.1 ± 0.0	10.1 ± 0.1	10.2 ± 0.1	10.0 ± 0.1	9.7 ± 0.1	9.3 ± 0.1	9.4 ± 0.1	9.6 ± 0.1	9.4 ± 0.1	9.0 ± 0.4
Conductivity (mS/m)	441.0 ± 1.7	840.5 ± 12.0	1379.0 ± 65.6	875.8 ± 3.6	1283.5 ± 6.4	442.8 ± 1.1	875.0 ± 4.9	1362.2 ± 76.4	873.2 ± 5.8	1282.2 ± 7.0
pH	7.4 ± 0.1	7.7 ± 0.0	7.9 ± 0.0	7.6 ± 0.0	7.7 ± 0.0	7.7 ± 0.0	8.0 ± 0.0	8.3 ± 0.0	8.1 ± 0.0	8.0 ± 0.2

[a]n=4

Appendix I Concentrations (Mean (µg/L) ± SEM) of pharmaceuticals and personal care products (PPCPs) in well water and municipal wastewater effluents 1 and 2 after AnMBR treatment (n=3)

		Timepoint					
		0 h			48 h		
Compound	Classification	Well Water	Effluent 1[a]	Effluent 2[b]	Well Water	Effluent 1	Effluent 2
Butylparaben	Antimicrobial	-	-	-	-	-	-
Caffeine	Stimulant	<0.05	0.53 ± 0.30	0.23 ± 0.00	<0.05	0.52±0.06	0.23 ± 0.00
Decamethylcylopentasiloxane (D5)	Emollient	-	-	-	-	-	-
Di (2-ethylhexyl) phthalate (DEHP)	Plasticizer	1.12 ± 0.17	0.91 ± 0.39	1.35 ± 0.02	1.41 ± 0.39	2.13 ± 0.58[c]	1.79 ± 0.22
Estrone	Estrogen	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
17α - Ethynlestradiol (EE_2)	Estrogen	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Ibuprofen	Analgesic	<0.05	1.34 ± 0.20	5.59 ± 0.03	<0.05	1.30 ± 0.10	5.18 ± 0.28

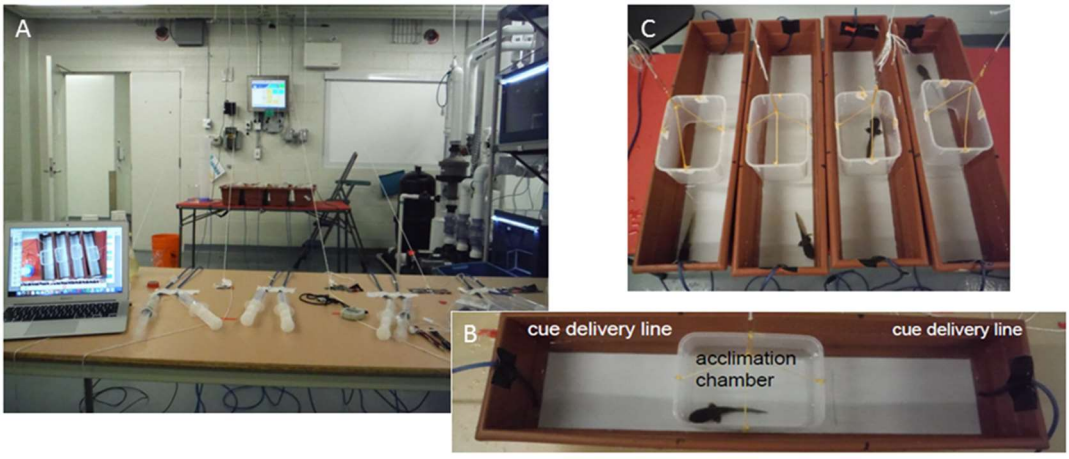
Ketoconazole	Antifungal	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Naproxen	Analgesic	<0.05	0.73 ± 0.09	1.86 ± 0.05	<0.05	0.72 ± 0.08	1.85 ± 0.03
4-Nonylphenol	Detergent	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Octamethylcyclotetrasiloxane (D4)	PPCP constituent	-	-	-	-	-	-
Thyroxine (T_4)	Thyroid hormone	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Tonalide	Musk	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Triclocarban (TCC)	Antimicrobial	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Triclosan (TCS)	Antimicrobial	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

[a]Influent spiked with vehicle and put through parallel AnMBR treatment train 1

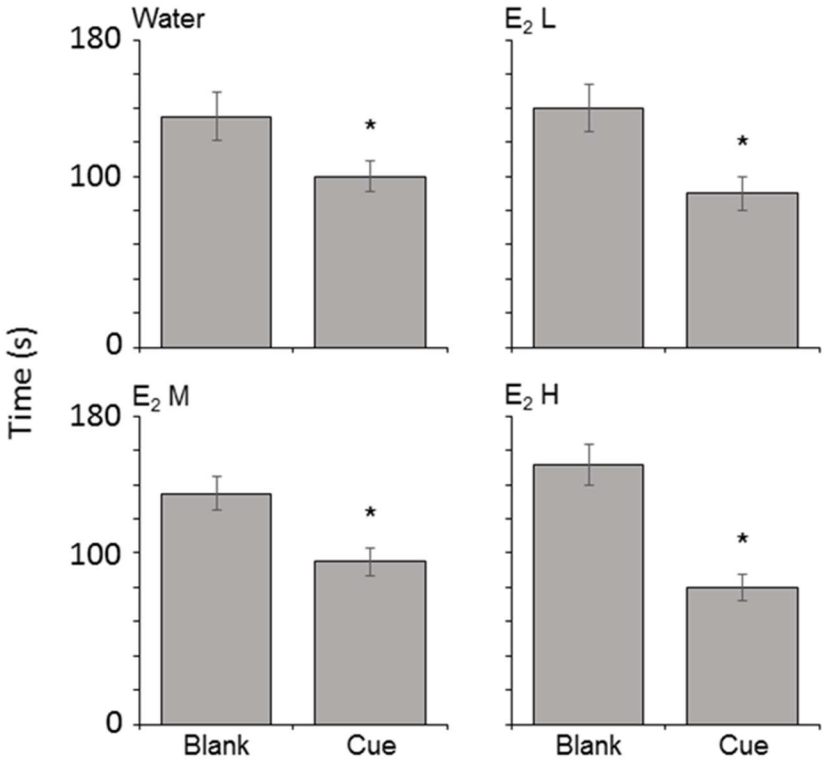
[b]Influent spiked with PPCP cocktail and put through parallel AnMBR treatment train 2

[c]n=2

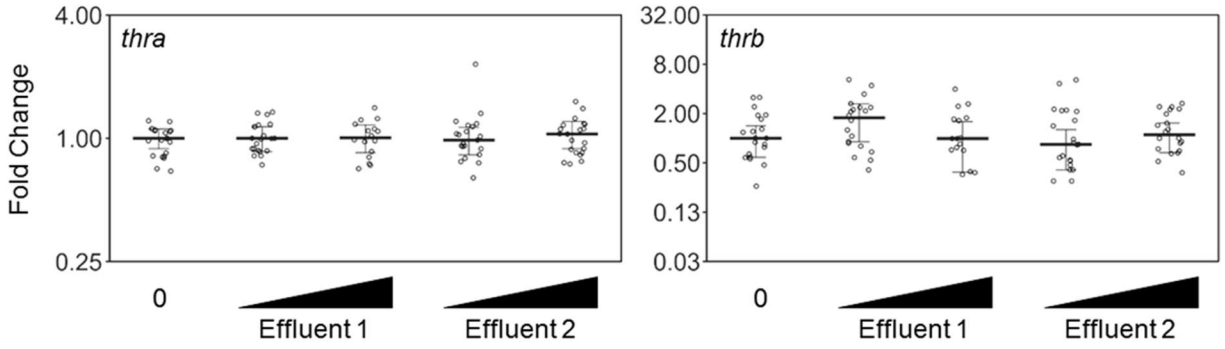
Appendix J Photographs of the I-maze set-up for the predator cue avoidance behavior experiments.



Appendix K Time *R. catesbeiana* tadpoles spent in the blank (water) and cue (amino acid mixture) arms of the linear-style choice I-maze after exposure to water (Water), 0.1 nM E_2(E_2L), 1.0 nM E_2 (E_2M), or 10 nM E_2 (E_2H).



Appendix L Premetamorphic tadpole olfactory epithelium transcript abundance for TH receptor α (*thra*) and TH receptor β (*thrb*) as measured by qPCR.



Appendix M Body morphology of tadpoles used in 17β-estradiol (E_2), thyroxine (T_4), and 3,5,3'-triiodothyronine (T_3) exposures (n=12 per group).

Exposure Set	Concentration (nM)	Total length (cm)[a]	TK stage[b]	Mass (g)[a]
E_2	Well Water	8.16 ± 0.22	III, I-VI	3.89 ± 0.32
	0.1	8.00 ± 0.22	III, I-VI	4.10 ± 0.20
	1	8.18 ± 0.22	II, I-IV	3.95 ± 0.26
	10	8.09 ± 0.26	I, I-VI	3.99 ± 0.43
T_4	Vehicle	8.15 ± 0.16	II, I-VI	4.21 ± 0.31
	0.5	8.08 ± 0.21	II, I-VI	3.96 ± 0.30
	5	8.43 ±0.20	V, I-VII	4.52 ± 0.31
	50	7.94 ± 0.39	II, I-IX	4.03 ± 049
T_3	Vehicle	7.99 ± 0.24	II, I-VI	4.00 ± 0.34
	0.1	7.93 ± 0.18	II, I-VI	4.12 ± 0.31
	1	8.12 ± 0.22	III, I-VI	4.22 ± 0.35
	10	7.90 ± 0.23	III, II-VI	3.77 ± 0.37

[a]Mean ± SEM

[b]Median, range

Appendix N Water quality measurements for the 17β-estradiol (E_2), thyroxine (T_4), and 3,5,3'-triiodothyronine (T_3) exposures. Mean ± SEM (n=30)

	Timepoint					
	0 h			48 h		
Parameter	E_2	T_4	T_3	E_2	T_4	T_3
Temperature (°C)	20.5 ± 0.1	20.6 ± 0.0	21.1 ± 0.0	20.1 ± 0.1	21.5 ± 0.0	21.3 ± 0.0
Dissolved oxygen (mg/L)	9.0 ± 0.0	9.0 ± 0.0	8.9 ± 0.0	9.1 ± 0.1	8.8 ± 0.0	8.9 ± 0.0
Conductivity (mS/m)	418.9 ± 0.2	421.1 ± 0.4	424.9 ± 0.2	422.9 ± 0.7	421.1 ± 0.6	423.5 ± 3.3
pH	7.7 ± 0.0	7.9 ± 0.0	7.9 ± 0.0	7.8 ± 0.0	7.8 ± 0.0	7.8 ± 0.0

Appendix O Body morphology of tadpoles used in the examination of natural metamorphosis

Exposure	n	Total length (cm)[a]	TK stage[b]	Mass (g)[a]
Premet	6	9.5 ± 0.35	V, IV-VI	8.25 ± 0.97
Early Promet	7	13.14 ± 0.30	XV, XV-XVI	19.78 ± 1.84
Late Promet	7	13.71 ± 0.32	XVII, XVII-XVIII	21.58 ± 2.36

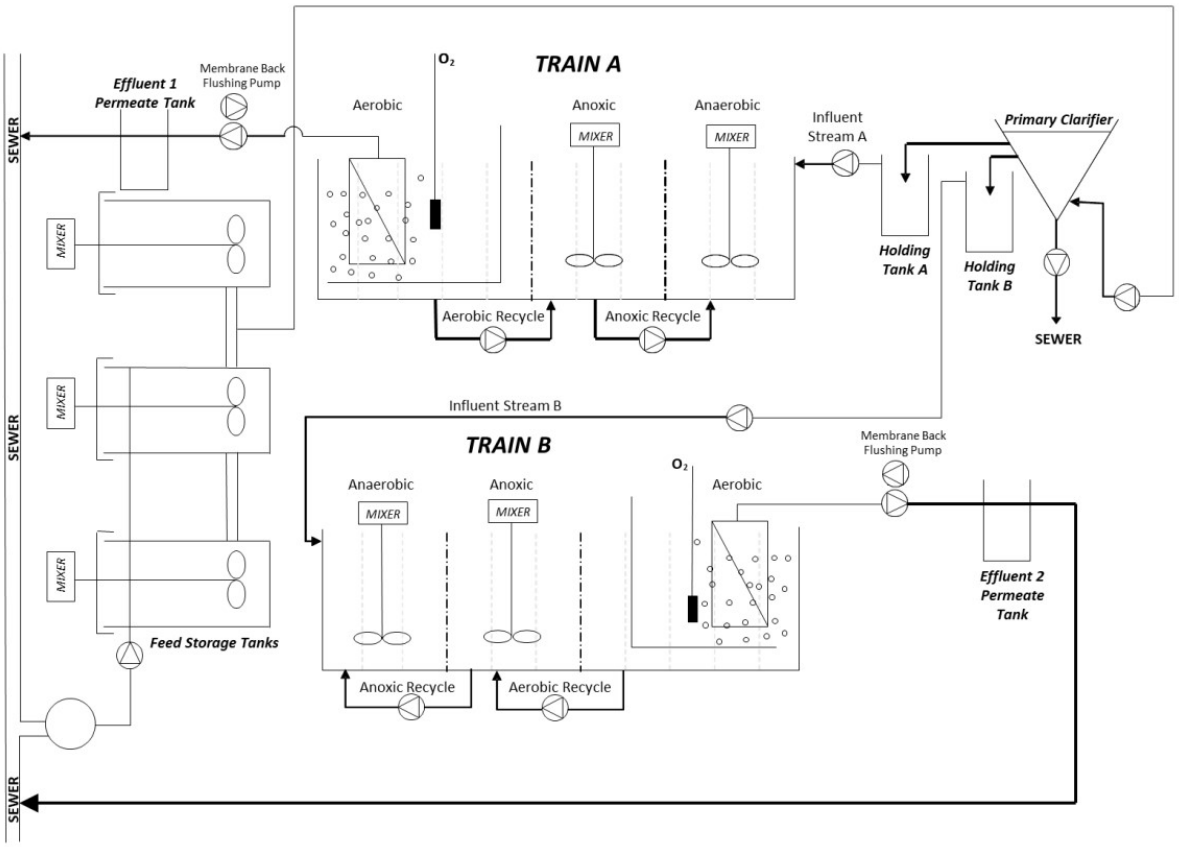
[a]Mean ± SEM

[b]Median, range

Appendix P Water quality measurements for the examination of natural metamorphosis. Mean ± SEM (n=20)

	Timepoint	
Parameter	0 h	48 h
Temperature (°C)	21.0 ± 0.0	20.4 ± 0.1
Dissolved oxygen (mg/L)	9.1 ± 0.0	7.4 ± 0.1
Conductivity (mS/m)	425.5 ± 0.3	420.1 ± 1.9
pH	8.0 ± 0.0	7.3 ± 0.0

Appendix Q Schematic of parallel MEBPR trains. Holding tank influent was spiked with vehicle for Train A or a PPCP cocktail for Train B.



Appendix R Design specifications of the biological zones of the MEBPR wastewater treatment pilot plant.

Biological Reactor	Vehicle-spiked Train A	PPCP cocktail-spiked Train B
Anaerobic zone	234 L	240 L
Anoxic zone	585 L	618 L
Aerobic zone	1311 L	1369 L
Total reactor volume	2130 L	2227 L

Appendix S Water quality data for MEBPR permeates, Mean ± SEM (n=4)

Parameter	Effluent 1	Effluent 2
pH	8.1 ± 0.0	8.2 ± 0.0
Ammonia (mg/L)	0.1 ± 0.0	0.1 ± 0.0
DO (mg/L)	8.5 ± 0.6	8.7 ± 0.4

Appendix T Body morphology of tadpoles used in the MEBPR effluent exposures. (n=21)

Exposure	Total length (cm)[a]	TK stage[b]	Mass (g)[a]
Well Water	9.97 ± 0.22	III, I-XI	7.79 ± 0.48
50% Effluent 1	10.07 ± 0.28	II, I-VI	8.26 ± 0.65
100% Effluent 1	10.20 ± 0.18	III, I-XI	8.41 ± 0.54
50% Effluent 2	9.54 ± 0.20	II, I-VII	6.89 ± 0.35
100% Effluent 2	9.49 ± 0.18	II, I-VII	6.97 ± 0.45

[a]Mean ± SEM

[b]Median, range

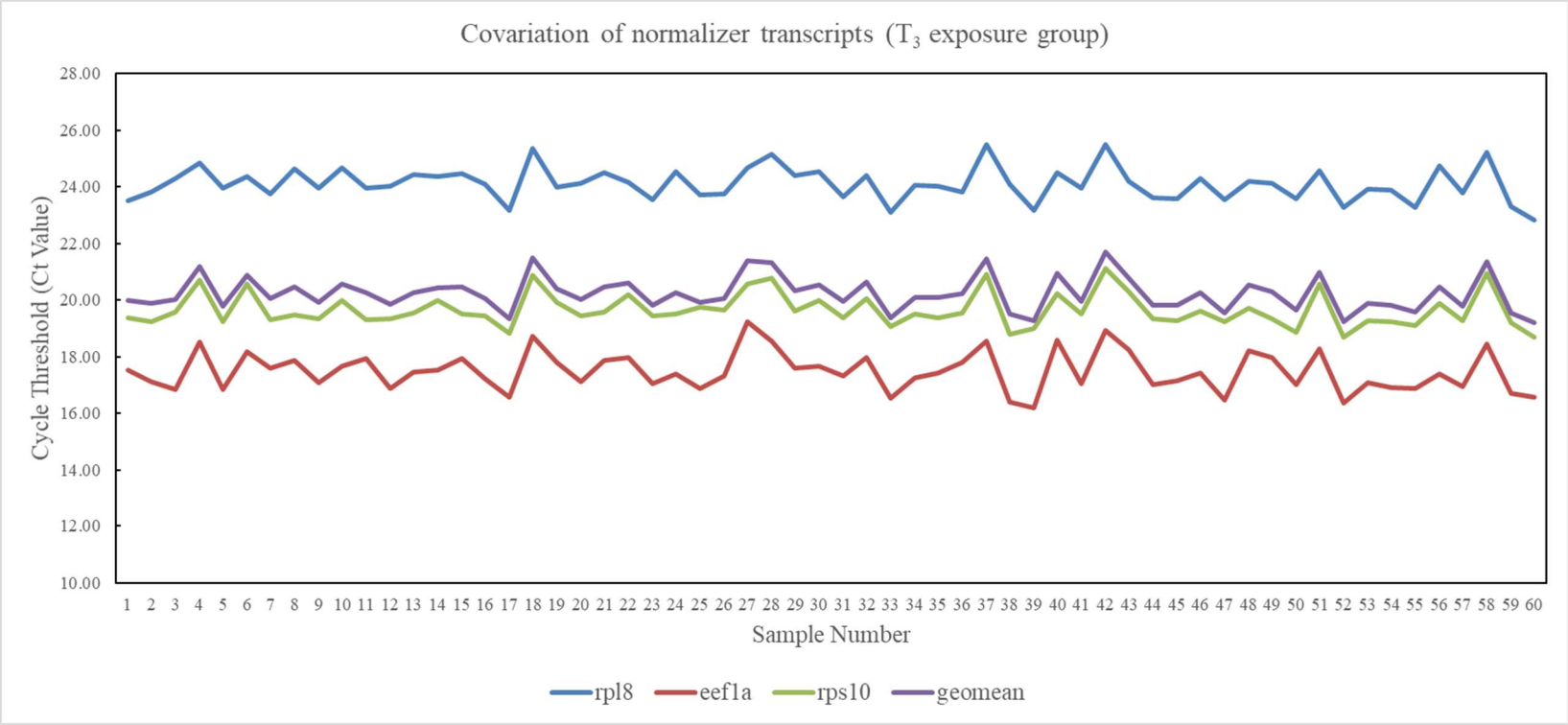
Appendix U Summary of olfactory epithelium RNA-seq results following 17β-estradiol (E_2), thyroxine (T_4), and 3,5',3-triiodothyronine (T_3) exposures

	Total contigs			Significant contigs		
Treatment	Overall	Annotated	Annotated with human Uniprot	Overall	Annotated	Annotated with human Uniprot
10 nM E_2	52699	51228 (97%)[a]	36887 (70%)[a]	267 (1%)[a]	259 (97%)[b]	192 (72%)[b]
50 nM T_4	59428	58034 (98%)[a]	43063 (72%)[a]	31439 (53%)[a]	30675 (98%)[b]	23480 (75%)[b]
10 nM T_3	59111	57740 (98%)[a]	44421 (75%)[a]	38830 (66%)[a]	38162 (98%)[b]	29542 (76%)[b]

[a]Percent values relative to overall total contigs

[b]Percent values relative to overall significant contigs

Appendix V Covariation analysis of normalizers used for qPCR analyses for the T_3 exposure dataset. Qualitative analysis was provided by graphing the cycle thresholds (Cts) of each normalizer along with the geomean. Co-efficients of correlation were determined for each transcript by BestKeeper. Cronbach's Alpha determination for the entire group was performed using reliabilitycalculator2. High quality scores are above 0.9.



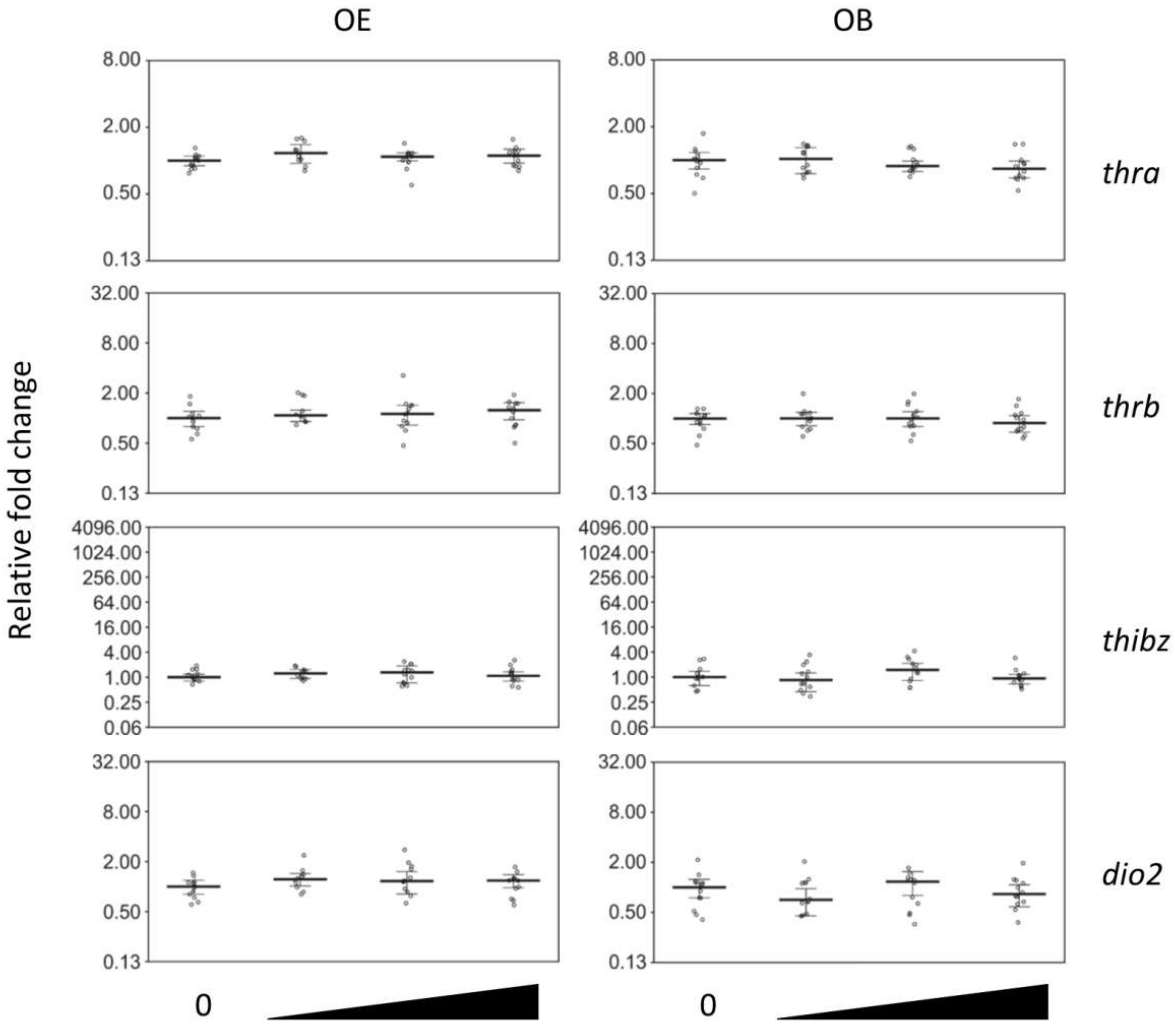
BestKeeper analysis (T3 exposure group)			
vs.	rpl8	eef1a	rps10
coeff. of corr. [r]	0.916	0.957	0.957
p-value	0.001	0.001	0.001

Reliabilitycalculator2 analysis (T3 exposure group)	
Cronbach's Alpha	0.936

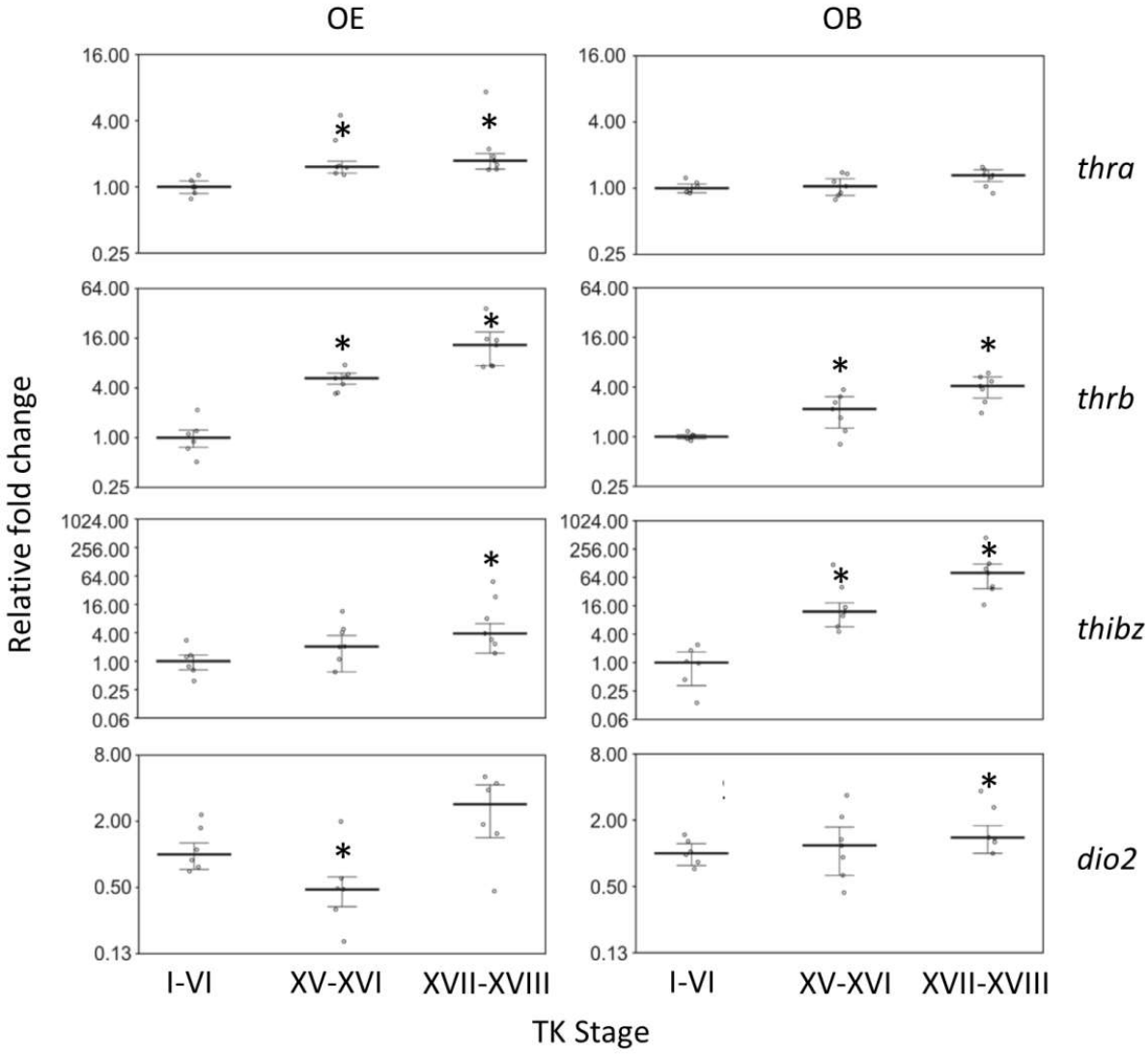
Appendix W Design details of forward and reverse primers created for each of the four additional qPCR tools.

Gene Target	Direction	Sequence 5'-3'	Tm °C	Amplicon length (bp)
st3	Forward	TGCGGTATTACACGGTCAA	52.2	158
	Reverse	TCAGTTGCGAGCCTTGTT	52.2	
thibz-all	Forward	CATCAATGCCGACCTCCT	51.8	156
	Reverse	TCACACTGTCCTGCTTCC	51.6	
heket	Forward	ACCTCCTCCTGTGTTAATCATA	52.0	168
	Reverse	GGTACAACTTGGTGGTAATTCT	52.1	
trpv1	Forward	ACTCACCTATGTTCTTCTTCTG	51.3	390
	Reverse	GGCTCTAACTCCTCTTCCTT	51.5	

Appendix X qPCR plots of four classical TH-responsive gene transcripts: TH receptor α (*thra*), TH receptor β (*thrb*), TH-induced basic region leucine zipper-containing transcription factor (*thibz*), and iodothyronine deiodinase 2 (*dio2*), from olfactory epithelium (OE) or olfactory bulb (OB) after premetamorphic *R. catesbeiana* tadpole exposure to E_2. The bevel represents increasing concentrations (0.1 nM, 1 nM, 10 nM). (n=12 per treatment group)



Appendix Y qPCR plots of four classical TH-responsive gene transcripts: TH receptor α (*thra*), TH receptor β (*thrb*), TH-induced basic region leucine zipper-containing transcription factor (*thibz*), and iodothyronine deiodinase 2 (*dio2*), in the olfactory epithelium (OE) and olfactory bulb (OB) of tadpoles in different stages during natural metamorphosis (n=6-7 per group)



Appendix Z Summary of RNAseq output as raw reads and their alignment efficacy with the Bullfrog Annotation Resource for the Transcriptome (BART).

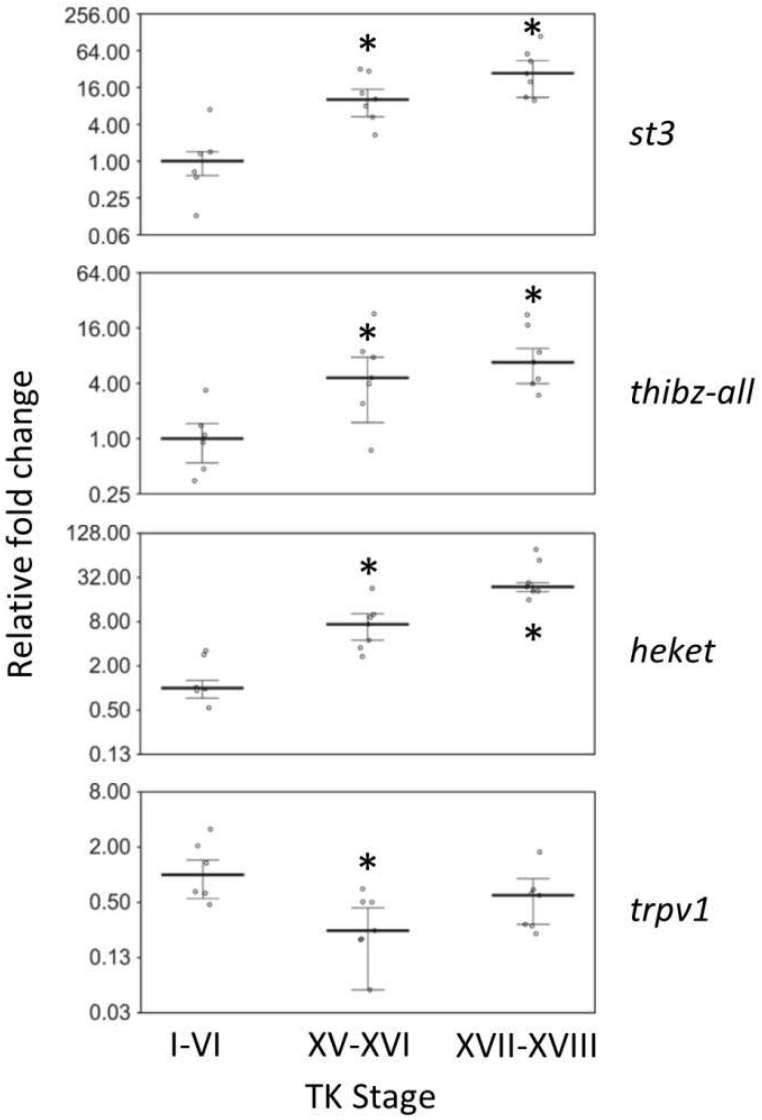
Olfactory Epithelium - E_2				
Sample	Reads in Input File	Unmapped	% Aligned	% BART Contigs w/ at least 1 aligned read
Vehicle 1	108069218	475705	99.6	89.8
Vehicle 2	111358471	486588	99.6	90.2
Vehicle 3	96881529	466507	99.5	90.4
Vehicle 4	99769379	419385	99.6	91.0
Vehicle 5	108138620	511122	99.5	91.4
Mean	104843443	471861	99.5	90.5
SD	6181578	33747	0.0	0.6
E_2 High 1	94552399	363236	99.6	87.1
E_2 High 2	104595320	622420	99.4	89.8
E_2 High 3	95964999	399499	99.6	89.6
E_2 High 4	115043689	606680	99.5	92.0
E_2 High 5	110628281	590665	99.5	91.7
Mean	104156938	516500	99.5	90.0
SD	8943529	124530	0.1	2.0

Olfactory Epithelium - T_4				
Sample	Reads in Input File	Unmapped	% Aligned	% BART Contigs w/ at least 1 aligned read
Vehicle 1	108592844	637095	99.4	90.4
Vehicle 2	111279502	560903	99.5	91.9
Vehicle 3	106318782	437308	99.6	90.2
Vehicle 4	97446400	440526	99.5	90.0
Vehicle 5	120186545	695714	99.4	91.4
Mean	108764815	554309	99.5	90.8

SD	8229501	115681	0.1	0.8
T_4 High 1	82433006	253621	99.7	86.1
T_4 High 2	94513421	381081	99.6	88.5
T_4 High 3	92751752	366873	99.6	88.3
T_4 High 4	103193114	504948	99.5	90.3
T_4 High 5	98549260	385596	99.6	89.7
Mean	94288111	378424	99.6	88.6
SD	7752599	89129	0.1	1.6

Olfactory Epithelium - T_3				
Sample	Reads in Input File	Unmapped	% Aligned	% BART Contigs w/ at least 1 aligned read
Vehicle 1	100860651	497398	99.5	90.3
Vehicle 2	97306293	424151	99.6	90.5
Vehicle 3	94373866	479854	99.5	91.2
Vehicle 4	95849705	469302	99.5	89.2
Vehicle 5	98017124	466748	99.5	90.4
Mean	97281528	467491	99.5	90.3
SD	2442153	27062	0.0	0.7
T_3 High 1	82430220	305295	99.6	87.6
T_3 High 2	99679649	351757	99.6	89.2
T_3 High 3	94138720	328618	99.7	88.5
T_3 High 4	98881169	332091	99.7	87.6
T_3 High 5	103716069	350315	99.7	88.6
Mean	95769165	333615	99.7	88.3
SD	8196273	18956	0.0	0.7

Appendix AA qPCR plots of stromelysin 3 (*st3; mmp11*), multiple splice variants of TH-induced basic region leucine zipper-containing transcription factor (*thibz-all*), an unannotated transcript (*heket*), and transient receptor potential cation channel subfamily V member 1 (*trpv1*) gene transcripts in the olfactory epithelium of tadpoles in different stages during natural metamorphosis (n=6-7 per group)



Appendix BB Concentrations (Mean (µg/L) ± SEM) of pharmaceuticals and personal care products (PPCPs) in well water and municipal wastewater effluents 1 and 2 after MEBPR treatment. (n=3)

	Timepoint:	0 h			48 h		
Compound	Classification	Well Water	Effluent 1[a]	Effluent 2[b]	Well Water	Effluent 1	Effluent 2
17α - Ethynylestradiol (EE2)	Estrogen	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
4-Nonylphenol	Detergent	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Butylparaben	Antimicrobial	-	-	-	-	-	-
Caffeine	Stimulant	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Decamethylcylopentasiloxane (D5)	Emollient	-	-	-	-	-	-
Di (2-ethylhexyl) phthalate (DEHP)	Plasticizer	0.42 ± 0.46	1.21 ± 0.69	0.80 ± 0.93	0.43 ± 0.14	0.26 ± 0.13	0.21 ± 0.08
Estrone	Estrogen	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Ibuprofen	Analgesic	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Ketoconazole	Antifungal	<0.05	0.06 ± 0.01	0.06 ± 0.00	<0.05	0.05 ± 0.00	0.06 ± 0.01

Naproxen	Analgesic	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Octamethylcyclotetrasiloxane (D4)	PPCP constituent	-	-	-	-	-	-
Thyroxine (T_4)	Thyroid hormone	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Tonalide	Musk	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Triclocarban (TCC)	Antimicrobial	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Triclosan (TCS)	Antimicrobial	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

[a]Feed stock spiked with vehicle and put through treatment train

[b]Feed stock spiked with cocktail and put through treatment train

Appendix CC Water quality measurements for MEBPR effluent exposures. Mean ± SEM (n=6)

	Timepoint									
	0 h					48 h				
Parameter	Well Water	50% Effluent 1	100% Effluent 1	50% Effluent 2	100% Effluent 2	Well Water	50% Effluent 1	100% Effluent 1	50% Effluent 2	100% Effluent 2
Temperature (°C)	19.4 ± 0.2	18.7 ± 0.3	17.7 ± 0.6	18.5 ± 0.4	17.8 ± 0.7	19.1 ± 0.1	19.1 ± 0.2	19.3 ± 0.0	19.3 ± 0.1	19.4 ± 0.1
Dissolved oxygen (mg/L)	9.2 ± 0.1	9.6 ± 0.1	10.2 ± 0.2	9.7 ± 0.1	10.2 ± 0.1	9.0 ± 0.1	8.6 ± 0.2	8.6 ± 0.1	8.6 ± 0.1	8.5 ± 0.1
Conductivity (mS/m)	430.0 ± 0.4	473.3 ± 0.4	518.7 ± 0.4	471.7 ± 0.5	513.7 ± 0.5	434.8 ± 0.8	477.8 ± 0.8	521.5 ± 1.3	474.7 ± 1.1	517.2 ± 1.0
pH	7.6 ± 0.1	7.9 ± 0.0	8.2 ± 0.0	8.0 ± 0.0	8.2 ± 0.0	7.8 ± 0.1	8.0 ± 0.1	8.2 ± 0.1	8.0 ± 0.0	8.2 ± 0.0

Appendix DD qPCR plots of TH-responsive gene transcripts that did not exhibit a significant effect when exposed to treated wastewater effluents from the A) AnMBR and B) MEBPR systems. Plots shown are of TH receptor α (*thra*), TH receptor β (*thrb*), stromelysin 3 (*st3; mmp11*), and transient receptor potential cation channel subfamily V member 1 (*trpv1)*. The bevel represents increasing concentrations of Effluent 1 or Effluent 2 (AnMBR: 7.5%, 15%; MEBPR: 50%, 100%).