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Transcriptomics investigation of thyroid hormone disruption in the olfactory system of the *Rana [Lithobates] catesbeiana* tadpole

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ABSTRACT

Thyroid hormones (THs) regulate vertebrate growth, development, and metabolism. Despite their importance, there is a need for effective detection of TH-disruption by endocrine disrupting chemicals (EDCs). The frog olfactory system substantially remodels during TH-dependent metamorphosis and the objective of the present study is to examine olfactory system gene expression for TH biomarkers that can evaluate the biological effects of complex mixtures such as municipal wastewater. We first examine classic TH-response gene transcripts using reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) in the olfactory epithelium (OE) and olfactory bulb (OB) of premetamorphic *Rana (Lithobates) catesbeiana* tadpoles after 48 h exposure to biologically-relevant concentrations of the THs, 3,5,3′-triiodothyronine (T_3) and L-thyroxine (T_4), or 17-beta estradiol (E_2); a hormone that can crosstalk with THs. As the OE was particularly sensitive to THs, further RNA-seq analysis found > 30,000 TH-responsive contigs. In contrast, E_2 affected 267 contigs of which only 57 overlapped with THs suggesting that E_2 has limited effect on the OE at this developmental phase. Gene ontology enrichment analyses identified sensory perception and nucleoside diphosphate phosphorylation as the top affected terms for THs and E_2, respectively. Using classic and additional RNA-seq-derived TH-response gene transcripts, we queried TH-disrupting activity in municipal wastewater effluent from two different treatment systems: anaerobic membrane bioreactor (AnMBR) and membrane enhanced biological phosphorous removal (MEBPR). While we observed physical EDC removal in both systems, some TH disruption activity was retained in the effluents. This work lays an important foundation for linking TH-dependent gene expression with olfactory system function in amphibians.

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 (in most) lifestyle of the juvenile frog. While the fundamentals of the olfactory system remain the same, what constitutes an appropriate response to different odorants differs depending upon life phase but is essential for survival in terms of both food location and predator evasion. These fundamentals include the binding of odorants to receptors on the surface of the olfactory epithelium (OE), and propagation of a signal to the olfactory bulb (OB) of the brain which elicits a behavioral response (Ache and Young, 2005; Gascuel and Amano, 2013).

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

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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 some classic TH-response gene transcripts (*thra*, *thrb*, and *thibz*) in the OE (Heerema et al., 2017). 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). The objective of the present study is to examine olfactory system gene expression for TH biomarkers that can evaluate the biological effects of complex mixtures such as municipal wastewater.

To address this objective, we first examined classic TH-response gene transcripts using reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) on OB and OE RNA from premetamorphic *Rana (Lithobates) catesbeiana* tadpoles after 48 h exposure to biologically-relevant concentrations of T_3, T_4, or E_2. The latter hormone was an important comparator to establish the extent of crosstalk with THs. We then used novel transcriptomic resources available for *Rana [Lithobates] catesbeiana* (Hammond et al., 2017) and generated transcriptomic profiles of the OE upon hormone exposure. These analyses provided a framework for the identification of additional hormone-responsive transcripts and the generation of specific, validated RT-qPCR tools for use as bioindicators of TH disruption in aquatic systems. Finally, we used a combination of classic and additional RNA-seq-derived TH-response gene transcripts to evaluate TH-disrupting activity in effluents produced from two different municipal wastewater treatment technologies, anaerobic membrane bioreactor (AnMBR) and membrane enhanced biological phosphorous removal (MEBPR).

2. Materials and methods

2.1. Experimental animals

Rana catesbeiana tadpoles of mixed sex were caught locally near Victoria (BC, Canada) within the Thetis Lake Park area (48.4710 °N, 123.4797 °W) by Westwind Sealab Supplies under permit from the Capital Regional District Parks and Recreation. 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 (Catalog #A6762C) at a ratio of ½ spirulina tablet per animal (9 tablets/tank) on the day that they were moved inside.

2.2. Experimental exposures

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 upon 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 in Supplementary Tables 1 and 2, respectively. As the present work was part of a larger project, the same hormone stock solutions were used as in Heerema et al. (2017) for the animal exposures, although the animals used were distinct from each other. Actual hormone measurements were taken from duplicate exposure water at 0 and 48 h time points, measured by liquid chromatography/mass spectrometry, and reported previously (Heerema et al., 2017). Since the actual measured hormone concentrations were similar to nominal, the nominal concentrations will be used in the present manuscript.

2.2.2. 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). AnMBR is an embryonic secondary treatment technology that is more sustainable than commonly-used secondary treatments from an energy perspective (Chang, 2014; Liao et al., 2006). The second experiment was conducted using an example of a commonly used secondary treatment, the 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 are in a companion paper (Heerema et al., 2017) and the MEBPR system in Supplementary Fig. 1.

Each treatment type utilized municipal wastewater feed stock which was then split into two parallel treatment trains. 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 pharmaceutical and personal care product (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, aliquotted, 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 Supplementary Table 3. 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 experiments 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