Comparison of transcriptome responses of the liver, tail fin, and olfactory epithelium of *Rana [Lithobates] catesbeiana* tadpoles disrupted by thyroid hormones and estrogen

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Abstract

Thyroid hormones (THs) are important developmental regulators in vertebrates, including during the metamorphosis of a tadpole into a frog. Metamorphosis is a post-embryonic developmental period initiated by TH production in the tadpole thyroid gland. The two main bioactive forms of TH are L-thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3); these hormones have overlapping but distinct mechanisms of action. Premetamorphic tadpoles are highly responsive to TH and can be induced to metamorphose through exogenous TH exposure, making them an important model for both the study of vertebrate TH signaling and endocrine disrupting chemicals (EDCs). It is important to differentiate TH-mediated responses from estrogenic responses in premetamorphic tadpoles when assessing dysregulation by EDCs as crosstalk between the two endocrine systems is well-documented. Herein, we compare the RNA-sequencing-derived transcriptomic profiles of three TH-responsive tissues (liver, olfactory epithelium, and tail fin) in premetamorphic bullfrog (*Rana [Lithobates] catesbeiana*) tadpoles exposed to T_3, T_4, and estradiol (E_2). These profiles were generated using the latest available genome assembly for the species. The data indicate that there is a clear distinction, and little overlap, between the transcriptomic responses elicited by E_2 and the THs. In contrast, within the THs, the T_3- and T_4-induced transcriptomic profiles generally show considerable overlap; however, the degree of overlap is highly tissue-dependent, illustrating the importance of distinguishing the two THs and the affected signaling pathways within the target tissue type when evaluating hormone active agents. The data herein also show that E_2 and TH treatment can uniquely induce significant changes in expression of their respective "classic" bioindicator transcripts *vtg* (E_2) and *thra, thrb,* and *thibz* (THs). However, care must be taken in the interpretation of increased *vep* or *esr1* transcripts as a change in transcript levels can be induced by THs rather than solely E_2.

Key Words

3,5,3’-triiodothyronine; endocrine disruptor; estrogen; RNA-Seq; thyroxine; tissue-specific response

INTRODUCTION

Thyroid hormones (THs) are critical for the modulation of vertebrate metabolism, growth, and development. The extent and impact of exposure to industrial, agricultural, or household sources containing exogenous THs or other endocrine disrupting chemicals (EDCs) that impact the TH axis are challenging to detect and establish (Thambirajah et al., 2019; Thambirajah et al., 2022). The metamorphosis of an amphibian tadpole to a frog is particularly susceptible to dysregulation by EDCs because of the total reliance on TH signaling (Thambirajah et al., 2019). Due to this sensitivity, tadpole metamorphosis has been used as a model system in the identification and study of EDCs targeting the TH system, largely through assessing perturbations in tadpole morphology, thyroid histology, and/or abundance of bioindicator gene transcripts (Thambirajah et al., 2019; Thambirajah et al., 2022).

TH-dependent metamorphosis, resulting in the transition from an aquatic tadpole to (semi-) terrestrial juvenile frog, is a postembryonic process involving the complete remodeling of the body plan and function. THs control metamorphosis primarily by binding to nuclear receptors α (TRα) and β (TRβ) which, when bound to TH responsive elements (TREs) within the gene promoters, activate or repress early response genes (Shi, 2000). Prior to metamorphosis, the tadpole thyroid gland is inactive and there are no circulating THs in the body (Shi, 2000). During this period, TRs (primarily TRα) are present in tissues at low levels and exposure to exogenous THs induces a precocious metamorphosis (Shi, 2000). As the tadpole develops, TH levels rise and these hormones promote complex and specific changes in gene expression in virtually every tissue resulting in whole-organism physiological and biochemical changes such as *de novo* development of the lungs and limbs, reprogramming of the liver, remodeling of the intestine and olfactory epithelium, and resorption of the tail fin.

The present study focuses on the liver, olfactory epithelium, and tail fin of premetamorphic *Rana [Lithobates] catesbeiana* (American bullfrog) tadpoles, which are TH-responsive tissues with distinct metamorphic outcomes (Gilbert et al., 1996). The liver is crucial for processing waste and general metabolism and must undergo major biochemical changes during metamorphosis to facilitate the transition from tadpole to frog (Gilbert et al., 1996; Shi, 2000). In contrast, olfaction is key to the survival and fitness of amphibians as it is used for food detection and predator avoidance (Gilbert et al., 1996; Shi, 2000). THs initiate structural changes in the olfactory epithelium that correspond to altered responses to odorants (Heerema et al., 2020). Other tissues, such as the tail fin, undergo TH-induced apoptosis in a time-, tissue-, and cell-specific manner, ensuring that the resorption of larval tissues is coordinated with the development of frog tissues (Ishizuya-Oka et al., 2010).

The two main biological forms of TH are L-thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3). The conventional dogma of TH action describes T_4 as a prohormone that is produced in the thyroid gland and converted by 5' deiodinases in the target tissues to the more bioactive T_3, which can interact with TRs (Mullur et al., 2014). However, some tissues, such as the tadpole liver, lack deiodinase 2 (Dio2), which converts T_4 to T_3. Maher et al. (2016) found that certain transcripts within the tadpole liver responded in a manner consistent with the direct action of T_4, suggesting that T_4 can directly bind TRs without prior conversion to T_3. The ratio of T_4 to T_3 and the presence of deiodinase 3 (Dio3), that inactivates T_4, have been theorized to time the metamorphosis of the Dio2-poor liver (Becker et al., 1997). Both forms of TH have distinct binding affinities to TRs and result in different dynamics between the liganded TR and nuclear coregulators (Maher et al., 2016). The degree to which these characteristics may facilitate distinct roles for T_3 and T_4 during metamorphosis remains unclear.

We used RNA-sequencing (RNA-Seq) to describe and compare transcriptomic changes in the premetamorphic *R. catesbeiana* tadpole liver, olfactory epithelium, and tail fin following whole organism exposure to biologically and environmentally relevant concentrations of T_3 and T_4. Given the interconnection between the TH and estrogen systems in other vertebrates (reviewed in Thambirajah et al., 2022), it is important to distinguish the responses of both systems to assess dysregulation by EDCs in the tadpole model. Premetamorphic tadpoles have not yet undergone sexual differentiation, thus providing an opportunity to observe the response of these naïve organisms to THs and their relationship to estrogens. We therefore also examined transcriptomic changes of premetamorphic tadpoles exposed to estrogen (E_2).

We performed differential expression analysis with DESeq2 to generate transcriptomic profiles and gene ontology (GO) term enrichment analysis to identify biological pathways associated with differentially expressed (DE) genes. To our knowledge, this is the first multi-tissue transcriptomic comparison of T_3, T_4, and E_2 treatment in amphibia. We show that E_2 exposure results in fewer transcriptomic changes than either T_3 or T_4 among the tissues studied. Furthermore, the transcriptomic changes and associated pathways elicited by E_2 exposure are highly dissimilar to those elicited by either TH in all three tissues. We also show tissue-specific differences in the transcriptomes induced by T_3 and T_4. Together, our results provide further evidence of discrete biological roles for T_3 and T_4 and suggest a clear distinction between an estrogenic response and a TH response in premetamorphic *R. catesbeiana* tadpoles. The present work stresses the importance of taking tissue context into account and helps delineate TH and estrogenic responses when evaluating EDC effects in the amphibian tadpole model.

MATERIALS AND METHODS

Experimental Animals, Exposure, and Tissue Isolation

Premetamorphic Taylor and Kollros (TK) stages VI-VIII (Taylor and Kollros, 1946) *R. catesbeiana* tadpoles of unknown sex were caught locally by Westwind Sealab Supplies in Victoria, British Columbia (BC, Canada) and housed at the University of Victoria Outdoor Aquatics Unit. Animals were housed in covered 100-gallon (378.54 L) fiberglass tanks containing recirculated dechlorinated municipal water at 15 ± 1°C, pH 6.8, and 96-98% dissolved oxygen (DO) and fed daily with Spirulina flakes (Nutrafin Max, Rolf C. Hagen, Montreal, PQ, Canada). Animals were then sent to Pacific Environmental Science Centre in North Vancouver, BC and housed in a covered outdoor facility prior to chemical treatments. Tadpoles were fed Nutrafin A6762C Max Spirulina meal tablets at a ratio of ½ tablet per individual every Monday, Wednesday, and Friday. Tanks were plumbed with on-site well water that was tempered to 15 ± 1°C with a 16 h light/8 h dark photoperiod. Tadpoles were brought indoors 96 h before the experiment and housed at 20.3 - 21.8°C (temperatures varied per hormone treatment) in 60 L tanks with a density of 10 tadpoles per tank 16 h light/8 h dark photoperiod. Tadpoles were fed at this time and no further food was given prior to testing.

Hormone exposure experiments were described previously (Heerema et al., 2018; Jackman et al., 2018). In brief, premetamorphic tadpoles were housed in aerated 20 L aquaria at a ratio of 10 L per tadpole, with two tadpoles per aquarium and 12 tadpoles per treatment condition. Animals were immersed in 20 - 21°C water containing 0.1, 1, or 10 nM T_3 (Sigma-Aldrich, Oakville, ON; Catalog # T2752, CAS 55-06-1), 0.5, 5, or 50 nM T_4 (Sigma, Catalog # T2501, CAS 6106-07-6), or 0.1, 1, or 10 nM E_2 (Sigma, Catalog # E4389, PubChem Substance ID 329799056), and NaOH vehicle (solvent) control (800 nM; TH control) or well water (E_2 control) for 48 hours as previously described in companion studies (Heerema et al., 2018;

Jackman et al., 2018). These concentrations are based upon observed environmental and physiological levels (Maher et al., 2016). The T_4 dose was matched to the T_3 dose based on a comparative analysis of the biological activity and binding affinity of T_4 and T_3 to TRs (Maher et al., 2016). Tadpole morphology details and water quality parameters for each exposure group have been reported previously (Jackman et al., 2018).

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 Committee of the University of Victoria (Protocol #2015-028). Tadpoles were euthanized in buffered tricaine methanesulfonate (1.0 g/L; Aqua Life, Syndel Laboratories, Nanaimo, BC, Canada). Liver, tail fin, and the rostrum tissues were isolated from each animal and placed in RNAlater solution (Ambion, Foster City, CA, USA) as per manufacturer's instructions for preservation. The olfactory epithelium was dissected later from the rostrum as described by Heerema et al. (2018). The preserved tissues were stored at -20°C and shipped on ice to the University of Victoria for RNA isolation.

Total RNA Isolation

Tissue samples were randomized prior to processing and extraction of RNA. Portions of the liver, tail fin, and olfactory epithelium were individually placed in 700 µL of TRIzol and mechanically mixed using a 3 mm tungsten carbide bead and Retsch MM301 Mixer Mill (Thermo Fisher Scientific, Ottawa, Canada) at 20 Hz for two three-minute intervals and rotated 180° between intervals. Samples were centrifuged at 12,000×g for 10 min at 4 °C to pellet the insoluble material and the supernatant was transferred to a new sterile, DNase-, and RNase-free microfuge tube. RNA was subsequently extracted using chloroform, isopropanol, and ethanol

161 treatments and dissolved in diethyl pyrocarbonate-treated water (Sigma-Aldrich) and stored at
162 −80 °C (Heerema et al., 2018).

163 **cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

164 Prior to the sequencing of RNA from select model chemical samples, qPCR analyses
165 were completed using validated primers for TH receptor α (*thra*), TH receptor β (*thrb*), and TH-
166 induced basic region leucine zipper-containing transcription factor (*thibz*) due to their established
167 TH-associated responses using three normalizer transcripts (*rpl8*, *eef1a*, and *rps10*) using the
168 primers and methods described previously (Heerema et al., 2018; Jackman et al., 2018).

169 **Illumina HiSeq2500 Sequencing**

170 The quality and concentration of the RNA samples were assessed using a Bioanalyzer
171 2100 (Agilent, Mississauga, Ontario, Canada) and RNA 6000 Nano Kit (Catalog # 5067-1511,
172 Agilent, Mississauga, Ontario, Canada). Five vehicle and five treatment RNA samples for each
173 of the three tissues with a RNA Integrity Number (RIN) ≥ 8 from each of 10 nM E_2, 10 nM T_3,
174 and 50 nM T_4 exposed tadpoles along with their matched controls (60 samples total) were
175 shipped at -20°C to the Michael Smith Genome Sciences Centre in Vancouver, BC for strand-
176 specific mRNA library construction and sequencing using the Illumina HiSeq2500 paired-end
177 platform (San Diego, California, USA) to generate 2x75 base pair reads as described previously
178 (Hammond et al., 2017).

179 **RNA-Seq analysis**

180 Raw reads were aligned to the *R. catesbeiana* genome version 3
181 (https://www.bcgsc.ca/downloads/supplementary/bullfrog/) using the STAR two-pass alignment
182 (version 2.6.1; Dobin et al., 2012). RNA-Seq analysis was performed as described in (Razmara

et al., 2021), with some minor changes. Briefly, StringTie (version 1.3.4) was used to assemble and count transcripts with a minimum read coverage of one for most transcripts, and 4.75 for single-exon transcripts (Pertea et al., 2015). Two sets of counts were generated; one for individual transcript abundance and a second which combined transcript isoforms counts to create gene counts. Both transcript and gene counts were tabulated across biological replicates for the subsequent comparison of treatments and controls. DESeq2 (version 1.28.1) (Love et al., 2014) was used for differential expression analysis from transcript counts ($p_{adj} \leq 0.05$, FDR $\leq$ 0.05). BLASTn (version 2.6.0) and BLASTx were used to annotate transcripts using the National Center for Biotechnology Information (NCBI) nucleotide (nt) and non-redundant (nr) protein databases.

DE genes (using gene counts) were annotated with gene ontology (GO) terms using Trinotate (version 3.2.0) (Bryant et al., 2017). The GO terms associated with the DE genes for each treatment were compared against the distribution of GO terms assigned to all the significantly expressed genes in each tissue (cpm > 0.1). GOseq (version 1.42.0) was then used to identify GO term enrichment ($p_{adj} \leq 0.05$, FDR ≤ 0.05) (Young et al., 2010). Due to the high abundance of enriched GO terms observed, an 'extremes' filter was applied using Gogadget (version 2.1) (Nota, 2017) to remove the GO terms that are highly specific or too general for better visualization of enriched processes. GO terms annotating <10 or >500 DEGs were excluded from analysis for most treatments. The olfactory epithelium treated with T_3 and T_4 had too many annotated GO terms for downstream visualization so cutoffs of <20 and >100 DEGs or <40 and >100 DEGs respectively were applied. Cytoscape was used to generate and visualize enrichment maps from the enriched GO terms (version 3.8.2) (Young et al., 2010).

Statistical Analysis

RNA-Seq data was analyzed using DESeq2 (version 1.28.1) (Love et al., 2014) was used for differential expression analysis from transcript counts ($p_{adj} \leq 0.05$, FDR ≤ 0.05). DESeq2 is part of the Bioconductor 3.15 statistical package that performs differential gene expression analysis based on the negative binomial distribution and also generates PCA and volcano plots (Love et al., 2014). Fold changes were calculated with statistical significance set at $p \leq 0.05$. GOseq (version 1.42.0) was used to identify GO term enrichment ($p_{adj} \leq 0.05$, FDR ≤ 0.05) (Young et al., 2010) and an 'extremes' filter applied using Gogadget (version 2.1) (Nota, 2017) to remove the GO terms that are highly specific or too general for better visualization of enriched processes as mentioned in the previous section.

RESULTS AND DISCUSSION

The raw RNA-Seq data generated from the olfactory epithelium has been previously analyzed and published using the assembled *R. catesbeiana* transcriptome (Jackman et al., 2018). Jackman *et. al* found > 30,000 TH-responsive transcripts in the olfactory epithelium. There was limited responsiveness to E_2, with only 267 differentially expressed transcripts. Gene ontology terms relating to sensory perception, potassium ion transport, transcription, and RNA processing were strongly enriched following TH treatment. There was minimal overlap between E_2 and THs, with only 57 transcripts similarly regulated between the hormones. In the present study, we reanalyzed the olfactory epithelium RNA-Seq data against the *R. catesbeiana* genome that has since been assembled (version 3; https://www.bcgsc.ca/downloads/supplementary/bullfrog/).

Mapping Reads

The average number of reads per library for the liver was 31.7 million, the olfactory epithelium had 31.8 million reads, and the tail fin had 31.9 million reads (Supplementary Table S1). RNA-Seq reads were mapped to the annotated *R. catesbeiana* genome version 3 [NCBI Genbank Accession No: LIAG00000000, BioProject PRJNA285814; (Hammond et al., 2017)]. An average of 79% of filtered reads from the liver were successfully mapped to the *R. catesbeiana* genome while the olfactory epithelium and tail fin both had 80% of reads successfully mapped (Supplementary Table S1).

Differential Expression Analysis Overview using DESeq2

The total number of assembled transcripts identified ranged from 12,516 to 12,589 in the liver, 16,999 to 17,173 in the olfactory epithelium, and 15,298 to 15,426 in the tail fin (Table 1). DESeq2 identified DE transcripts following hormone treatment in the three tissues (Table 1). On average, 90% of all DE transcripts in the liver were successfully annotated and 89% were successfully annotated in the olfactory epithelium and the tail fin using BLASTx against the NCBI non-redundant database (Table 1).

Principal component analysis (PCA) of the DE transcripts showed strong separation between the control and treatment samples within the first component and accounted for 67-84% of variation in all groups (Figure 1). This highlights the robust and reproducible transcriptomic responses that tadpoles exhibit upon hormone exposure.

Our analysis shows prominent differences in the transcriptomic responses of each tissue type to E_2, T_3, and T_4 treatment (Figure 2). T_3 and T_4 exposure resulted in substantially more DE transcripts than E_2 exposure in all three tissues. E_2 exposure resulted in only 5 DE transcripts in the tail fin, 28 in the olfactory epithelium, and 70 in the liver (Table 1, Figure 3). None of these

E_2-responsive transcripts overlapped between the three tissue types (Figure 4). Very few, if any, E_2-responsive transcripts overlapped with TH-responsive transcripts (7/28 – 25% in olfactory epithelium; 29/70 – 41% in liver; 0/5 – 0% in tail fin; Figure 3).

The differential ability of T_3 and T_4 to elicit distinct changes in gene expression in each tissue is striking (Table 1). In the highly TH-responsive olfactory epithelium, a similar number of DE transcripts were significantly affected by T_3 (7,653) and T_4 (6,478; Table 1, Figures 3 and 4). Of these, a considerable proportion (5,321/8,810 – 60%) was shared between both THs (Figure 3). Approximately twice as many transcripts were unique to olfactory epithelium from T_3-treated tadpoles (2,332/8,810 – 26%) compared to T_4-treated tadpoles (1,157/8,810 – 13%; Figure 3). Similar results were obtained using a reference transcriptome for read alignment in a previous analysis of these RNA-Seq data (Jackman et al., 2018) compared to the present read alignments to the *R. catesbeiana* genome assembly (version 3) used herein. During metamorphosis, the olfactory epithelium undergoes changes in structure and olfactory sensory neuron type, and exposure to T_3 has been associated with significant changes in olfaction-mediated behavior (Heerema et al., 2020). Further analysis is needed to explore how the distinct transcriptomic profile of this sensitive tissue relates to behavior, especially adverse behavioral outcomes when EDCs disrupt TH signaling.

While 335/1,865 (18%) DE transcripts were common between the liver transcriptomes from T_4- and T_3-treated tadpoles (Figure 3), there was a marked imbalance in transcripts uniquely affected differentially by the two THs. Approximately 11-fold more transcripts were unique to liver from T_4-treated tadpoles (1,346/1,865 – 72%) compared to T_3-treated tadpoles (143/1,865 – 8%; Figure 3). The liver lacks Dio2 for conversion of T_4 to T_3, which suggests that T_4 has intrinsic biological activity independent of conversion to T_3 (Maher et al., 2016). Moreover, T_4

has greater affinity for TRα than TRβ and the *R. catesbeiana* premetamorphic tadpole liver has previously been shown to express significantly more transcripts encoding TRα than TRβ (Maher et al., 2016). Our results provide further evidence that T_4 may play a more extensive role in modulating the transcriptome of the liver than T_3.

The tail fin response to THs demonstrated a third relationship between T_3 and T_4 where 1,405/3,949 (36%) DE transcripts were shared between the two hormones (Figure 3). The tail fin from tadpoles treated with T_3 had ~4-fold greater unique DE transcripts (1,994/3,949 – 50%) compared to tadpoles treated with T_4 (545/3,949 – 14%; Figure 3) suggesting that this tissue is more sensitive to T_3 than T_4. This observation is in contrast with previous work on a limited number of specific transcripts (Maher et al., 2016) that demonstrated equivalency of responses to T_3 and T_4 when corrected for differential nuclear receptor binding. However, the expanded scope of the present study provides a further dimension in understanding the impact of T_3 and T_4 exposure in this tissue.

Comparison of the tissue responses to each of the TH treatments revealed that the number of DE transcripts in common across tissues was proportionally low across all tissues (141/9,060 – 1.6%, T_3-treated tadpoles; 234/7,938 – 2.9%, T_4-treated tadpoles; Figure 4). The relative proportion of DE transcripts shared between olfactory epithelium and tail fin (2,134/8,771 – 24%; Figure 4) was ~5-fold greater in T_3-treated tadpoles compared to those shared between the olfactory epithelium and the liver (289/5,849 – 5%; Figure 4) or the tail fin and liver (189/3,689 – 5%; Figure 4). This may be due to the shared epithelial origin of the olfactory epithelium and tail fin.

In contrast, T_4-treated tadpoles exhibited a different pattern of shared DE transcripts between tissues (Figure 4). The relative proportion of DE transcripts shared between olfactory

epithelium and tail fin (1,099/6,598 – 17%; Figure 4), the olfactory epithelium and the liver (965/6,329 – 15%; Figure 4), and the tail fin and liver (341/3,290 – 10%; Figure 4) in T_4-treated tadpoles were comparable to one another.

These tissue- and hormone-specific transcriptomic differences may reflect the distinct metamorphic outcomes of the three tissues. It has previously been posited that factors including TR isoform expression, deiodinase activity, coregulator expression, and epigenetic mechanisms could facilitate the tissue-specific actions of THs (Helbing et al., 2010; Maher et al., 2016). The remarkable tissue-specificity observed in our data suggests that certain tissues or cell types employ one or more of these factors, resulting in distinct TH-mediated outcomes. Taken together, these results support the marked sensitivity of premetamorphic tadpole tissues to THs and the relatively "muted", but present response to E_2.

Assessment of TH and E_2 Classic Indicator Transcripts

We queried our dataset for transcripts that are commonly used as indicators of hormone action and dysregulation by EDCs. TH-responsive transcripts TH receptor α (*thra*), TH receptor β (*thrb*), and TH-induced basic region leucine zipper-containing transcription factor (*thibz*) are established TH-responsive transcripts and have been used in previous studies to identify potential TH disruption (Thambirajah et al., 2019; Thambirajah et al., 2022). In the liver, olfactory epithelium, and tail fin, *thra*, *thrb*, and *thibz* transcripts significantly increased in abundance following exposure to either T_3 or T_4 (Table 2) while none of these transcripts were differentially expressed in any of the three tissues following E_2 treatment (Table 2). These results are consistent with qPCR results obtained from tissues of tadpoles exposed to three different concentrations of hormones (n=12 per treatment; Supplementary Figure S1 and S2 for the tail fin and liver; olfactory epithelium results were published previously in Jackman et al., 2018). Our

results support the use of these gene transcripts in the liver, olfactory epithelium, and tail fin of *R. catesbeiana* tadpoles to identify exposure to biologically relevant concentrations of T_3 or T_4.

In fish and frogs, exposure to estrogens is often assessed by measurement of vitellogenin (*vtg*) gene transcripts. Vitellogenins are phospholipoglycoproteins produced primarily in the liver that are involved in yolk production in mature females and are used as biological indicators of estrogenic activity (Huang et al., 2005). Liver from E_2-exposed tadpoles had significant upregulation of two *vtg* transcripts with 2,135- and 1,961-fold increases (Supplementary Table S3). Increases in these transcripts were not observed for other tissues or treatment conditions (Supplementary Table S3). In addition to fish and frogs, these data highlight the suitability of using expression of vitellogenin mRNA in the tadpole liver for identifying E_2 exposure exclusively, as these transcripts were not responsive to THs.

While no increase in the abundance of vitellogenin mRNA was identified in response to TH treatment (Supplementary Table S3), a transcript encoding a vitelline membrane outer layer protein 1 precursor homolog was increased by 13±0.7-fold in the liver following E_2 treatment and a transcript encoding another vitelline membrane protein homolog was increased by 37±0.9-fold in the same tissue following T_4 treatment (*vep;* Supplementary Table S3). Vitelline envelope proteins have been extensively studied and all are extremely responsive in the liver to estrogen exposure. In fact, the transcripts encoding these proteins are more sensitive to estrogen exposure than vitellogenin (Osachoff et al., 2016). Both E_2 and T_4 treatment leading to increased abundance of different transcripts involved in vitellogenesis presents a possible intersection of TH and estrogenic action wherein similar processes are upregulated without upregulating the same transcripts (Thambirajah et al., 2022).

To continue to elucidate possible TH and estrogenic cross-regulation, the datasets were queried for differential expression of estrogen receptor (ER) transcripts following TH treatment. While ERs are not commonly used as indicator genes due to their variable expression following E_2 exposure, previous work examining hormone cross-regulation in the brain of *R. pipiens* tadpoles found that 50 nM T_3 treatment led to a significant increase in *esr1* transcript abundance (Hogan et al., 2008). We found that *esr1* was detected but not differentially expressed in all olfactory epithelium and tail fin groups. A 4±0.4-fold increase of *esr1* mRNA was seen in the liver of tadpoles exposed to T_3 (Supplementary Table S5). This suggests that T_3 can influence estrogen signaling pathways in a tissue-specific manner in the liver. E_2 treatment did not cause a change in TR transcript abundance in any of the tissues (Table 2).

Taken together, we show that E_2 and TH treatment can uniquely induce significant changes in expression of their respective "classic" bioindicator transcripts *vtg* (E_2) and *thra, thrb,* and *thibz* (THs). However, care must be taken in the interpretation of increased *vep* or *esr1* transcripts as a change in transcript levels can be induced by THs rather than solely E_2.

Deiodinase Transcript Abundance Demonstrates Tissue- and Hormone-Specificity

Notable differences in the transcriptomic response to T_3 or T_4 were seen in all three tissues. Deiodinases are known to play a role in controlling TH levels by regulating the proportion of T_3 to T_4 during metamorphosis. Thus, deiodinases can influence intracellular TH availability and, in turn, TR binding and transcription of target genes (Brown and Cai, 2007). We queried our dataset for the deiodinases *dio1*, *dio2*, and *dio3* transcripts, to assess whether deiodination may be involved in a tissue-specific manner. Deiodinase 2 (Dio2) converts T_4 to T_3 by outer ring deiodination. Deiodinase 3 (Dio3) converts T_3 to T_2 as well as converting T_4 to reverse triiodothyronine (rT_3), which are both inactive and do not bind to TRs (Bianco et al.,

2002). Deiodinase 1 (Dio1) can convert T_4 into T_3 and convert T_4 into rT_3 (Bianco et al., 2002). Both Dio2 and Dio3 are known to regulate developmental timing during metamorphosis. In the tail fin, *dio3* is expressed throughout metamorphosis, which ensures low levels of bioactive TH. At metamorphic climax, *dio3* is downregulated and *dio2* upregulated thereby stalling tail regression until the peak levels of T_3 are present (Brown and Cai, 2007). Upregulation of *dio2* is similarly highest in the intestine directly preceding its remodeling (Ishizuya-Oka and Shimozawa, 1992).

However, not all tissues express *dio2* when they undergo their greatest developmental changes. Tadpole gills do not express *dio2* and must rely on circulating T_3 reaching a sufficiently high level in the bloodstream to induce reabsorption at metamorphic climax (Cai and Brown, 2004). The liver also lacks *dio2*-mediated conversion of T_4 to T_3 and would similarly need to rely on circulating T_3 to induce its metamorphic program (Becker et al., 1997). However, recent research shows evidence of transcriptomic changes in the liver consistent with direct action of T_4 (Maher et al., 2016). T_4 also has "non-genomic action" outside of binding to the nuclear TR receptors and can bind to membrane receptors and extracellular TRs (Taylor and Heyland, 2017). T_4 can bind to membrane receptor integrin αVβ3 and initiate the mitogen-activated protein kinase (MAPK) signaling cascade (Davis et al., 2008) by stimulating MAPK association with and phosphorylation of TRβ1 thereby influencing the transcription of certain genes (Davis et al., 2000). Through the MAPK signaling cascade, T_4 can promote angiogenesis as well as sensory neuron and osteoblast development (Taylor and Heyland, 2017). Deiodinase activity and differences in T_3- and T_4-mediated transcriptomic changes may partially account for differences in the metamorphic responses of different tissues.

385 Dio2 converts T_4 to T_3 and is highly expressed during the metamorphic climax in Dio2-
386 rich tissues such as the intestine and back skin (Cai and Brown, 2004). In the present study, *dio2*
387 mRNA was only expressed in the tadpole olfactory epithelium and was upregulated after both
388 TH treatments (Table 2), consistent with previous analyses (Jackman et al., 2018). This increase
389 suggests that the olfactory epithelium then develops the capacity to convert T_4 to T_3. Given the
390 experimental design of compensating TH dosage to account for the ~5-fold difference in
391 affinities for their TRs (50 nM T_4 against 10 nM T_3; Maher et al., 2016), if there is direct binding
392 of the hormone to TRs, then a similar fold transcript response is expected compared to controls.
393 On the other hand, if Dio2 activity influences intracellular T_4 conversion to T_3 and this newly
394 generated T_3 interacts with TRs, then a greater TH-mediated response would be expected (Maher
395 et al., 2016). The present study demonstrates that in the olfactory epithelium that evidence for
396 both mechanisms exist. The increase in *thrb* transcripts relative to the controls are similar in the
397 olfactory epithelium of T_3- and T_4-treated tadpoles (8±0.2-fold and 9±0.2-fold, respectively;
398 Table 2), supporting the direct binding of the THs to the TRs. In contrast, the increase in *thibz*
399 transcripts relative to the controls was substantial but lower in the olfactory epithelium of T_3-
400 compared to T_4-treated tadpoles (206±0.3-fold and 304±0.2-fold, respectively; Table 2),
401 supporting the influence of Dio2. Moreover, our data demonstrate the absence of *dio2* in the
402 liver, aligning with previous findings that T_4 to T_3 conversion is poor in this tissue and that T_4
403 has independent, direct action in this tissue (Hammond et al., 2017; Maher et al., 2016).

404 Dio3 converts T_3 to T_2 and T_4 to rT_3, which do not bind TRs (Bianco et al., 2002). In the
405 current study, *dio3* was upregulated in the T_3- and T_4-treated tail fin but was only upregulated in
406 the liver of tadpoles exposed to T_4. An upregulation of *dio3* may increase the conversion of
407 active TH to its inactive forms, thus contributing to the TH-mediated regulation of metamorphic

timing in these tissues. One isoform of *dio3*, bullfrog transcript *LICA5447*, was downregulated in the olfactory epithelium, which may increase the availability of T_3 or T_4 in this tissue. Changes in *dio3* abundance in these tissues may play a role in regulating the concentrations of either TH for timing metamorphosis.

Lastly, Dio1 can catalyze both conversion of T_4 to T_3, and T_4 to rT_3 (Bianco et al., 2002). Its role in TH metabolism is still somewhat debated. In mammals it is considered to contribute to circulating T_3 levels by converting T_4 to T_3, albeit with lower catalytic efficiency compared to Dio2 (Bianco et al., 2002). Dio1 is also considered a "scavenger" enzyme which removes inactive iodothyronines from circulation (Dentice et al., 2013). The role of Dio1 in amphibians is still unclear. Localized *dio1* expression has been reported in the eyes, brain ventricles, and branchial arches of late-stage embryonic *Xenopus laevis* (Morvan Dubois et al., 2006) as well as in the liver and kidneys of young adult *X. laevis* (Kuiper et al., 2006). Our study provides novel evidence for the tissue-specific presence of *dio1* transcripts in *R. catesbeiana* tadpoles exposed to T_3 or T_4. *Dio1* was significantly downregulated in the tail fin of tadpoles exposed to T_3 and T_4 and in the liver of tadpoles exposed to T_4 (Table 2). As Dio1 can catalyze conversion of T_4 to T_3 as well as T_4 to rT_3, it is unclear what affect its downregulation has on the two tissues. It could potentially increase or decrease the amount of available T_4 in the tail fin or liver, which could influence gene expression associated with this specific hormone. It is not clear what role *dio1* downregulation plays in these tissues, but this result provides intriguing evidence that *R. catesbeiana* do express *dio1* in response to TH treatment. It is possible that tissue- and stage-dependent expression of *dio1*, like *dio2* and *dio3*, regulates T_3 and T_4 levels during amphibian metamorphosis.

Our work shows tissue- and hormone-specific differences in deiodinase expression, which may aid in regulating local availability of T_3 and T_4. T_3 and T_4 have different affinities for nuclear TRs as well as other membrane receptors (Taylor and Heyland, 2017); T_3 and T_4 treatment resulted in different transcriptomic profiles in our study. None of the deiodinase transcripts were differentially expressed in any tissue following E_2 exposure, suggesting that estrogen does not affect deiodinases associated with the regulation of THs. Deiodinase activity may influence T_3 and T_4 levels, and thus influence timing and developmental outcome of tissues during metamorphosis.

Closely regulated metamorphic timing is critical given that mistiming of lung, gill, or limb developmental changes could be deleterious to an aquatic tadpole transitioning to a terrestrial frog. Tissues may respond differently to disruption of T_3- or T_4- mediated gene expression programs by EDCs, given that not all tissues undergo the same deiodination processes. In the olfactory epithelium, the predator cue avoidance response to an olfactory stimulus was abolished in *R. catesbeiana* tadpoles treated with exogenous T_3 but not T_4 or E_2 (Heerema et al., 2018). Our work shows that deiodinase expression is tissue-specific in response to TH treatment, which has implications for both metamorphic timing and EDC disruption.

GENE ONTOLOGY (GO) ANNOTATION

Unique Organellar Functions Associated with the Liver from E_2-Treated Tadpoles

As E_2 exposure resulted in substantially fewer DE genes than TH exposures (Supplementary Table S6), there were correspondingly few GO enriched terms following E_2 treatment. There were no GO terms associated with either the olfactory epithelium or tail fin from E_2-treated tadpoles. The E_2-treated liver DE transcripts were associated with GO terms

relating to golgi and endoplasmic reticulum transport (Figure 5). These terms were unique to E_2 treatment and were not enriched following treatment with either TH, although T_3 and T_4 treatment did result in enrichment of related terms (Figure 5). These results emphasize the vastly distinct responses to THs and estrogens as observed in our transcriptomic analysis (Figure 3).

Urea Cycle Enrichment Associated with the T_3-Treated Liver

Consistent with the transition from an ammoniotelic to ureotelic liver during metamorphosis, we found enrichment in GO terms related to the urea cycle and nitrogen metabolism following T_3 treatment in the liver (Figure 6). These GO terms were not enriched in the T_4-treated liver, providing further evidence for distinct biological roles of T_3 and T_4.

Fatty Acid Metabolism Associated with the TH-Treated Liver

Lipids are important energy and signaling molecules and THs are known to affect lipid metabolism in vertebrates (Ichu et al., 2014). In the liver, both T_3 and T_4 treatments were associated with fatty acid metabolism and arachidonic acid metabolism gene ontologies (Figure 7). Oxidation of arachidonic acid generates eicosanoids, which are a family of signaling molecules that are involved in a diversity of biological processes including inflammation, immune system modulation, and tissue repair and regeneration (Esser-Von Bieren, 2019). Eicosanoid levels have been shown to change in abundance during metamorphosis with levels generally decreasing once metamorphosis is complete (Ichu et al., 2014). The association of arachidonic acid metabolism could therefore be indicative of eicosanoid signaling in the liver during TH-dependent metamorphosis.

RNA Regulation Associated with the TH-Treated Olfactory Epithelium

Spliceosome activity and micro (mi) RNA-mediated gene silencing ontologies were found to be associated with the T_3- and T_4-treated olfactory epithelium (Figure 8). These are both important mechanisms of transcriptional regulation with established roles in neurodevelopment (Park et al., 2022). GO terms related to other aspects of RNA metabolism were also associated with the TH-treated olfactory epithelium, including RNA polymerase activity, nuclear export, and RNA methylation (Figure 8). These associated pathways were not enriched in the olfactory epithelium of E_2 treated tadpoles, nor in the liver or tail fin of tadpoles exposed to any of the three hormones. The olfactory epithelium may exhibit TH-dependent mechanisms of transcriptional regulation which are distinct from that of the liver or tail fin.

Immune Cell Migration and Cellular Adherence in the TH-Treated Tail fin

Pathways related to cell motility and leukocyte recruitment were ontologically associated with the tail fin of T_3- and T_4- treated tadpoles (Figure 9). This may reflect immune cells being recruited to the tail fin to clean up the cellular debris following apoptosis, as has been shown in the apoptotic tadpole intestine (Ishizuya-Oka et al., 2010). The resorption of the tail fin also requires the breakdown of extracellular matrix (ECM) components and of cell-to-cell and cell-to-ECM adhesions (Ishizuya-Oka et al., 2010). Our GO analysis revealed associations to ECM organization and to cellular adhesions, with the former enriched following only T_3 treatment, again supporting distinct regulatory roles for each form of TH (Figure 9).

CONCLUSION

Our transcriptomic analysis showed remarkable differences in the DE transcript profiles following T_3 and T_4 treatment in the liver, olfactory epithelium, and tail fin, supporting distinct regulatory roles for each TH in the tissues studied. The ability of T_3 and T_4 to elicit discrete

transcriptomic changes in a tissue-specific manner may contribute to the temporal and spatial coordination of metamorphosis and reveal potential differential vulnerabilities to EDC effects. More research is required to investigate the mechanisms behind this hormone tissue specificity.

E_2 treatment resulted in a transcriptomic response that was distinct from that of either TH in all three tissues. We found that E_2 treatment did not induce expression of classic TH response transcripts *thra*, *thrb*, or *thibz*. Similarly, transcript abundance of the classic E_2 bioindicator *vtg* was only significantly upregulated in the liver of E_2-treated tadpoles. However, care must be taken in the interpretation of increased *vep* or *esr1* transcripts as a change in transcript levels can be induced by THs rather than solely E_2.

Our data demonstrate that premetamorphic *R. catesbeiana* tadpoles have distinct transcriptomic responses to T_3, T_4, and E_2. These unique changes may be useful bioindicators for assessing the targets and mechanisms of perturbation by potential EDCs. The importance of amphibian metamorphosis as a model for identifying perturbations in TH signaling, and as a sentinel for the health of humans and other vertebrates cannot be overstated. Moreover, successful metamorphosis is critical for amphibian survival, and with amphibian species in rapid decline, it is crucial that we deepen our understanding of anthropogenic factors negatively affecting their health.

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TABLES

Table 1. Total number of transcripts sequenced from liver, olfactory epithelium (OE), and tail fin libraries from E_2-, T_3-, or T_4-exposure experiments. Each exposure experiment consisted of a control and the indicated hormone treatment groups (n=5 individual tadpoles/group). Total transcripts include those detected in any of the control or treatment animals for each respective hormone exposure group. Transcripts were annotated using BLASTn (version 2.6.0, National Centre for Biotechnology Information). A transcript was considered differentially expressed (DE) if there was a statistically significant ($p_{adj} \leq 0.05$) difference in transcript abundance between the control and treatment groups with a false discovery rate (FDR) of 0.05.

Treatment	Tissue	Transcripts	% Annotated	DE Transcripts	% DE Annotated
E_2	Liver	12,516	90	70	90
	OE	17,051	89	28	82
	Tail fin	15,289	89	5	100
T_3	Liver	12, 589	91	478	93
	OE	17,173	89	7,653	92
	Tail fin	15,426	89	3,400	92
T_4	Liver	12,542	90	1,681	94
	OE	16,999	90	6,478	92
	Tail fin	15,406	89	1,950	92

Table 2. RNA-Seq-derived mean fold changes in thyroid hormone-responsive transcripts as across tissue and treatment groups relative to the paired controls (n=5 individual tadpoles/group). Increased fold changes (↑), decreased fold changes (↓) and standard error of the mean (SEM) are denoted for each sample group. All indicated fold changes were statistically significant (p_{adj}<0.05). Detailed transcript counts are in **Supplementary Table S2**. '-', no change; *dio1*, deiodinase 1; *dio2*, deiodinase 2; *dio3*, deiodinase 3; ND, not detected; *thibz*, TH-induced basic region leucine zipper-containing transcription factor; *thra*, TH receptor α; *thrb*, TH receptor β.

Treatment	Tissue	*thra*	*thrb*	*thibz*	*dio1*	*dio2*	*dio3* LICA 5446[a]	*dio3* LICA 5447[a]
E_2	Liver	-	ND	-	-	ND	-	-
	OE	-	-	-	-	-	-	-
	Tail fin	-	-	-	-	ND	ND	-
T_3	Liver	↑ 4 ± 0.2	↑ 46 ± 0.7	↑ 47 ± 0.2	-	ND	-	-
	OE	↑ 3 ± 0.1	↑ 8 ± 0.2	↑ 206 ± 0.3	-	↑ 4 ± 0.1	-	↓ 2 ± 0.1
	Tail fin	↑ 2 ± 0.1	↑ 13 ± 0.1	↑ 157 ± 0.1	↓ 36 ± 0.7	ND	↑ 110 ± 0.4	↑ 100 ± 0.4
T_4	Liver	↑ 4 ± 0.2	↑ 143 ± 0.6	↑ 116 ± 0.3	↓ 4 ± 0.4	ND	↑ 2 ± 0.1	↑ 2 ± 0.2
	OE	↑ 3 ± 0.2	↑ 9 ± 0.2	↑ 304 ± 0.2	-	↑ 3 ± 0.2	-	-
	Tail fin	↑ 2 ± 0.1	↑ 7 ± 0.2	↑ 123 ± 0.3	↓ 29 ± 0.7	ND	↑ 256 ± 1	↑ 56 ± 0.5

[a]Two separate *dio3* isoforms having different sequences and potential protein products are referred to by their identity in the *R. catesbeiana* genome.

FIGURE LEGENDS

Figure 1. Principal Component Analysis (PCA) of differentially expressed transcripts from the liver, olfactory epithelium, and tail fin of premetamorphic tadpoles treated with E_2, T_3, or T_4. Diluent control biological replicates (800 nM NaOH for TH, or well water for E_2) are indicated by open circles. Biological replicates treated with hormone (10 nM E_2, 10 nM T_3 or 50 nM T_4) are indicated by closed circles.

Figure 2. Volcano plots of differentially expressed transcripts in the liver, olfactory epithelium, and tail fin of premetamorphic tadpoles treated with 10 nM E_2, 10 nM T_3, or 50 nM T_4. Differential expression of transcripts was determined by DESeq2 analysis (Love et al., 2014). Red dots indicate differentially expressed (DE) transcripts and grey dots are non-DE transcripts ($p_{adj} < 0.05$).

Figure 3: Comparison of numbers of differentially expressed transcripts in liver, olfactory epithelium, and tail fin treated with 10 nM E_2, 10 nM T_3, or 50 nM T_4. Transcripts differentially expressed ($p < 0.05$) in response to E_2 (pink), T_3 (orange) or T_4 (blue) were identified by DESeq2 (Love et. al, 2014). Circles were approximately scaled to represent the difference in total transcripts expressed in response to each hormone.

Figure 4: Comparison of numbers of differentially expressed transcripts in response to treatment with 10 nM E_2, 10 nM T_3, or 50 nM T_4 in liver, olfactory epithelium, and tail fin. Transcripts

differentially expressed ($p < 0.05$) in the olfactory epithelium (pink), tail fin (orange), and liver (blue) were identified with DESeq2 (Love et. al, 2014). Circles were approximately scaled to represent the difference in total transcripts expressed in each tissue.

Figure 5. Gene ontology (GO) enrichment analysis of organellar functions in the liver following treatment with 10 nM E_2, 10 nM T_3, or 50 nM T_4. A) GO term enrichment visualized using the Cytoscape Enrichment Map plugin. Each GO term is represented by a single node split into thirds, each indicating a hormone treatment. If a third is orange, that GO term is enriched in that hormone treatment with shade of orange indicating p-value (grey = no enrichment). Node size indicates the number of genes associated with that GO term, line thickness indicates the relatedness of adjacent nodes, and the numbers associated with each node correspond to the GO terms indicated in Part B. The black circle highlights the nodes involved in E_2 mediated ER Golgi transport. B) The negative $\log_{10}$ of the p-value associated with each GO term. Bar color corresponds to E_2 (pink), T_3 (orange), or T_4 (blue) treatment. Only bars are shown where GO term enrichment was detected.

Figure 6. Gene ontology (GO) enrichment analysis of the urea cycle in the liver following 10 nM T_3 or 50 nM T_4 treatment. A) GO term enrichment visualized using the Cytoscape Enrichment Map plugin (see Figure 5 legend for additional details). Each GO term is represented by a single node split in half with each side indicating a different TH treatment. B) The negative $\log_{10}$ of the p-value associated with each GO term. Bar color corresponds T_3 (orange) or T_4 (blue) treatment.

Figure 7. Gene ontology (GO) enrichment analysis of fatty acid metabolism in the liver following 10 nM T_3 or 50 nM T_4 treatment. A) GO term enrichment visualized using the Cytoscape Enrichment Map plugin. The black circle highlights the nodes involved arachidonic acid metabolic processes. B) The negative $\log_{10}$ of the p-value associated with each GO term. See the Figure 6 legend for additional details.

Figure 8. Gene ontology (GO) enrichment analysis of five clusters of enriched GO terms implicated in RNA regulation in the olfactory epithelium following 10 nM T_3 or 50 nM T_4 treatment. These clusters include spliceosome activity, micro (mi) RNA-mediated gene silencing, RNA polymerase (RNAP) activity, nuclear export, and RNA methylation. Due to the size of the spliceosome cluster, only the nodes with the most significant p-values were included here. Complete GO term visualization and p-value figures for this cluster are available in Supplementary Figure S3. A) GO term enrichment visualized using the Cytoscape Enrichment Map plugin. B) The negative $\log_{10}$ of the p-value associated with each GO term. See the Figure 6 legend for additional details.

Figure 9. Gene ontology (GO) enrichment analysis of three clusters of enriched GO terms implicated in immune cell migration and resorption in the tail fin following 10 nM T_3 or 50 nM T_4 treatment. If the cluster contained more than five nodes, only the nodes with the most significant p-values were included. A) GO term enrichment visualized using the Cytoscape Enrichment Map plugin. B) The negative $\log_{10}$ of the p-value associated with each GO term. See

721 the Figure 6 legend for additional details. Complete GO term visualization and p-value figures
722 for all nodes are available in Supplementary Figure S4.