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

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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 olfactory epithelium and identify further molecular bioindicators linked to behavioral response disruption.

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

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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 olfactory epithelium (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 endocrine disrupting chemicals (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. 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.

3. 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 gal 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 24 h.

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.

4. Experimental exposures

4.1. 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). 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 Supplementary Table 1. 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