

**EFFECTS OF THYROID HORMONES, THYROID HORMONE DISRUPTORS,
AND TREATED WASTEWATER ON BULLFROG TADPOLE
CHEMOSENSORY FUNCTION AND BEHAVIOUR**

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Dedication

To my parents: Thank you for supporting me through every moment of life. I love you both.

Abstract

Endocrine disrupting compounds (EDCs) are persistent in wastewater effluent and discharged into receiving waters. Some EDCs can interact with the thyroid system in vertebrates. The present study investigated the effects of TH disruption on olfactory responses in *Lithobates (Rana) catesbeiana* tadpoles. A neurophysiological and a behavioural technique were developed to study olfactory acuity in tadpoles. To investigate the effects of thyroid hormone (TH) disruption, tadpoles were exposed to either 3,5',3-triiodothyronine (T₃), thyroxine (T₄), 17β-estradiol (E₂), known EDCs, or wastewater effluent. Olfactory-mediated avoidance responses to a chemosensory stimulus were measured. Exposure to T₃ and treated wastewater resulted in impaired avoidance responses. Exposures to T₄, E₂ and known EDCs had no effect on behavioural responses. These results indicated that EDCs in wastewater effluent may contribute to olfactory impairment through TH disruption. Chemosensory-mediated behaviour may serve as an environmentally relevant endpoint to determine possible effects of TH disruption in wastewater effluent receiving waters.

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List of Symbols and Abbreviations

Symbol	Definition
E ₁	estrone
E ₂	17 β -estradiol
EE ₂	ethinyl estradiol
BPA	bisphenol A
EDC	endocrine disrupting compound
EOG	electro-olfactography
MC	medial cavity
PC	principal cavity
OB	olfactory bulb
OE	olfactory epithelium
OMP	olfactory marker protein
OR	olfactory receptor
OSN	olfactory sensory neuron
SC	sustentacular cell
SE	standard error
SEM	scanning electron microscopy
T ₃	3,5', 3-triiodothyronine
T ₄	thyroxine
TH	thyroid hormone
TK	Taylor and Kollros staging guide
TMS	tricaine methane-sulfonate
TR α	thyroid receptor alpha
TR β	thyroid receptor beta
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
UBC	University of British Columbia
UWat	University of Waterloo
VNO	vomer nasal cavity

CHAPTER 1: Introduction

Endocrine disrupting compounds (EDCs) are exogenous organic compounds that have the potential to cause adverse health effects by disruption of the normal function of the endocrine system (World Health Organisation 2012). The proper function of the endocrine system is important for vertebrates, because the endocrine system regulates growth and development (reviewed in Quanrud and Propper 2010; World Health Organisation 2012). A multitude of everyday products including pharmaceuticals and personal care products (PPCPs), flame-retardants, antimicrobials, detergents, and pesticides contain EDCs. Given their widespread use, EDCs are deposited into municipal wastewater and the wastewater undergoes treatment. Conventional wastewater treatment processes were not originally designed to remove EDCs from wastewater, and therefore are ineffective in doing so. As a result, low concentrations of EDCs have been measured in treated effluent around the world (Brun et al. 2006; Lishman et al. 2006; Ryu et al. 2011). Conventionally the efficacy of wastewater treatment processes was determined by measuring parameters such as chemical oxygen demand and microorganisms, but is now starting to include the identification and quantification of EDCs (Castillo et al. 2013). Although EDC concentrations in effluent are rarely considered in water quality guidelines, some countries are working towards doing so. For example, the United Kingdom is expected to revise their wastewater effluent quality guidelines to include regulations for some EDCs that have been identified as biologically active at low concentrations (reviewed in Petrie et al. 2015), but in Canada and USA there are no guidelines. Consequently, EDCs in treated effluent are discharged into receiving waters. Therefore, it is not surprising that many studies have reported EDCs in wastewater

effluent receiving streams around the world (Boyd et al. 2003; Benotti et al. 2009). For example, after surveying 100 streams in the USA Kolpin et al. (2002) reported that 80% contained one or more organic wastewater contaminants (some may have endocrine disrupting activity). As a result, the presence of EDCs in wastewater effluent receiving water is a growing concern in water quality today as it can affect aquatic ecosystems.

Determining the efficacy of wastewater treatment plants' removal of EDCs for the protection of aquatic health is a complex task. Simply identifying and quantifying EDCs in effluent provides no indication of the possible biological effects (reviewed in Snyder and Benotti 2010). The endocrine system naturally responds to low concentrations of endogenous hormones. Therefore, low concentrations (from nanogram to microgram per litre) of exogenous EDCs are also able to elicit biological responses from organisms inhabiting receiving waters (reviewed in Sumpter 2005; Vandenberg et al. 2012; Wojnarowicz et al. 2013). Biological activity of EDCs can negatively affect growth and development in organisms (reviewed in Sumpter, 2005). For example, Kusk et al. (2011) sampled wastewater from two Danish WWTPs and reported persistent EDCs in effluent hindered the development of the copepod, *Acartia tonsa*. Predicting the organismal effects of EDCs in effluent is challenging. Some EDCs do not follow a linear dose-response curve, but a non-monotonic dose-response curve. Therefore, toxicity data generated at higher concentrations of EDCs cannot be used to predict the responses in organisms exposed to lower concentrations (reviewed in Vandenberg et al. 2012; Wojnarowicz et al. 2014). Also, wastewater effluent is temporally variable in composition and often contains complex mixtures of EDCs (Petrie et al. 2015). Synergistic biological activity is often observed in EDC mixtures and therefore effects on aquatic organisms are difficult to quantify (reviewed in Sowers et al. 2009; Quanrud and Propper 2010; Petrie et

al. 2015). Further complicating the issue, EDCs undergo biodegradation both during the treatment process and in the environment. Biodegradation of EDCs results in transformation products that are hard to identify, but can be more toxic than their parent compounds. Overall transformation products of EDCs are not well understood, and further research is required to understand their persistence and toxicity in the aquatic environment (Petrie et al. 2015). Therefore, the effects of EDCs at environmentally relevant concentrations in receiving waters must be determined using sensitive tests in order to identify potential biological effects.

Past EDC research has focused on their effects on the reproduction system in wildlife. Many studies have reported estrogenic activity of EDCs (Waring and Harris 2005; Sowers et al. 2009; Wojnarowicz et al. 2014). For example, fish in English rivers exposed to low concentrations of estrogenic hormones including 17 β -estradiol (E₂), estrone (E₁), and ethinyl estradiol (EE₂) experienced feminization (Jobling et al. 1998). Vajda et al. (2008) reported skewed sex ratios towards females, gonadal intersex, disrupted testicular function, and disrupted ovarian development in white suckers (*Catostomus commersonii*) sampled downstream of a wastewater effluent discharge site in Boulder, Colorado.

Recently EDC research has focused on their effects on the thyroid system because some EDCs can disrupt the synthesis and secretion of thyroid hormones (THs) in vertebrates (Crofton 2008). In vertebrates proper function of the thyroid system is crucial for normal growth and development of many organs including the brain, lungs, and heart; as well as for energy homeostasis and metabolic pathways (Jugan et al. 2010). Overall the thyroid system is conserved across vertebrates (reviewed in Brown and Cai 2007). Thyroid hormone synthesis (Figure 1.1) is regulated in the hypothalamic-pituitary axis by

iodine uptake (reviewed in Kudo and Yamauchi 2005). Thyrotropin-releasing hormone (TRH) is produced in the hypothalamus and stimulates the pituitary gland to synthesize a large protein thyroid-stimulating hormone (TSH), thyrotropin. Thyrotropin binds to receptors in the thyroid gland and stimulates the synthesis and secretion of TH in the form of thyroxine (T_4). Transport proteins carry T_4 to target tissues where it is converted to 3,5',3-triiodothyronine (T_3) through deiodinase activity. Nuclear thyroid hormone receptors ($TR\alpha$ and $TR\beta$) bind to T_3 to mediate TH response elements and alter gene expression within target tissues. Circulatory concentrations of THs are controlled by a negative feedback mechanism of T_4 and TSH (Tata 2006). Some environmental toxicants interfere with the TH system at multiple levels such as blocking iodine uptake, binding to transport proteins and nuclear receptors (Figure 1; reviewed in Boas et al. 2006; Gilbert et al. 2012). For example previous research reported bisphenol A (BPA) antagonized T_3 and resulted in decreased TH-mediated gene expression, as measured in tadpoles and rats (reviewed in Crofton 2008; Gilbert et al. 2012). Hydroxylated polychlorinated biphenyls have also been reported with antagonistic TH action because they can bind to TH transport proteins and be transported directly to target tissues. This antagonistic action results in excess unbound T_4 that is then metabolized and excreted (reviewed in Heimeier and Shi 2010). Therefore there is substantial potential for the EDCs in wastewater effluent to alter the signalling and concentrations of endogenous THs.

Previous studies have reported that altered TH concentrations can have serious negative effects in wildlife, particularly in early developmental stages

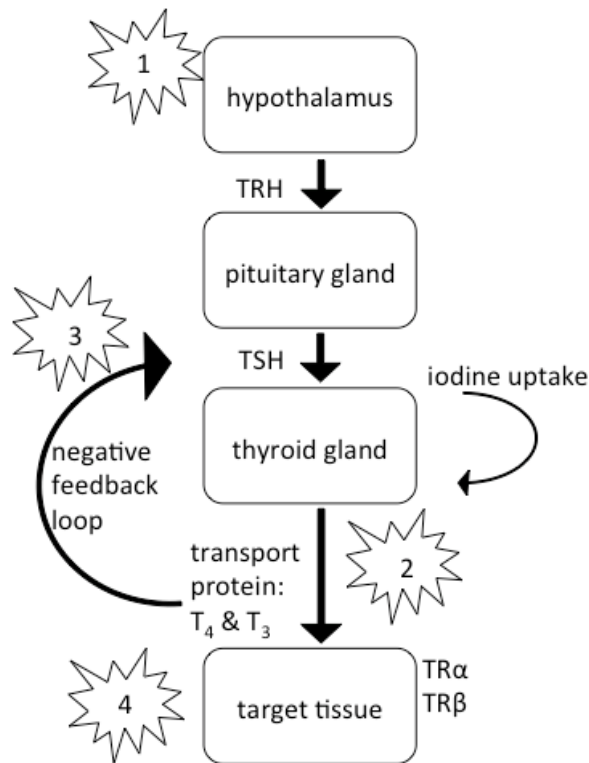


Figure 1.1-

Thyroid hormone (TH) synthesis in the hypothalamic-pituitary axis and targets of thyroid system disruption from endocrine disrupting compounds (EDCs). The hypothalamus produces thyrotropin-releasing hormone (TRH). Thyrotropin-releasing hormone stimulates the pituitary gland to produce thyrotropin (TSH), which binds to the thyroid gland. The thyroid gland uptakes iodine and synthesizes and secretes thyroid hormones (THs), thyroxine (T₄) and the more active 3,5',3-triiodothyronine (T₃) in circulation. Transport proteins carry the THs to target tissues. Thyroid hormones bind to TH receptors (TRs; TRα and TRβ) and mediate TH response elements and alter gene expression. Thyroid hormone production is regulated by a negative feedback loop between circulating TH concentrations and TSH. Endocrine disrupting compounds can interfere with the thyroid system through (1) blocking iodine uptake, (2) competitively bind to transport proteins, (3) impact the negative feedback loop between circulating TH concentrations and production of TSH, or (4) competitively bind to TRs at target tissues (adapted from Boas et al. 2006; and Gilbert et al. 2012).

(reviewed in Weiss et al. 2015). The brain is especially vulnerable to TH disruption and in mammals TH disruption has been linked to retardation and neurological effects (reviewed in Jugan et al., 2010). Other negative health effects include physical abnormalities. For example, herring gulls (*Larus argentatus*) and mummichogs (*Fundulus heteroclitus*) exhibited abnormal thyroid glands as a result of EDC exposure in the wild (reviewed in Zoeller and Rovet 2004; Gilbert et al. 2012). In laboratory studies three-spined sticklebacks (*Gasterosteus aculeatus*) exhibited altered behaviour and rodents experienced hearing loss and reduced circulatory concentrations of THs after exposure to EDCs (reviewed in Waring and Harris 2005).

Anurans have proven to be valuable sentinels for studying the effects of EDCs in aquatic environments. They undergo metamorphosis which is almost exclusively driven by THs (reviewed in World Health Organisation 2012). Three types of metamorphic changes are initiated by THs: (1) remodelling of existing organs including the intestine, skin, liver, and brain; (2) organogenesis of bone marrow, limbs, and stomach gland; and (3) apoptosis of cells and tissues that are not needed in the juvenile animal including the tail and gills (reviewed in Tata 2006; Grimaldi et al. 2013). Metamorphosis is divided into three stages with respect to TH activity (Figure 1.2). Premetamorphosis precedes thyroid function; therefore there is no TH in circulation. During prometamorphosis the thyroid gland begins secreting THs, which initiates some metamorphic changes such as limb bud growth. At metamorphic climax maximal TH concentrations are reached and extensive changes in the tadpole body occur as it transitions into the juvenile form (reviewed in Tata 2006). Given the dependency anurans have on THs for proper development, TH disruption from EDCs can have adverse morphological and behavioural

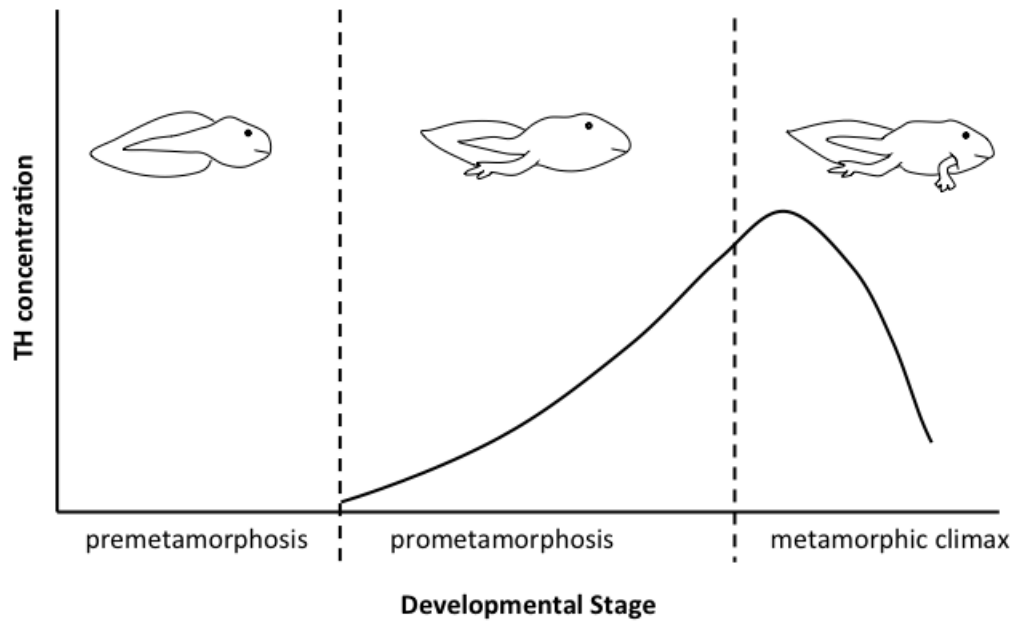


Figure 1.2 – Amphibian metamorphosis driven by endogenous thyroid hormone (TH) concentrations. During premetamorphosis there are no endogenous THs. During prometamorphosis THs begin to rise, triggering some developmental changes including hind limb bud development. At metamorphic climax THs reach their maximal concentration and trigger developmental changes including forelimb eruption and tail regression (adapted from Brown and Cai 2007).

implications. Therefore, anurans provide an excellent model to study the effects of EDCs on the thyroid system in aquatic environments (Opitz et al. 2006).

Molecular approaches have been utilized to identify effects of EDCs in anurans. Many previous studies have quantified TH dependent gene expression in anurans as a biomarker to identify effects on EDCs on TH dependent genes (Jelaso et al. 2002; Veldhoen et al. 2006; Jugan et al. 2009). For example ibuprofen, a known EDC, disrupted gene expression in the liver and tail fin of North American bullfrog (*Lithobates (Rana) catesbeiana*) tadpoles (Wojnarowicz et al. 2014). Physiological endpoints have also been used to study the effects of EDCs on the thyroid system in tadpoles. Exposure to butachlor increased whole-body TH concentrations in African clawed frog (*Xenopus laevis*) tadpoles (Li et al. 2016). Effects on time to metamorphosis have been reported in northern leopard frog (*Rana pipiens*) and *L. catesbeiana* tadpoles and *X. laevis* (Sowers et al. 2009; Veldhoen et al. 2014) as well as effects on mass (Veldhoen et al. 2006; Sowers et al. 2009; Searcy et al. 2012). Given that in tadpoles changes in these endpoints are mediated by THs, these effects are indicative of the agonistic or antagonistic action of EDCs on the thyroid system.

Although effects of TH disruption on molecular and physiological endpoints have been identified, these are not indicative of possible implications to ecological health. However, behavioural endpoints can provide a clearer picture of the ecological effects of TH disruption. To date few studies have investigated the effects of TH disruption on tadpole behaviour. Studies that have measured tadpole behaviour reported effects of EDCs on behavioural endpoints including startle response, tactile responsiveness, and activity in various tadpole species (Fraker and Smith 2004; Smith and Burgett 2005).

However, the effects of TH disruption specifically on behavioural endpoints have not been studied.

Another approach to studying the ecologically relevant effects of EDCs and other toxicants is to measure olfactory responses to chemical stimuli in organisms. Amino acids, gonadal steroids, bile acids, and prostaglandins are sensed by organisms at low concentrations and inform about potential food, mates, and predation threats (Scott and Sloman 2004; Waring and Harris 2005). To study the effects of toxicants on olfaction, previous studies have used electro-olfactography (EOG) (reviewed in Tierney et al. 2010). Electro-olfactography is a neurophysiological technique that measures the extracellular field potential generated at the olfactory epithelium during olfaction. Previous studies have reported reduced EOG responses to chemical stimuli after exposure to toxicants in fish (reviewed in Lurling and Scheffer 2007), but no studies to date have used EOG on tadpoles. Another approach to study the effects of toxicants on olfaction is the use of olfactory-mediated behaviour endpoints. For example, reduced anti-predator responses to alarm stimuli have been measured after exposure to toxicants in both fish (Moore and Waring 2001; Azizishirazi et al. 2014; Dew et al. 2014; Ehram et al. 2016) and tadpoles (Ferrari et al. 2008). It is important that organisms are able to respond appropriately to chemical stimuli, as this response is essential for their survival (Rohr et al. 2009; Troyer and Turner 2015). Therefore, olfaction-based endpoints have proven to be ecologically relevant endpoints to detect the effects of toxicants.

In the case of TH disruption, effects on olfaction in tadpoles provide a sensitive and relevant endpoint. In tadpoles THs regulate major remodelling of the olfactory system during metamorphosis (Figure 1.3; reviewed in Dittrich et al. 2016). In general, the tadpole olfactory system comprises one olfactory pit and is equipped to detect aquatic

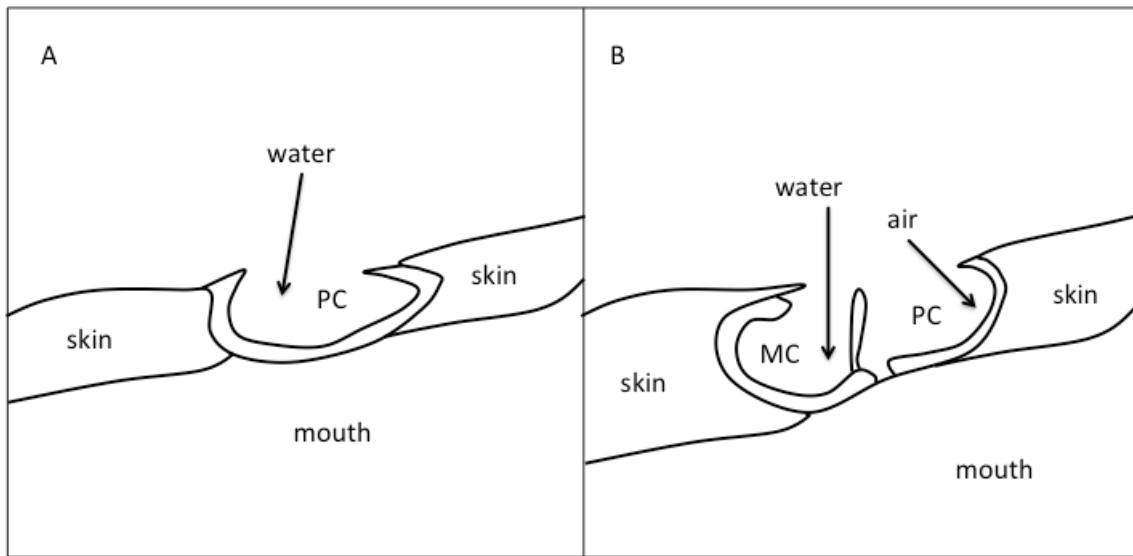


Figure 1.3 –

Transformation of tadpole olfactory system during metamorphosis. (A) The tadpole olfactory system has one olfactory pit (principal cavity; PC) that is equipped to detect aquatic stimuli. (B) A second olfactory pit arises during metamorphosis. One olfactory pit functions to detect aquatic olfactory stimuli (medial cavity; MC) and the second pit detects aerial olfactory stimuli (PC) (adapted from Gascuel and Amano 2013).

stimuli. During metamorphosis a second olfactory pit arises and the olfactory system detects both aquatic and aerial stimuli (reviewed in Gascuel and Amano 2013). Additionally, there are substantial changes in the olfactory bulb during metamorphosis such as developing olfactory bulb (OB) layers and increasing axonal growth (Higgs and Burd 2001). Considering these major developmental changes that are driven by TH, there is potential that TH disruption will disrupt the development and function of the olfactory system in tadpoles. Changes in behaviour or olfaction can have serious ecological implications to tadpole populations because their survival is dependent on their ability to successfully locate food and avoid predators. Therefore, behavioural and olfactory effects could provide a clearer picture of the effects of EDCs on the thyroid system in an ecologically relevant context.

Thesis Project

This thesis investigates the effects of THs and EDCs on olfactory acuity in premetamorphic *L. catesbeiana* tadpoles in a controlled laboratory experiment. Conducting this experiment in a laboratory provides greater control than a field study over potential external influences including temperature, sunlight, and developmental tadpole stages. Premetamorphic *L. catesbeiana* tadpoles were chosen because they are a relevant species to North America and have a long larval period of approximately two years. Additionally, the use of premetamorphic tadpoles ensures a baseline of no endogenous THs.

The objective of this research was to characterize and compare the effects of THs, EDCs and wastewater effluent exposure on olfactory acuity in premetamorphic *L. catesbeiana* tadpoles. To meet these objectives I will:

- 1) Develop techniques to measure olfactory acuity in tadpoles
- 2) Measure olfactory acuity after exposure to THs, EDCs, and wastewater.

During preliminary experiments techniques will be developed to measure olfactory acuity in tadpoles. Specifically, an existing EOG methodology developed for fish will be adapted for use with *L. catesbeiana* tadpoles. A behavioural assay will also be developed to test changes in olfactory-mediated behaviour. In separate experiments, tadpoles will be exposed to one of T₃, T₄, E₂, and EDC cocktail, or wastewater effluent and changes to olfactory acuity and TH-mediated molecular responses will be investigated.

CHAPTER 2: Electro-olfactogram of the North American bullfrog (*Lithobates (Rana) catesbeiana*) tadpole

Introduction

In aquatic environments complex mixtures of chemical stimuli play a vital role in an organism's ability to gain information from its surroundings regarding mates, food, and predators (reviewed in Laberge and Hara 2001; Ache and Young 2005; Kaupp 2010). Larval amphibians are no exception, and previous studies have demonstrated that olfaction of chemical stimuli can result in specific behavioural responses to food and predator stimulus, such as attraction, refuge use, and altered activity (Ferrari et al. 2007; Garcia et al. 2012).

The structure and function of the tadpole olfactory system has been well documented in *Xenopus laevis* tadpoles, and overall is conserved across anuran species (Ache and Young 2005; Gascuel and Amano 2013). Olfactory epithelium lines paired olfactory pits, which are exposed to the outside environment via nares. Three cell types comprise the olfactory epithelium including sustentacular cells (SCs), which provide support; basal cells, which enable the epithelium to regenerate; and bipolar olfactory sensory neurons (OSNs), which mediate odour detection (Hansen et al. 1998; Manzini and Schild 2010). Ciliated or microvillous OSNs are projected onto the apical surface of the olfactory epithelium. Each OSN expresses one type of odour receptor (OR) that is specified for particular odorants, and together the OSNs expressing the same ORs comprise a class of OSNs (reviewed in Manzini and Schild 2010). Odorants bind to ORs and trigger a molecular signal transduction cascade, which opens gated ion channels and results in an influx of extracellular cations causing a bulk depolarization of OSNs as

action potentials are propagated to the brain for information processing (reviewed in Ache and Young 2005; Gascuel and Amano 2013).

Electro-olfactography (EOG) is a neurophysiological technique that measures the odour-evoked extracellular field potential from the surface of the olfactory epithelium. The magnitude of the resultant depolarization is indicative of olfactory acuity (reviewed in Scott and Scott-Johnson 2002). This technique has been developed for use with many different organisms including salamanders (Van As et al. 1985), daphniids (Simbeya et al. 2012), adult bullfrogs (Ottoson 1956), and has been utilized most extensively with various fish species (Byrd Jr and Caprio 1982; Green et al. 2010; Azizishirazi et al. 2013; Dew et al. 2014). Electro-olfactography is often used in aquatic toxicology studies to measure the effects of toxicants on olfactory acuity in fish. For example, Azizishirazi et al. (2013) reported reduced EOG responses to olfactory stimuli in *Perca flavescens* after exposure to metal-contaminated water. Many toxicological studies use tadpoles as sentinels and study endpoints including growth, morphology, time to metamorphosis, and survival are commonly measured (reviewed in Fraker and Smith 2004; Hopkins 2007; Ruiz et al. 2010). However, there is currently no EOG methodology for tadpoles. Olfactory acuity (as measured by EOG) is an ecologically relevant endpoint because it has direct implications on the survival of organisms. Therefore, development of a tadpole EOG method would provide a useful endpoint to further study the effects of toxicants on tadpoles.

The objective of the present study was to develop an EOG method to measure olfactory acuity in *L. catesbeiana* tadpoles in response to standard olfactory stimuli. Tadpole EOG is a novel technique that measures olfactory responses of larval individuals to an olfactory stimulus. Here, we describe a modified EOG method for use with bullfrog

tadpoles and establish tadpole olfactory responses to L-alanine and *Spirulina* spp. extract. We also investigate tadpole responses to various other standard olfactory stimuli, as measured by EOG.

Materials and Methods

Experimental animals

Lithobates (Rana) catesbeiana tadpoles were wild caught in Victoria, British Columbia and housed in a re-circulatory system at 15°C on a 16 h: 8 h light: dark photoperiod in the University of Lethbridge Aquatic Research Facility. Tadpoles were fed *Spirulina* flakes *ad libitum* daily. All procedures involving tadpoles were reviewed and approved by the Animal Welfare Committee at the University of Lethbridge for compliance under the Canadian Council on Animal Care guidelines. Tadpoles were staged according to Taylor and Kollros (1946) (TK; Appendix A). As demonstrated by Heerema et al. (Chapter 3), *L. catesbeiana* tadpoles must be \geq TK IX for successful EOG measurements.

Electro-olfactography (EOG)

The experimental set-up used was modified from standard techniques developed for fish and *Daphnia* spp., as described by Green et al. (2010) and Simbeya et al. (2012), respectively. Borosilicate glass electrodes (0.5 μ m pore diameter) were pulled from 7.62 cm capillary tubes (World Precision Instruments, Inc., Sarasota, FL, USA) using a micropipette puller (Model PMP-102, MicroData Instruments, Inc., South Plainfield, NJ, USA). Electrodes were filled with a 4% gelatin and 0.9% NaCl solution, and submerged in a 3 M KCl solution and stored at 4 °C until needed. The day the experiment started,

two microelectrode holder half-cells (World Precision Instruments, Inc.) were filled with 3 M KCl and electrodes were inserted into the half-cell. Tadpoles were anesthetised in buffered tricaine methane-sulfonate (TMS; 500 mg/L; Aqua Life, Syndel Laboratories, Nanaimo, BC, Canada), until their heart rate slowed to approximately one beat per two seconds (measured visually). In contrast to EOG with fish, where the nasal septum is removed to expose the olfactory rosette (Green et al. 2010), tadpole EOG is more invasive. To expose the olfactory pit, the left naris was snipped inferiorly and superiorly. The two initial snips were then connected medially, making a small “C” shaped incision and the skin from the incision site was removed to expose the olfactory pit (Figure 2.1 A). The tadpole was wrapped in damp paper towel to prevent desiccation, with the head and tail exposed. Inside a Faraday cage, the tadpole was placed in a holding trough and a grounding clip was attached to the tail to reduce electrical interference. To allow for proper probe placement, the exposed olfactory pit was angled towards the researcher in the trough. The recording electrode was micromanipulated into the olfactory pit, positioning it medially in the pit, but not touching the olfactory epithelium. The reference probe was micromanipulated inferior to the exposed olfactory pit, touching the skin (Figure 2.1 B). An irrigation line was micromanipulated onto the olfactory pit so that it was not touching any electrodes, but supplied the olfactory pit with dechlorinated water without forming droplets. The difference between the recording electrode and the reference electrode was measured at a sampling rate of 20 samples per second. The signal was amplified by a DC headstage and a differential amplifier (settings: high pass 0.1, low pass 100, gain 10; DP-311, Warner Instruments), and recorded using Lab Chart & Scope 5 (ADInstruments, Sydney, AUS) software on a Windows desktop computer.

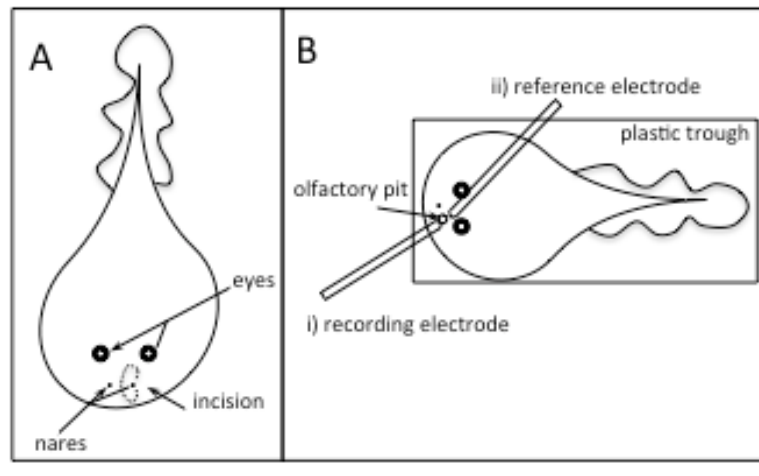


Figure 2.1 –

Preparation of *Lithobates (Rana) catesbeiana* tadpoles for EOG and electrode placement. A: To prepare bullfrog tadpoles for EOG, the olfactory pit was exposed. The dashed line signifies the shape of the incision, where the skin is then removed. B: The tadpole is placed in a plastic trough, with the exposed olfactory pit angled towards the researcher. Probe placement: (i) recording electrode is lowered into the olfactory pit, not touching the olfactory epithelium; (ii) reference electrode is positioned posteriorly to the olfactory pit, touching the skin.

Once a stable baseline was obtained, the test began. Olfactory stimuli were added into the olfactory pit irrigation line via a switch-controlled valve box system (VC-6 Six Channel Valve Controller, Warner Instruments) in pulses of five seconds. The raw EOG response was determined by measuring the difference between the baseline signal and the response to the stimulus. The stimulus delivery order was fully randomized, and a minimum of two minutes was allowed to pass between each stimulus pulse to mitigate against olfactory fatigue. Each stimulus was delivered three times throughout the duration of the test. Across each individual, mean EOG responses were blank corrected by subtracting any response to the blank. At the end of each test, the tadpole was euthanized with buffered TMS (1000 mg/L).

Responses to various olfactory stimuli

To identify appropriate olfactory stimuli for tadpoles, preliminary experiments were completed to test EOG responses to the following stimuli: 1) 10^{-2} M L-alanine (Bio-ultra, Sigma Aldrich, Oakville, ON, Canada), 2) 10^{-2} M L-serine (USP grade, Sigma Aldrich), 3) 10^{-2} M glycine (proteomics grade, Amresco, Solon, OH, USA), 4) 10^{-2} M L-histidine (98 %, Acros Organics, Waltham, Massachusetts, USA), 5) 10^{-2} M L-lysine (99 %, Acros Organics), 6) 10^{-2} M taurocholic acid (TCA; ≥ 95 %, Sigma Aldrich), and 7) 2 g/L *Spirulina* extract. Additionally all olfactory stimuli were tested on a dead individual to ensure the stimulus itself was not generating an electrical charge that would mimic a response.

Based on EOG responses to the above-mentioned stimuli, L-alanine and *Spirulina* extract were selected as appropriate olfactory stimuli. To determine olfactory acuity to these standard olfactory stimuli, EOG responses to *Spirulina* extract (2 g/L) and L-alanine

(10^{-2} M) from tadpoles TK XII – XVI (mean \pm SE; mass 6.1 ± 0.3 g, body length 9.8 ± 0.1 cm, n = 5) were characterized.

Spirulina extract was used to characterize concentration-dependent EOG responses. Electro-olfactography responses to graded concentrations of *Spirulina* extract (0.5, 1.0, and 2.0 g/L) from tadpoles TK XV – XVIII (mean \pm SE; mass 25.1 ± 2.3 g, body length 13.9 ± 0.4 cm, n = 4) were measured.

All stimuli were prepared in dechlorinated water. To prepare the *Spirulina* extract, *Spirulina* flakes (Nutrafin Max, Hagen, Montreal, QB, Canada) were stirred for 30 minutes in dechlorinated municipal water (mean \pm SE; alkalinity 136 ± 3 mg/L as CaCO₃; hardness 172 ± 2 mg/L as CaCO₃; median, range; pH 8.29, 8.11 – 8.54) and filtered through filter wool (Aqua-Fit, Hagen).

Statistical analyses

All analyses were conducted using R version 3.2.2 (R Core Team 2015). For each individual blank corrected EOG responses were calculated by subtracting responses to the blank from responses to the olfactory stimuli. Mean blank-corrected EOG responses were calculated. Parametric assumptions were tested using the Shapiro-Wilk normality test and the Bartlett test for homogeneity of variance on the mean responses to stimuli. Blank-corrected mean EOG responses to *Spirulina* extract and L-alanine were compared to the blank-corrected responses to the blank with a one-sample t-test. P-values were corrected with a Bonferoni correction for multiple comparisons. Mean blank-corrected responses to graded *Spirulina* extract concentrations did not satisfy parametric assumptions and therefore were compared using a permutation one-way analysis of variance (ANOVA) (Legendre 2007) followed by a nonparametric relative contrast effects post hoc analysis

using the ‘nparcomp’ package (Konietschke et al. 2015). Mean differences were considered to be significant when $p \leq 0.05$.

Results

In preliminary experiments, tadpole responses to various olfactory stimuli were measured using a modified EOG method, and are summarized in Table 2.1. L-alanine and *Spirulina* extract elicited consistent and repeatable responses. L-serine evoked responses from some tadpoles, but responses were unreliable across individuals. The remaining stimuli tested were unsuccessful in evoking measurable EOG responses in tadpoles. As measured by EOG on a dead individual, none of the olfactory stimuli tested produced an electrical charge that would mimic response.

L-alanine and *Spirulina* extract elicited consistent and reliable EOG responses from tadpoles (Figure 2.2). Mean EOG responses to *Spirulina* extract were 10 mV higher than responses to the blank ($t_4 = 3.7$, $p = 0.04$). Responses to L-alanine however, were not significantly different from 0 ($t_4 = 2.1$, $p = 0.18$). Tadpoles exhibited concentration-dependent EOG responses to *Spirulina* extract (Figure 2.3). The magnitude of EOG responses to 2.0 g/L *Spirulina* extract were dramatically greater than EOG responses to 0.5 and 1.0 g/L *Spirulina* extract ($F_{(2, 9)} = 28.34$, $p < 0.001$). However, there was no difference in the magnitude of responses between 0.5 and 1.0 g/L of *Spirulina* extract.

Discussion

In the present study we developed an EOG method for use with *L. catesbeiana* tadpoles and established EOG responses to standard olfactory stimuli. Tadpoles exhibited consistent EOG responses to *Spirulina* extract and L-alanine (Figure 2.2).

Table 2.1 –
 Olfactory stimuli, including a number of amino acids, tested on *Lithobates (Rana)*
catesbeiana tadpoles for electro-olfactography (EOG) responses.

Stimulus	Response
L-alanine	Yes
L-serine	Variable
Glycine	No
L-histidine	No
L-lysine	No
TCA	No
<i>Spirulina</i>	Yes

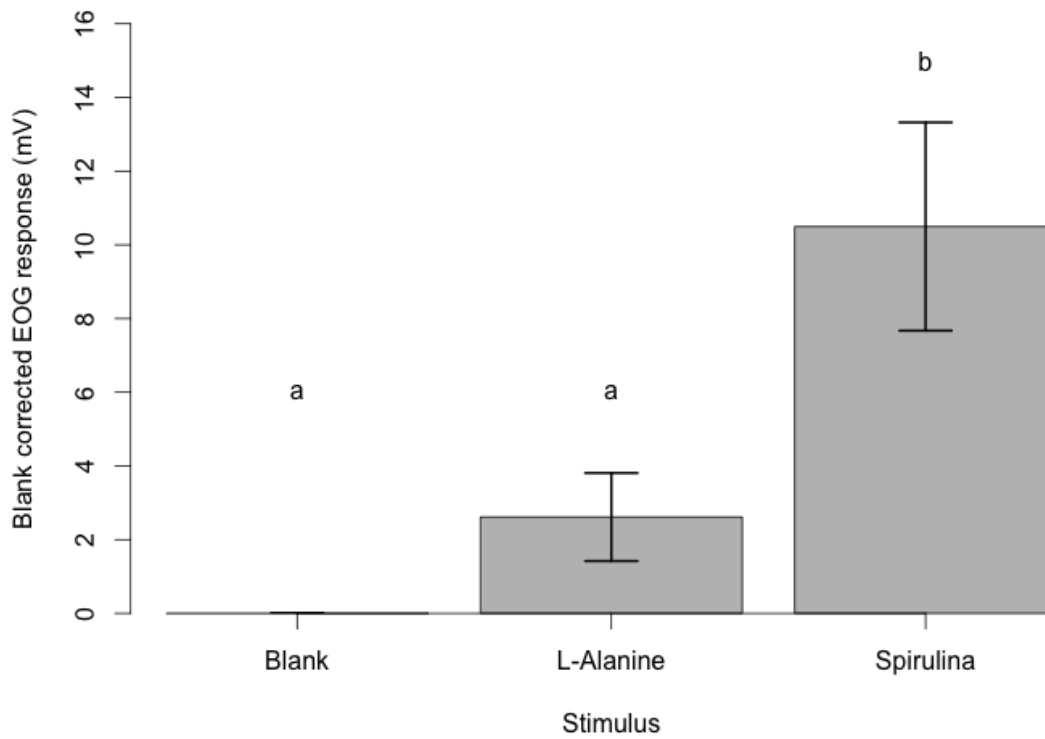


Figure 2.2 – Blank corrected EOG responses (mean \pm standard error) of *Lithobates (Rana) catesbeiana* tadpoles to the blank (dechlorinated water), 10^{-2} M L-alanine and 2 g/L *Spirulina* extract. Different letters above bars indicate responses to olfactory stimuli were significantly greater than 0 ($p \leq 0.05$), $n = 5$.

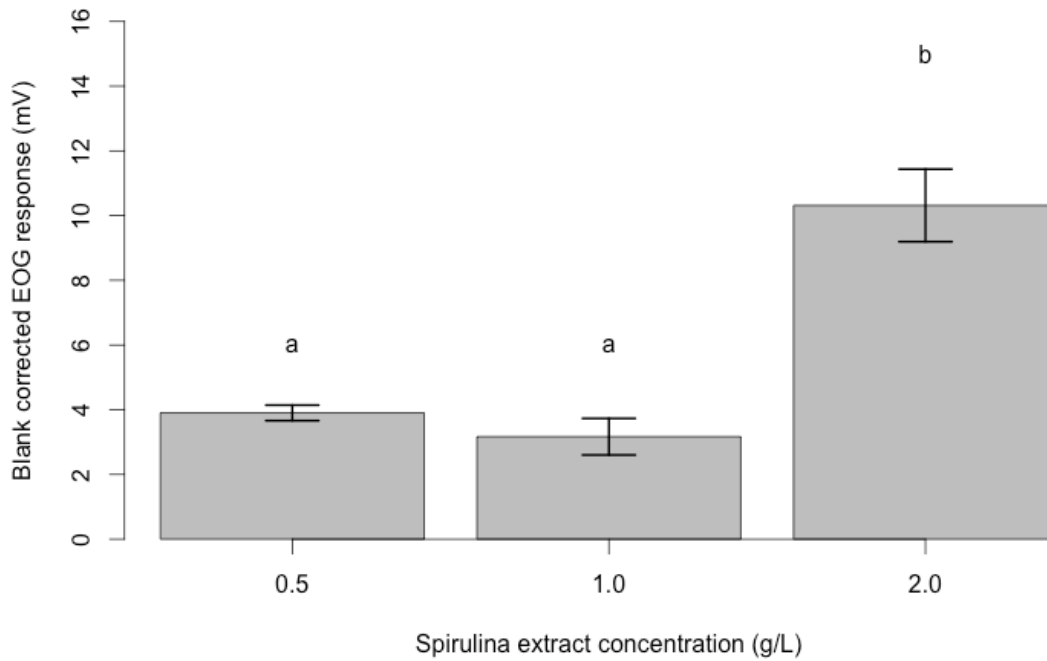


Figure 2.3 –
Blank corrected electro-olfactography (EOG) responses (mean \pm standard error) of *Lithobates (Rana) catesbeiana* tadpoles to a concentration series of *Spirulina* extract (0.5, 1.0, 2.0 g/L). Letters above bars indicate significant differences in EOG responses among *Spirulina* concentrations ($p \leq 0.05$), $n = 4$.

Electro-olfactography responses to L-alanine and *Spirulina* extract are supported by previous studies that have also measured olfactory responses to these stimuli. It is widely accepted that amino acids represent food stimuli and previous studies have used them as olfactory stimuli in EOG experiments with fish (Azizishirazi et al. 2013) and in electrophysiological studies with tadpoles (Vogler and Schild 1999; Hassenklover et al. 2012). Electrophysiology techniques such as calcium imaging and patch-clamp have measured OSN responses to L-alanine in *X. laevis* tadpoles (Vogler and Schild 1999; Manzini et al. 2002). *Spirulina* has also been previously used to elicit olfactory responses in electrophysiology studies with *X. laevis* tadpoles (Manzini et al. 2002). Given that tadpoles are opportunistic feeders and consume detritus and organic matter (Altig et al. 2007), *Spirulina* extract is a good representation of a food stimulus. Therefore, L-alanine and *Spirulina* extract both represent relevant and effective olfactory stimuli for EOG with *L. catesbeiana* tadpoles, as evidenced by EOG responses measured in the present study.

Apart from L-alanine, other amino acids tested in the present study were unsuccessful in evoking measurable EOG responses from *L. catesbeiana* tadpoles (Table 2.1). This result was unexpected given that these amino acids have been used previously as olfactory stimuli for *X. laevis* tadpoles in electrophysiology experiments (Hassenklover et al. 2012). Taurocholic acid is a bile salt that generally represents a social cue, and previous fish studies have reported EOG responses to it (reviewed in Hamdani and Døving 2007). However no studies have reported that bile salt is an effective olfactory stimulus for tadpoles.

An EOG technique for use on *L. catesbeiana* tadpoles introduces the potential to study olfactory physiology in intact tadpoles. In comparison, patch-clamp and calcium imaging electrophysiology techniques require tadpoles to be euthanized and the olfactory

epithelium isolated. Given that the tadpole olfactory system is considerably remodelled during metamorphosis (reviewed in Gascuel and Amano 2013), tadpole EOG provides an opportunity to study olfaction on a larval organism, and the ontogeny of olfaction through development. Additionally, there is potential to use tadpole EOG to study the effects of toxicants on tadpole olfaction from a neurophysiological standpoint, as with previous fish studies (Dew et al. 2014). With tadpoles, previous studies have investigated the effects of toxicants, such as wastewater contaminants and pesticides, on chemosensory-mediated behavioural endpoints (Fraker and Smith 2004; Smith and Burgett 2005; Hanlon and Relyea 2013), and few have used behavioural endpoints as a measure of olfactory acuity (Lefcort et al. 1998; Troyer and Turner 2015).

To conclude, we have developed an EOG method for use with *L. catesbeiana* tadpoles and tested responses to various amino acids and other chemosensory stimuli. Based on our findings, L-alanine and *Spirulina* extract are appropriate stimuli for tadpole EOG, and produce consistent and reliable responses.

CHAPTER 3: Ontogenetic changes in chemosensory structures and associated acuity in North American bullfrog (*Lithobates (Rana) catesbeiana*) tadpoles

Introduction

The postembryonic development of anurans includes the metamorphosis of the free-living aquatic, herbivorous tadpole to a (semi-) terrestrial, carnivorous frog (Gilbert et al. 1996). During metamorphosis, there are major developmental changes in the anuran olfactory system as it shifts from functioning in water environments alone in the larval stage to functioning primarily in terrestrial environments in the adult life stage (reviewed in Gascuel and Amano 2013). These metamorphic changes may affect chemosensory acuity in larval anurans.

The olfactory epithelium, the portion of the olfactory system exposed to the environment, is comprised of three cell types: sustentacular cells (SCs), which provide support for the epithelium and remove dead neurons (reviewed in Schwob 2002); basal cells, which are involved in the regeneration of the epithelium; and olfactory sensory neurons (OSNs), which function to detect odours (reviewed in Manzini and Schild 2010). The OSNs are bipolar and project ciliated or microvillous olfactory receptors (ORs) on the apical surface of the olfactory epithelium (reviewed in Manzini and Schild 2010). The development of the *Xenopus laevis* (African clawed frog) olfactory system throughout metamorphosis has been well studied, while other species remain less well documented (Gascuel and Amano 2013). However, it is important to note that *Xenopus* are unique because although they become air-breathing as frogs, they remain aquatic rather than emerge on land as the majority of anurans do. Therefore there are likely inter-species differences in structural and cellular aspects of olfactory systems.

Generally, the olfactory epithelium originates as two placodes, each giving rise to an olfactory pit. Each olfactory pit differentiates into a principal cavity (PC) and vomeronasal organ (VNO). In the tadpole, the olfactory epithelium lies within the PC and expresses both ciliated OSNs and microvillous OSNs, which are specialized to detect airborne odorants and aquatic odorants, respectively. Despite the presence of both types of OSNs, the tadpole PC detects only waterborne odorants (Hansen et al. 1998). The VNO contains microvillus OSNs and is involved in the detection of pheromones (reviewed in Døving and Trotier 1998). As development proceeds towards metamorphosis, the PC gives rise to a medial cavity (MC; Figure 1.3).

After metamorphosis, the PC expresses only ciliated OSNs (Mezler et al. 1999) and is specialized to detect volatile odours, which are typical of a terrestrial environment (Higgs and Burd 2001; Gascuel and Amano 2013). In most species, the MC contains only non-sensory epithelium, with the exception of *X. laevis*. In the case of *X. laevis*, the adult MC resembles the larval PC, and expresses both microvillous OSNs and ciliated OSNs, and functions to detect odours in aquatic environments (Hansen et al. 1998; Wang et al. 2008; Gascuel and Amano 2013; Dittrich et al. 2016). The frog VNO does not change from the tadpole form, and continues to express only microvillous OSNs (Taniguchi et al. 1996; Hansen et al. 1998).

The development of the olfactory system and many other tissues during metamorphosis is driven by thyroid hormones (THs) thyroxine (T_4) and 3,3',5-triiodothyronine (T_3) (Brown and Cai 2007). Metamorphic changes include cell apoptosis resulting in tail resorption and loss of gills; organogenesis of limbs, stomach, and bone marrow; and the complete remodelling of the intestine, brain, and spinal cord (Brown and Cai 2007). Metamorphosis is divided into three stages with respect to endogenous TH.

During premetamorphosis the thyroid gland is inactive and no TH circulates through the anuran body. During prometamorphosis the thyroid gland becomes active and begins secreting TH and initiating metamorphic changes. At metamorphic climax, endogenous TH is at its highest concentration, triggering extensive changes and remodelling throughout the anuran body (Tata 2006; Brown and Cai 2007). Given the substantial changes that occur in the tadpole olfactory system during development, changes in chemosensory function are expected. However, very little is known about the nature of these changes at a structural and functional level in frogs that more completely transition to a terrestrial existence such as the North American bullfrog, *Lithobates (Rana) catesbeiana*.

Previous studies have utilized chemosensory cues to evoke tadpole behavioural responses in a variety of species using many different assays (Mirza et al. 2006; Smith et al. 2008; Takahara et al. 2012). Another approach to studying chemosensation is to measure the odour-evoked extracellular field potentials using electro-olfactography (EOG). Odour molecules interact with surface receptors in the olfactory epithelium triggering a molecular signal transduction cascade that leads to bulk depolarization of the olfactory sensory neurons (OSNs). The EOG measures the loss of cations from the extracellular environment resulting from this bulk depolarization. The amplitude of the resulting electro-olfactogram is related to olfactory acuity. Previous studies have used EOG to measure chemosensory acuity in fish (Green et al. 2010; Dew et al. 2014), and the technique has recently been modified and developed for use with *L. catesbeiana* tadpoles (Chapter 2).

The purpose of the present study was to investigate ontogenetic changes in the chemosensory system, as measured in the ultrastructure of the olfactory epithelium and

chemosensory responses of *L. catesbeiana* tadpoles. The present study was conducted in two parts. Part one aimed to investigate changes in chemosensory acuity in premetamorphic *L. catesbeiana* tadpoles. We used EOG to examine the progression of measurable chemosensory responses to standard food cues, *Spirulina* spp. and L-alanine. The structural development of the olfactory tissues from these individuals was examined using scanning electron microscopy (SEM). To further investigate chemosensory function during premetamorphosis, tadpoles were rendered anosmic and attraction responses were measured with a behavioural assay. Part two of the present study compared behavioural and neurophysiological (EOG) responses to *Spirulina* extract in early and late prometamorphic and metamorphic *L. catesbeiana* tadpoles. Responses to L-alanine were also measured with EOG. Additionally, the surface anatomy of the olfactory tissues from these individuals was examined using SEM.

Materials and Methods

Experimental animals

Lithobates (Rana) catesbeiana tadpoles were wild caught in Victoria, British Columbia, Canada, and housed on a re-circulatory system in the Aquatic Research Facility (ARF) at the University of Lethbridge, AB, Canada. Tadpoles were housed at 15°C on a light: dark 16:8 h photoperiod and fed daily *Spirulina* flakes *ad libitum*. Prior to experiments tadpoles were fasted and temperature-acclimated to 24 °C for 72 hours in aerated 15 L polypropylene buckets. During the temperature acclimation water quality including temperature (mean, range; 24.2, 21.4 – 25.0 °C, n = 16), dissolved oxygen (mean, range; 6.7, 4.1 – 7.9 mg/L, n = 15) was measured daily and pH (median, range; 8.27, 7.79 – 8.39, n = 9) every other day. Hardness (mean, range; 178, 175 - 185 mg/L as

CaCO₃, n = 30) and alkalinity (mean, range; 136, 130 – 145 mg/L as CaCO₃, n = 30) are routinely measured in the ARF and are stable through time. All procedures involving tadpoles were approved by the Animal Welfare Committee at the University of Lethbridge and the University of Victoria Animal Care Committee, for compliance under the Canadian Council on Animal Care guidelines.

Tadpoles were staged according to (Taylor and Kollros 1946; TK; Appendix A) and will be referred to by developmental group for ease of comparison according to Table 3.1.

Stimulus preparation

For both behavioural and EOG experiments all chemosensory stimuli were prepared fresh daily. To prepare the *Spirulina* extract for behavioural and EOG experiments, *Spirulina* flakes (2 g/L; Nutrafin Max, Hagen, Montreal, QC, Canada) were stirred into dechlorinated water for 30 minutes and filtered with aquarium filter wool (Aqua-Fit, Hagen). For EOG experiments, L-alanine (USP grade, Sigma-Aldrich, Oakville, ON, Canada) was prepared in dechlorinated water. To prepare the amino acid mixture for the anosmic behavioural experiment, equal molarity (0.022 M) L-alanine (USP grade, Sigma-Aldrich), L-serine (USP grade, VWR, Radnor, PA, USA), and glycine (proteomics grade, AMRESCO, Cleveland, OH, USA) were stirred into dechlorinated water.

Table 3.1 –
Classification of Taylor and Kollros (1946; TK) into developmental groups in the present study.

TK Stages	Developmental Group
I - X	Premetamorphic
XI - XVI	Early prometamorphic
XVII	Late prometamorphic
XVIII - XX	Metamorphic climax

Premetamorphic and early prometamorphic tadpole chemosensory acuity and olfactory epithelium surface anatomy

Methodology for *L. catesbeiana* tadpole EOG as described in Chapter 2 was used to measure the olfactory responses to 2 g/L *Spirulina* extract and 10^{-2} M L-alanine of premetamorphic and early prometamorphic tadpoles (Table 2.1). Tadpoles (mean \pm 1 SE; body mass: 7.0 ± 0.4 g; median, range: TK VI, I – XIII, n = 7) were anaesthetized in buffered tricaine methanesulfonate (500 mg/L; TMS, Aqua Life, Syndel Laboratories, Nanaimo, BC, Canada) until the heartbeat slowed to approximately one beat every two seconds. Chemosensory stimuli were delivered to the exposed olfactory pit in pulses of five seconds and a minimum of two minutes was allowed to pass between each stimulus delivery to prevent habituation. For each tadpole tested the delivery order of the cues was randomized and responses to each cue were measured three times throughout the EOG test. L-alanine and *Spirulina* have been established as appropriate chemosensory stimuli for tadpoles and have been used for other electrophysiological techniques, including patch-clamp and calcium imaging (Manzini et al. 2002; Hassenklover et al. 2012). Responses to each stimulus were averaged for each individual. Mean EOG responses to chemosensory stimuli were blank corrected by subtracting any response elicited by the blank across each individual.

Immediately following EOG experiments, tadpoles were euthanized in buffered TMS (1000 mg/L) solution. Olfactory pits were collected and fixed by immersion in Karnovsky's fixative (Karnovsky 1965) for 24 hours at room temperature. Fixed tissues were successively rinsed twice in cacodylate buffer (0.2 M; pH 7.2; Ted Pella Inc., Redding, CA, USA) for 15 minutes each, and dehydrated in a series of graded ethanol dilutions. Tissues were dried and mounted on 15 mm aluminum stubs, sputter coated with

platinum, and observed with an SEM (S-3400N SEM, Hitachi, Tokyo, Japan) at the Lethbridge Research and Development Centre, Lethbridge, AB, Canada.

Induced anosmia

To determine whether olfaction is used to behaviourally respond to chemical stimuli at early stages, tadpoles (mean \pm 1 SE; body mass 4.8 ± 0.4 g; median, range; TK III/IV, I – IIX, n = 23; Table 3.1) were rendered anosmic and their response to a chemosensory stimulus was tested in a choice maze. To induce anosmia, tadpoles were anaesthetised in buffered TMS (500 mg/L) and the olfactory pits were exposed in the same manner used for tadpole EOG (Chapter 2). Surgical glue (Vetbond, 3M Canada, London, ON, Canada) was applied to the olfactory pits, completely capping them, and allowed to dry for approximately 30 seconds. The tadpoles were placed in dechlorinated water and allowed to recover for 48 hours before running the experiment. Subsequently, behavioural responses to a chemosensory stimulus were measured. To measure behavioural responses, a previously described experimental protocol from Dew et al. (2014) was modified as follows. Linear troughs (75 x 20 x 15 cm) were divided into three zones (by length). The two distal ends received chemosensory stimuli (stimulus-delivery zones) while the middle section served as the acclimation zone. A clear plastic bottomless container (21.5 x 15 x 11.5 cm) was installed in the acclimation zone of the maze and was used as an acclimation chamber where tadpoles could adjust to maze conditions while being prevented access to either of the distal reaches of the maze. For each trial, a single tadpole was placed in the acclimation chamber and allowed to acclimate to maze conditions for 20 minutes prior to the start of the choice assay. To each stimulus-delivery zone, 50 mL of blank (dechlorinated water) or stimulus (mixture of L-alanine, L-serine, glycine, 0.022

M for each amino acid) were remotely administered via silicone tubing and syringes. The assignment of the blank or chemosensory stimulus to one end of the maze or other was randomized for each successive trial. Once administered to the maze the cue and blank were allowed to diffuse for one minute before the acclimation chamber was remotely lifted via an overhead pulley system. The lifted acclimation chambers allowed the tadpole to swim freely throughout the maze. All trials were recorded with a webcam (HD 720p, Logitech, Romanel-sur-Morges, Switzerland) and viewed via a laptop computer (MacBook Air, Apple, Cupertino, CA, USA) so as to not influence trials. Four tadpoles in separate mazes were tested simultaneously, and the position of each tadpole in the maze was recorded every 10 seconds for five minutes. Each tadpole tested was used in one behavioural trial. Prior to running behavioural experiments, a number of preliminary tests were completed. To determine the diffusion time used in this experiment, 50 mL of dechlorinated water mixed with food colouring was administered into each arm of the maze and the time needed for a gradient of chemosensory stimulus to fill the arm was measured. To ensure tadpoles did not inherently favour one arm of the maze, 50 mL of dechlorinated water was administered to each arm of the maze and behavioural trials were run as described above. Also to reduce bias, the researcher was blind to which end of the choice maze the blank and chemosensory stimulus were administered.

Prometamorphic and metamorphic tadpole chemosensory ontogeny

To establish behavioural responses to *Spirulina* extract (2 g/L) in early prometamorphosis (mean \pm SE; mass 10.1 ± 0.9 g; median, range; TK 12, 11 – 12, n = 9), attraction responses were measured with the linear trough-style maze, as described in Section 2.3.

To study the ontogeny of the olfactory epithelium in later developmental stages, *L. catesbeiana* tadpoles (mean \pm SE; mass 22.5 ± 1.1 g; median, range; TK 17.5, 17 – 20, n = 23) were divided into two experimental groups: late prometamorphic and metamorphic climax tadpoles (Table 3.1). Behavioural responses to *Spirulina* extract (2 g/L) were recorded from a linear trough-style choice maze, as described above. Directly following behavioural experiments, EOG responses to *Spirulina* extract (2 g/L) and L-alanine (10^{-2} M) were measured on the same tadpoles as described in Section 2.3. Lastly, olfactory pits were excised from these same individuals and prepared for observation with SEM using the methods described above.

Statistical analyses

All statistical analyses were completed using R version 3.2.2 (R Core Team, 2015). For behavioural trials, tadpoles that failed to leave the acclimation chamber for the duration of the test were removed from the dataset. The average time spent in the stimulus arm and the control arm was compared for each experimental group. Parametric assumptions were tested using the Shapiro-Wilk normality test on the paired differences of time spent in the stimulus and blank arms of the maze. Data meeting parametric assumptions were analyzed with a paired t-test. Data not meeting parametric assumptions even after transformation were analyzed with a paired Wilcoxon Rank Sum test. Effect sizes (Cohen's D) of behavioural responses to *Spirulina* extract early prometamorphic, late prometamorphic, and metamorphic tadpoles were calculated (Torchiano 2016).

For EOG experiments, parametric assumptions were tested using the Shapiro-Wilk normality test and the Bartlett's test for homogeneity of variances. When parametric assumptions were not met, data were transformed with a $\log_{10}(1 + x)$ transformation to

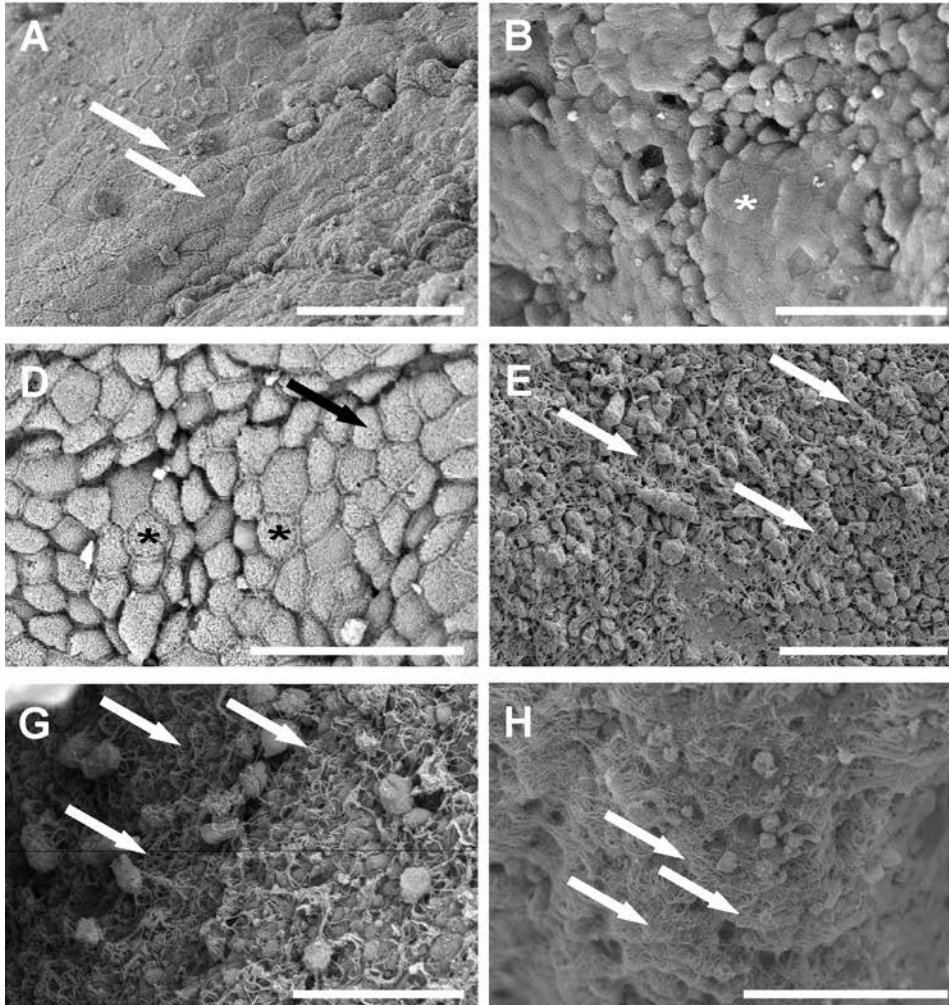
reclaim assumptions. Therefore, the transformed corrected mean EOG responses were compared with an independent-samples t-test. Where parametric assumptions could not be reclaimed after transformation, the Wilcoxon Rank Sum test was used to compare mean EOG responses. Mean differences were considered to be significant when $p \leq 0.05$.

Results

Premetamorphic tadpole olfactory surface anatomy and EOG responses

Premetamorphic tadpoles ranging from TK I-VIII did not provide detectable EOG signals in response to *Spirulina* extract or L-alanine. The morphology of the PC olfactory epithelium throughout these stages was observed with SEM and results with the corresponding EOG traces are presented in Figure 3.1. At TK V, the olfactory epithelial cells have a polygonal shape and short microvilli protrusions are evident (Figure 3.1 A & B). By TK VI, the polygonal cells are domed and continue to bear short microvilli (Figure 3.1 C). Few specimens at TK VI exhibited further development of a common sensory structure: olfactory knobs with longer ciliated projections than observed at previous stages (Figure 3.1 D). Overall EOG responses were undetectable at TK VI, although one individual did exhibit variable EOG responses to *Spirulina* extract. At TK IX EOG responses to *Spirulina* extract and L-alanine are detectable, but still inconsistent across individuals. By early prometamorphosis (TK XIII), the olfactory epithelium is densely covered by long cilia, and consistent responses to *Spirulina* extract and L-alanine were measured with EOG (Figure 3. 3). Overall, the ciliated projections become longer and more prominent with increasing developmental stage, until they completely cover the entire epithelial surface.

Micrographs



EOG Traces

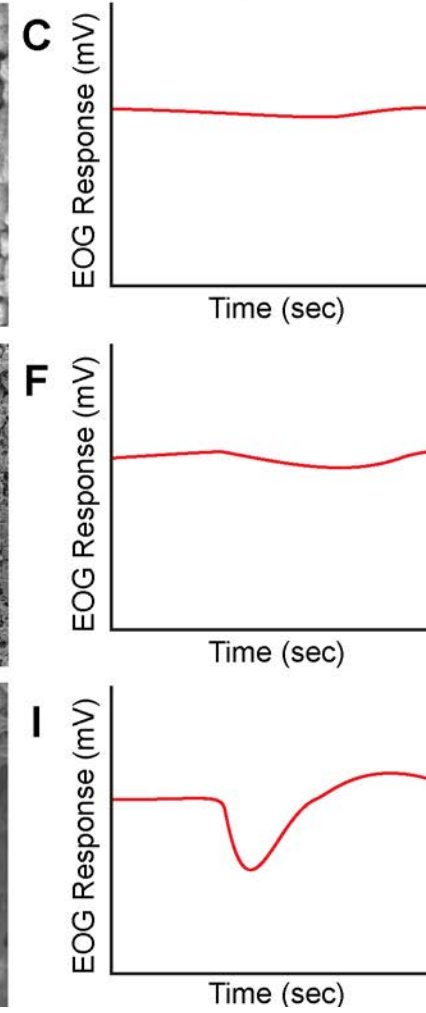


Figure 3.1 –

Representative scanning electron micrographs of the principal cavity (PC) olfactory epithelium of *Lithobates (Rana) catesbeiana* tadpoles and corresponding electro-olfactogram (EOG) responses to *Spirulina* extract. **A - B:** Developmental stage Taylor and Kollros (1946; TK) V. Polygonal cells (asterisks) are evident in the olfactory epithelium, and some appear domed. Short microvilli (arrows) are also present. **C:** TK V. EOG responses to *Spirulina* extract are undetectable. **D:** TK VI. Polygonal cells (asterisks) are evident with short microvilli projections (arrows). **E:** TK VI. Olfactory knobs (asterisks) with ciliated projections (arrows) cover the olfactory epithelium in only a few individuals. **F:** Lack of EOG response to *Spirulina* extract at TK IV. One TK VI individual did, however, exhibit a variable EOG response to *Spirulina* extract. **G:** TK IX. Olfactory knobs (asterisks) with ciliated projections (arrows) cover the olfactory epithelium. **H:** TK XIII: Entire surface of the olfactory epithelium is densely covered with olfactory knobs (asterisks) and cilia (arrows). **I:** Beginning at TK IX, EOG measurements of responses to *Spirulina* extract are successful. Scale bars: A, C - E = 30 μm , B, F = 50 μm .

Premetamorphic tadpoles with induced anosmia

Tadpole responses to an amino acid mixture of equal parts glycine, L-alanine, and L-serine (0.022M for each) were measured using tadpoles with intact olfactory pits and tadpoles that were rendered anosmic. When olfactory pits were left intact, tadpoles avoided the amino acid mixture, spending significantly more time in the blank arm than the stimulus arm of the linear trough-style choice maze ($t_{10} = 2.17$; $p = 0.05$; Figure 3.2 A). Conversely, there was no significant difference between time spent in the blank and stimulus arms when tadpoles were rendered anosmic ($t_{11} = 0.81$, $p = 0.43$; Figure 3.2 B).

Ontogeny of prometamorphic and metamorphic tadpole chemosensory acuity

Attraction responses to *Spirulina* extract from early prometamorphic tadpoles were measured with a linear trough-style choice maze and are presented in Figure 3.3. Tadpoles spent significantly more time in the stimulus arm than the blank arm of the maze ($t_9 = -2.5$, $p = 0.03$, $d = 0.9$; Figure 3.3 A).

Attraction responses from late prometamorphic (TK XVII) and metamorphic climax tadpoles were measured with the linear trough-style choice maze and results are presented in Figure 3.3. The same individuals were tested with EOG and the surface anatomy of their olfactory epithelium was observed under SEM, and results are presented in Figures 3.4 and 3.5, respectively. Late prometamorphic tadpoles spent 3-fold more time in the stimulus arm than in the blank arm of the linear trough-style choice maze; however, this difference was not statistically significant ($W = 6$, $p = 0.19$, $d = 0.7$; Figure 3.3 B). Metamorphic climax tadpoles were also not attracted to *Spirulina* and spent the same amount of time in the stimulus and blank arms of the maze ($t_8 = -0.37$, $p = 0.71$, $d = 0.1$; Figure 3.3 C). Conversely, late prometamorphic tadpoles exhibited significantly

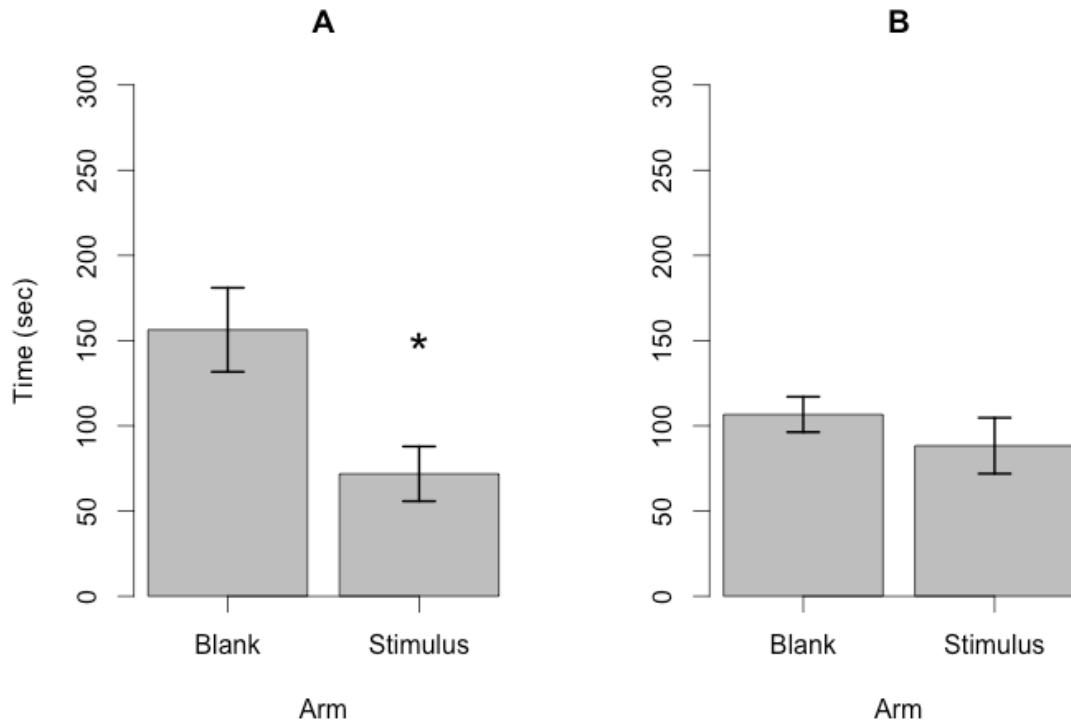


Figure 3.2 –

Time premetamorphic *Lithobates (Rana) catesbeiana* tadpoles spent in the blank (dechlorinated water) and stimulus (0.022 M each of L-alanine, L-serine, and glycine) arms of the linear trough- style choice maze when (A) olfactory pits were left intact and (B) olfactory pits were rendered anosmic. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 11 - 12$. Error bars represent mean \pm standard error.

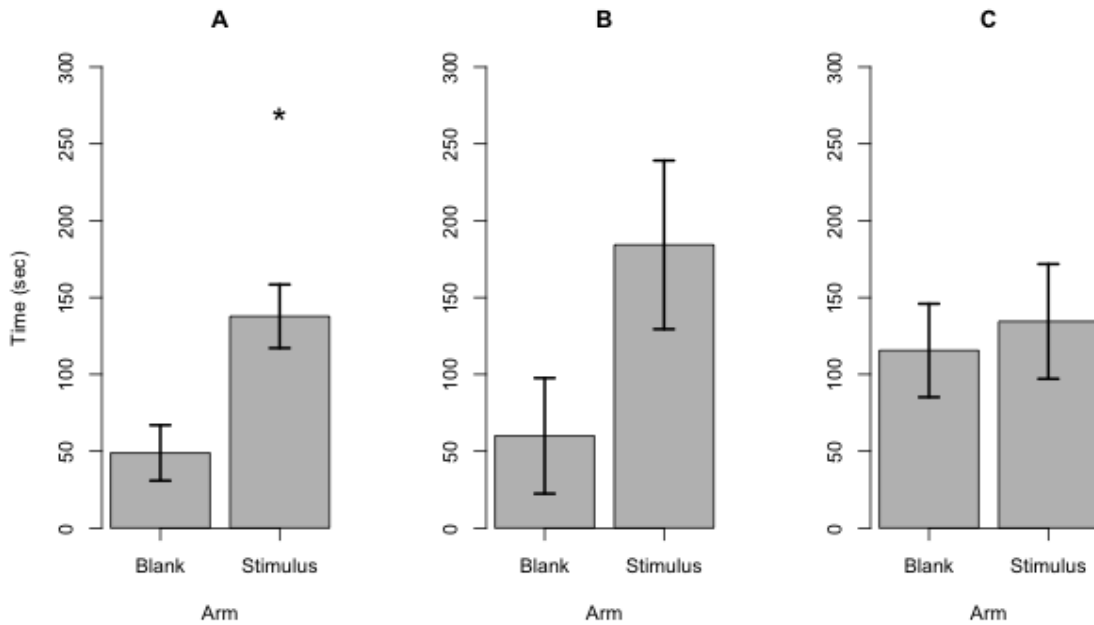


Figure 3.3 –
 Time spent in the blank (dechlorinated water) and stimulus (*Spirulina* extract) arms of the choice maze in: (A) early prometamorphic tadpoles, (B) late prometamorphic tadpoles (TK XVII) and (C) metamorphic climax *Lithobates (Rana) catesbeiana* tadpoles. Error bars represent mean \pm standard error. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 7 - 9$.

higher EOG responses to *Spirulina* extract than metamorphic climax tadpoles. *Spirulina* extract EOG responses were over two-fold higher from late prometamorphic tadpoles when compared to metamorphic climax tadpoles ($t_{16} = -2.4$, $p = 0.02$, Figure 3.4 A). Late prometamorphic tadpole EOG responses to L-alanine were also two-fold higher when compared to metamorphic tadpoles; however, this difference was not significant ($W = 43$, $p = 0.16$; Figure 3.4 B). Scanning electron micrograph observation of the olfactory epithelium showed no qualitative change in the sensory structures between late prometamorphic and metamorphic climax tadpoles. In all specimens observed, long ciliated projections densely covered the olfactory epithelium and olfactory knobs were evident (Figure 3.5 A – C).

Discussion

An ontogenetic shift in the olfactory system of *L. catesbeiana* tadpoles is evident at cellular and functional levels throughout larval development. The results of the present study demonstrate that discrete ontogenetic changes happen at specific stages of the larval period, as observed with EOG, behaviour, and scanning electron micrographs. To our knowledge, this is the first study to compare chemosensory responses between developmental stages in *L. catesbeiana* with behaviour and EOG. Additionally, the surface anatomy of *L. catesbeiana* olfactory epithelium has not previously been studied with SEM.

The amphibian olfactory system undergoes substantial remodelling during metamorphosis. The sensory structures observed in the olfactory epithelium of tadpoles in the present study are similar to those observed in studies of *X. laevis* (Hansen et al. 1998). Polygonal-shaped cells observed in TK V – VI (Figure 3.1 A – C) in the present study are

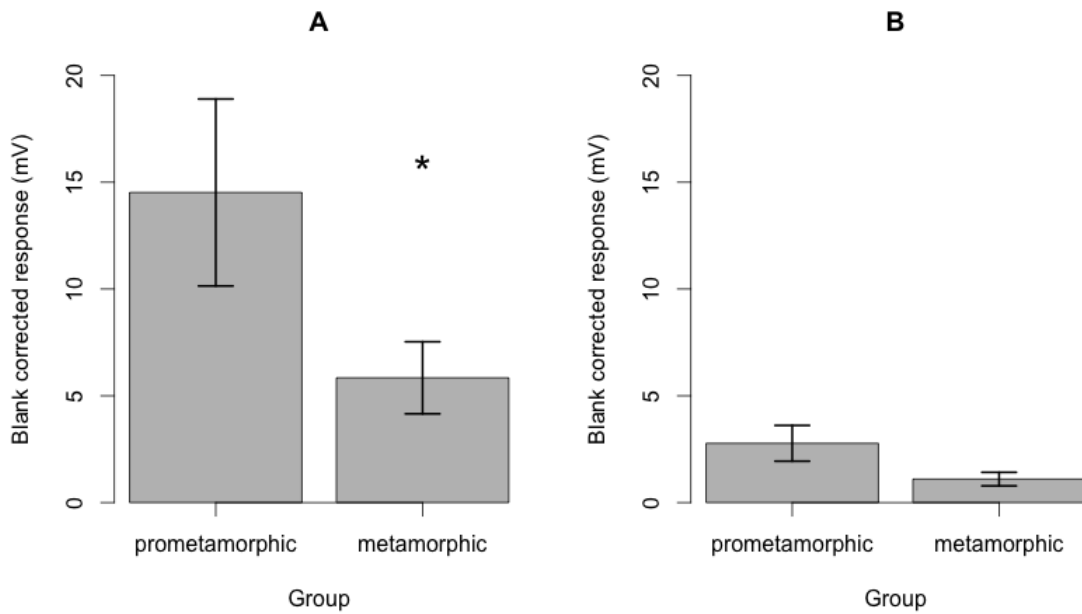


Figure 3.4 – Prometamorphic and metamorphic climax *Lithobates (Rana) catesbeiana* tadpole responses to (A) *Spirulina* extract and (B) L-alanine as measured by electro-olfactogram. An asterisk denotes a significant difference ($p \leq 0.05$), $n = 11 - 12$. Error bars represent mean \pm standard error.

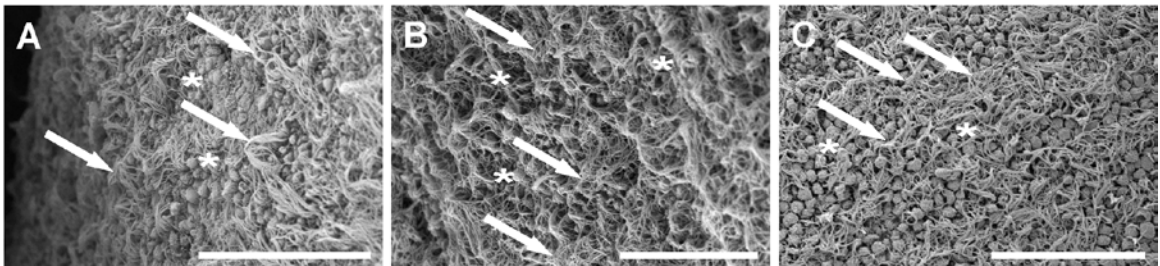


Figure 3.5 –

Scanning electron micrographs of the principal cavity olfactory epithelium of (A) late prometamorphic tadpoles (Taylor and Kollros 1946; TK XVII) and (B - C) metamorphic climax tadpoles (TK XVIII – XX). In late prometamorphic and metamorphic climax tadpoles, dense cilia (arrows) cover the olfactory epithelium and olfactory knobs (asterisks) are present. Scale bars: A = 50 μm , B = 30 μm , C = 40 μm .

similar to those Hansen et al. (1998) observed in the developing PC of *X. laevis* at early developmental stages. The cilia and microvilli observed at all TK stages in the present study (Table 3.1 & 3.5) are typical of the larval amphibian olfactory epithelium (Hansen et al. 1998). However, the cilia and microvilli observed could be projections from OSNs or SCs, as these cell types have been reported in the larval PC in other species including *X. laevis*, *Rana japonica*, and *Ascaphus truei* (Taniguchi et al. 1996; Hansen et al. 1998; Benzekri and Reiss 2012).

The lack of detectable EOG olfactory responses to chemosensory stimuli in premetamorphic tadpoles is seemingly due to the underdeveloped sensory structures in the olfactory epithelium. These data (Figure 3.1) clearly show that there is a close relationship between EOG responses to chemosensory stimuli and the cellular structures present on the olfactory epithelium, including cilia and olfactory knobs. Few specimens at TK VI and all at TK IX had developed olfactory knobs and sparse cilia and the EOG responses were variable (Figure 3.1 D – E). Comparatively, at TK XIII, the dense cover of cilia over the olfactory epithelium allowed for consistent EOG responses (Figure 3.1 F).

The development of the olfactory epithelium observed here is supported by previous studies, which have mainly focused on *X. laevis* (Klein and Graziadei 1983; Hansen et al. 1998). One main finding from these studies is that not all cell types of the mature olfactory epithelium are present at early developmental stages, but rather cells differentiate gradually throughout development. For example, Klein and Graziadei (1983) and Hansen et al. (1998) observed that microvilli develop before cilia in the PC, but both are present by the equivalent of TK III (Klein and Graziadei 1983; Hansen et al. 1998).

Similar results were also observed in *R. japonica* tadpoles (Taniguchi et al. 1996). However, the lack of EOG responses to chemosensory stimuli at earlier developmental stages in our study brings into question when the olfactory epithelium begins to function. For *X. laevis*, Hansen et al. (1998) suggests the olfactory system begins functioning prior to the equivalent stage of TK I., when OSNs first become functional and the tadpole begins feeding. Given the various staging regimes used, it can be difficult to compare both developmental stage (McDiarmid and Altig 1999) and the characteristics that might be unique to specific stages across species. Even considering these discrepancies, the tadpoles used in the present study were further developed than those in Hansen et al. (1998), and therefore the olfactory epithelium of tadpoles in the present study should be functioning. Conversely, the lack of EOG responses at early stages in *L. catesbeiana* tadpoles in our study suggests that the olfactory epithelium is not yet functional. However, when tested in a linear trough-style choice maze, TK I – VIII tadpoles with their olfactory senses intact avoided the chemosensory stimulus, but those rendered anosmic did not exhibit the same avoidance response to the chemosensory stimulus (Figure 3.2). Therefore, premetamorphic *L. catesbeiana* tadpoles have a functional olfactory system and were using it to respond to chemosensory stimuli. This chemosensory-driven behavioural response indicates first, that early stage olfactory sensory structures are not developed enough to generate a detectable EOG response and second, EOG analysis is not sensitive enough to record olfactory responses at early developmental stages.

Further ontogenetic changes in chemosensory function were observed in *L. catesbeiana* tadpoles in later developmental stages. The drastic change in attraction responses observed among early and late prometamorphic and metamorphic climax stages

is supported by the dietary shift tadpoles undergo as they transition into frogs (reviewed in Hourdry et al. 1996). Generally, tadpoles are opportunistic feeders and will forage on herbivorous detritus and organic matter, as well as some protein-based food (reviewed in Altig et al. 2007; Trakimas et al. 2011). As juvenile and adult frogs, they will have transitioned into a carnivorous diet. In particular, juvenile and adult *L. catesbeiana* are generalist predators, and consume a variety of invertebrates and vertebrates (Leivas et al. 2012; Liu et al. 2015).

In the present study, early prometamorphic tadpoles exhibited a significant attraction response to *Spirulina* extract (Figure 3.3 A), but this response was considerably reduced in late prometamorphic and metamorphic climax tadpoles (Figure 3.3 B and C). Comparison of olfactory-mediated attraction responses between late and prometamorphic tadpoles also demonstrates that responses were diminishing in tadpoles at later stages of development. Therefore we can conclude that late prometamorphic tadpoles were in a transitional stage and had started to make their dietary shift towards protein, but were still interested in *Spirulina*. Metamorphic climax tadpoles were further transitioned to preferring protein however, and showed no interest in *Spirulina*.

The substantial remodelling of the gut that occurs during metamorphosis further supports the observed behavioural results in the present study. The tadpole gut is specialized for a diet rich in carbohydrates, with long and coiled thin walls. At metamorphic climax, the increased endogenous T₃ and T₄ concentrations trigger apoptosis of the tadpole intestinal epithelium (Hourdry et al. 1996) and the proliferation and differentiation of a new intestinal epithelium, resulting in a shorter intestinal tract with thick walls, which is appropriate for a protein-rich diet (reviewed in Hourdry et al. 1996; Brown and Cai 2007; Ishizuya-Oka et al. 2010). Additionally, tadpoles stop feeding

during the metamorphic climax because their newly formed gut is not yet equipped for digestion (Hourdry et al. 1996). Therefore, in the present study the reduced attraction responses to *Spirulina* extract observed in metamorphic tadpoles are indicative of a dietary shift from an herbivorous to a carnivorous diet.

An ontogenetic shift in chemosensory acuity, as measured by EOG, was observed between prometamorphic and metamorphic climax *L. catesbeiana* tadpoles (Figure 3.4). Like the intestine, the olfactory system of a larval anuran undergoes extensive remodelling during metamorphosis, while it prepares for a partially terrestrial habitat. In larval anurans, the PC olfactory epithelium expresses both ciliated and microvillus sensory neurons, and functions to detect airborne and aquatic odours, respectively. In contrast, the adult PC olfactory epithelium expresses only ciliated OSNs, and functions to solely detect airborne odorants (Taniguchi et al. 1996; Hansen et al. 1998). Previous studies have demonstrated that during the transition of the PC olfactory epithelium, from the larval to the adult form, all OSNs suffer apoptosis and are replaced (Higgs and Burd 2001; Dittrich et al. 2016). Interestingly, Dittrich et al. (2016) found that in *X. laevis*, the highest rate of OSN apoptosis coincides with the maximum concentrations of plasma THs during metamorphic climax. In *L. catesbeiana*, THs increase to their maximal concentrations after TK XVII (reviewed in White and Nicoll 1981). Therefore, the reduced chemosensory acuity to *Spirulina* extract in metamorphic climax tadpoles when compared to prometamorphic tadpoles may be attributed to the widespread apoptosis of OSNs in the larval PC olfactory epithelium. Metamorphic climax is also the period where larval OSNs are replaced by ciliated OSNs and the PC prepares to detect airborne odorants. Therefore, PC OSNs in metamorphic climax tadpoles are likely not tuned to detect aqueous *Spirulina* extract and L-alanine stimuli.

There is a disparity between stage-specific responses to *Spirulina* extract when considering behavioural and EOG measurements in late prometamorphic and metamorphic climax tadpoles. Measured EOG responses represent the depolarization of an odour binding to many OSNs. A substantial depolarization forms an action potential that is propagated to the brain and could induce a behavioural response (Baldwin and Scholz 2005). However, EOG does not indicate if the depolarization leads to propagation of an action potential to the brain. Furthermore, behavioural responses to the stimulus could be more subtle, and not measureable with the current behavioural assay. Despite this, our findings suggest that the cessation of feeding at metamorphic climax, as discussed above, is partially driven by an ontogenetic change in chemosensory acuity. The reduced ability to detect a food stimulus through chemosensation during metamorphosis is perhaps a mechanism to protect the reorganizing intestine during a sensitive period when digestion is not possible (reviewed in Hourdry et al. 1996). Unfortunately, SEM limits our study of the ontogeny of olfactory epithelium to the surface only. Techniques such as transmission electron microscopy and histology would allow for the identification of supporting cells, basal cells, and OSNs, and provide a more detailed picture of the ontogenetic changes in the olfactory epithelium, including cell turnover.

In summary, the results of the present study demonstrate the ontogenetic changes in the olfactory system in *L. catesbeiana* tadpoles as they progress through metamorphosis. It is evident that the chemosensory structures on the PC olfactory epithelium develop gradually. At early stages of premetamorphosis, sensory structures are underdeveloped, and therefore EOG is unsuccessful. In addition, metamorphic climax

tadpoles also exhibit chemosensory ontogenetic changes, with a decreased chemosensory acuity when compared to early and late prometamorphic tadpoles.

Chapter 4: Effects of thyroid hormones on olfactory-mediated behaviour in *Lithobates (Rana) catesbeiana* tadpoles

Introduction

Extensive changes occur throughout the larval anuran body plan during metamorphosis and almost all of these changes are initiated by the thyroid system (Brown and Cai 2007). There are three stages of anuran larval development and they are delineated with respect to the function of the thyroid gland (Figure 1.2).

Premetamorphosis is characterized by an inactive thyroid gland and no endogenous thyroid hormones (THs) in circulation. With prometamorphosis the thyroid gland begins to secrete THs and minor metamorphic changes including hind limb-bud development are initiated. The metamorphic climax is characterized by a dramatic increase in TH secretion and endogenous THs reach their maximal concentrations. Consequently during metamorphic climax major developmental changes, including tail regression and forelimb eruption occur (reviewed in Fort et al. 2007).

Overall the anuran thyroid system is comparable to thyroid systems in other vertebrates (reviewed in Furlow and Neff 2006). Briefly, the thyroid gland secretes mainly, thyroxine (T_4), which is transported to target tissues. At target tissues T_4 is converted to 3,5',3-triiodothyronine (T_3) through 5' deiodinases. Classically, T_3 is known as the most active form of TH (reviewed in Brown and Cai 2007). Metamorphosis is mediated by two nuclear TH receptors (TRs), $TR\alpha$ and $TR\beta$. Thyroid hormones bind to $TR\alpha$ and $TR\beta$ to facilitate the expression of TH responsive-genes to trigger the remodelling of the entire anuran body plan (reviewed in Brown and Cai 2007). Metamorphic changes are extensive and range from total cell death in tissues including

the tail fin, to organogenesis of tissues such as limbs, or complete remodelling of some existing tissues including the intestine (reviewed in Brown and Cai 2007). In premetamorphic tadpoles, prior to the function of the thyroid gland, TR α and TR β are present in tissues at low levels (reviewed in Tata 2006; Grimaldi et al. 2013). Thus although no endogenous THs are circulating, premetamorphic tadpoles are equipped to respond to THs, and exposure to exogenous THs has induced precocious metamorphosis in premetamorphic tadpoles in a number of studies (reviewed in Tata 2006; Brown and Cai 2007).

One of the systems that undergoes major remodelling during anuran metamorphosis is the olfactory system (Hansen et al. 1998; Wang et al. 2008). Generally, the olfactory system comprises paired olfactory cavities that are lined with olfactory epithelia. Ciliated or microvillous olfactory sensory neurons (OSNs) project onto the apical surface of the olfactory epithelium and express odour receptors (ORs). Odourants 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 behavioural responses to the odorant (reviewed in Ache and Young 2005; Gascuel and Amano 2013). In the tadpole olfactory system each olfactory cavity is made up of two olfactory pits, the principal cavity (PC) and vomeronasal cavity (VNO). The VNO detects pheromones and remains unchanged throughout metamorphosis (Hansen et al. 1998). The tadpole PC (Figure 1.3) detects only aqueous odorants but transitions during metamorphosis to the juvenile frog PC, which detects volatile odorants exclusively. During metamorphosis a second olfactory pit develops, the medial cavity (MC; Figure 1.3), which is equipped to detect aquatic odorants in the juvenile frog (Hansen et al. 1998; Wang et al. 2008; 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 metamorphosis in *Xenopus laevis* tadpoles. For example, Manzini and Schild (2004) reported that the number of olfactory stimuli to which OSNs responded decreased as metamorphosis progressed, as measured by calcium imaging. Therefore, 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 (Reiss and Burd 1997; Dittrich et al. 2016).

Considering the major changes triggered by THs that take place in the tadpole olfactory system, exposure to exogenous THs may also affect olfactory acuity. Exogenous TH effects on olfactory acuity may have important implications for ecological health. Many consumer products contain endocrine disrupting compounds (EDCs), which are not always fully removed by municipal wastewater treatment processes. As a result, EDCs have been measured in wastewater effluent receiving waters globally (Kolpin et al. 2002; Boyd et al. 2003). Some EDCs share similar structures with T₃ and T₄, and can consequently interfere with the normal function of the thyroid system. For example, previous studies have reported accelerated or delayed metamorphosis in tadpoles as a result of EDC exposure (Crump et al. 2002; Veldhoen et al. 2006; Sowers et al. 2009). For this reason exposure to EDCs may accelerate metamorphic changes in the olfactory system and affect olfactory acuity in tadpoles. Detection of olfactory stimuli informs aquatic organisms about potential threats and food, and therefore influences behaviour (reviewed in Laberge and Hara 2001). Thus, EDCs that interact with the thyroid system may have important ramifications on ecological health.

The purpose of this study was to characterize the effects of exposures to physiologically relevant concentrations of THs on olfactory-mediated avoidance

responses in *Lithobates (Rana) catesbeiana* tadpoles and to link those responses to molecular endpoints. Premetamorphic tadpoles were exposed to one of T₃, T₄, or 17β-estradiol (E₂) and olfactory-mediated avoidance responses in premetamorphic *L. catesbeiana* tadpoles were measured. Previous studies have measured olfaction in tadpoles using olfactory-mediated behavioural assays, using endpoints such as activity and refuge use (Ferrari et al. 2007; Garcia et al. 2012). In the present study, olfaction is measured by quantifying tadpole chemosensory-mediated responses in a linear trough-style maze. Estrogenic compounds are often measured in wastewater effluent receiving waters (Kolpin et al. 2002), but have not been shown to have direct effects on the thyroid system. In the present study exposure to E₂ served as a negative control to determine whether effects on the olfactory system are specific to THs.

Materials and Methods

Experimental animals

Wild *L. catesbeiana* tadpoles of mixed sex were obtained from the University of Victoria, British Columbia and housed at the University of Lethbridge in the Aquatic Research Facility (ARF) on a re-circulatory system. Upon arrival to the University of Lethbridge tadpoles were quarantined for two weeks. Tadpoles were fed *Spirulina* flakes (Nutrafin Max, Rolf C. Hagen, Montreal, QC, Canada) *ad libitum* daily. All procedures were approved by the Animal Welfare Committee at the University of Lethbridge for compliance under the Canadian Council on Animal Care guidelines. Tadpoles were staged according to Taylor and Kollros (1946; TK; Appendix A).

Hormone exposures

Tadpoles (Table 4.1) were exposed to physiologically relevant concentrations of T₃, T₄, or physiologically relevant concentrations of E₂ at 24 °C for 48 hours. For the T₃ exposure set, tadpoles were exposed to one of the concentrations of T₃ (0.1, 1, 10 nM), NaOH vehicle control (800 nM) or dechlorinated water. For the T₄ exposure set, tadpoles were exposed to one of the concentrations of T₄ (0.5, 5, 50 nM), NaOH vehicle control (800 nM) or dechlorinated water. For the E₂ exposure set, tadpoles were exposed to a series of concentrations of E₂ (0.1, 1, 10 nM) or dechlorinated water. 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 Table 4.2. Temperature and dissolved oxygen were measured on a daily basis. Ammonia (test strips, Nutrafin, Hagen) and pH were measured were measured at the start and end of each exposures. Treatments had no effect on hardness and alkalinity; therefore these parameters were measured on the final day of the exposures.

Behavioural experiments

Avoidance responses to an amino acid mixture (0.022 M each of L-alanine, L-serine, and glycine) were measured using a linear trough-style maze, as previously described in Chapter 3. *Lithobates (Rana) catesbeiana* tadpoles have exhibited consistent avoidance responses to this amino acid mixture, as reported in Chapter 2. The amino acid mixture was administered to one end of the linear trough-style maze and dechlorinated water (blank) to the other. All trials were recorded via webcam (Logitech, Romanel-sur-Morges, Switzerland) and viewed remotely.

Table 4.1 –
Body morphology of tadpoles used in 3,5',3-triiodothyronine (T₃), thyroxine (T₄), and
17β- estradiol (E₂) exposures.

Exposure	n	Body length* (mm)	Tail length* (mm)	Mass* (g)	Stage** (TK)
T ₃ exposure					
water	38	25.8 ± 0.4	52.7 ± 1.2	4.2 ± 0.2	III, I - XI
vehicle	38	26.2 ± 0.5	53.2 ± 1.6	4.7 ± 0.4	III, I - XI
0.1 nM T ₃	38	26.5 ± 0.6	52.6 ± 1.8	4.8 ± 0.4	II, I - XI
1 nM T ₃	38	26.7 ± 0.5	53.9 ± 1.2	5.3 ± 0.6	III, I - XI
10 nM T ₃	38	26.5 ± 0.6	54.1 ± 1.5	4.2 ± 0.3	IV, I - XII
T ₄ exposure					
water	16	28.3 ± 0.9	56.7 ± 3.9	6.6 ± 0.7	VI, I - XII
vehicle	16	27.6 ± 1.1	56.2 ± 3.7	6.2 ± 0.7	VII, I - XII
0.5 nM T ₄	16	29.0 ± 1.3	60.9 ± 3.1	7.7 ± 1.0	VII, I - XII
5 nM T ₄	16	29.6 ± 0.8	63.2 ± 2.1	7.9 ± 0.6	X, I - XII
50 nM T ₄	16	28.3 ± 0.8	61.4 ± 2.3	6.6 ± 0.7	X, I - XII
E ₂ exposure					
water	16	26.0 ± 0.9	60.1 ± 2.2	5.8 ± 0.6	VI, I - XI
0.1 nM E ₂	16	27.0 ± 0.9	58.3 ± 2.0	5.9 ± 0.6	IV, I - XIII
1 nM E ₂	16	29.6 ± 0.8	65.4 ± 2.2	8.4 ± 0.9	VIII, I - XIII
10 nM E ₂	16	29.4 ± 1.2	62.9 ± 2.3	7.4 ± 0.8	VIII, I - XII

* = mean ± SE; ** = median, range.

Table 4.2 –
Water quality measurements for 3,5',3-triiodothyronine (T₃) , thyroxine (T₄), and 17β-
estradiol (E₂) exposures.

Parameter	T ₃	T ₄	E ₂
Temperature (°C)*	23.7 ± 0.1 (116)	23.5 ± 0.1 (59)	24.5 ± 0.1 (45)
Dissolved oxygen* (mg/L)	7.2 ± 0.04 (116)	7.1 ± 0.1 (59)	6.7 ± 0.1 (41)
Ammonia (ppm)*	0.3 ± 0.0 (86)	0.3 ± 0.0 (31)	0.0 ± 0.0 (13)
pH**	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)	178 ± 1 (30)	172 ± 1 (35)	170 ± 1 (35)
Alkalinity* (mg/L as CaCO ₃)	136 ± 2 (30)	141 ± 1 (35)	142 ± 2 (35)

* = mean, SE (sample size); ** = median, range (sample size)

on a laptop computer (MacBook Air, Apple, Cupertino, CA, USA). The researcher was blind to which arm of the maze amino acid and blank cues were administered.

The amino acid mixture was prepared fresh on the days of behavioural experiments. To prepare the amino acid mixture equal molarity (0.022 M each) 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) were stirred into dechlorinated water.

Statistical analysis

All statistical analyses were completed using R version 3.2.2 (R Team 2015). For behavioural experiments, tadpoles that failed to leave the acclimation zone for the duration of the test were removed from the dataset, as determined *a priori*. Shapiro-Wilk test for normality was used to check that the paired differences of time spent in the blank and stimulus arm of the maze were normally distributed to meet parametric assumptions. Where assumptions were not met data were transformed with a square transformation (x^2) to reclaim assumptions. Mean time spent in the blank and stimulus arm was calculated for each treatment and compared using a paired t-test. Where parametric assumptions could not be reclaimed, mean time was compared with a Permutation t-test (Legendre and Blanchet 2015). For the E₂ exposure, effect sizes (Cohen's d) of the time spent in the blank arms of the maze were calculated for each treatment (Torchiano 2016).

Results

T₃ exposure

Olfactory-mediated avoidance responses in bullfrog tadpoles were measured with a linear trough-style maze and are presented in Figure 4.1. After exposure to the water and NaOH treatments tadpoles spent approximately 1.5-fold more time in the blank arm than the stimulus arm of the maze (water: $t_{26} = 2.3$, $p = 0.02$ vehicle: $t_{29} = 1.9$ $p = 0.05$). Conversely, after exposure to physiologically relevant concentrations of T₃, tadpoles failed to avoid the stimulus and therefore spent a similar amount of time in the blank and stimulus arms of the a linear trough-style maze (0.1 nM T₃: $t_{29} = 1.3$, $p = 0.18$; 1 nM T₃: $t_{24} = 1.0$, $p = 0.29$; 10 nM T₃: $t_{30} = 0.5$, $p = 0.59$).

T₄ exposure

After exposure to T₄, olfactory-mediated avoidance responses to the amino acid mixture were measured with a linear trough-style maze, and are presented in Figure 4.2. Tadpoles in the control groups (dechlorinated water and NaOH) avoided the amino acid mixture, and thereby spent significantly less time in the stimulus arm than the blank arm (water: $t_{14} = 2.15$, $p = 0.04$; NaOH: $t_{15} = 2.37$, $p = 0.03$). Similarly, after exposure to physiologically relevant concentrations of T₄, tadpoles avoided the amino acid mixture (0.5 nM T₄: $t_{14} = 2.19$, $p = 0.04$; 5 nM T₄: $t_{13} = 2.39$, $p = 0.03$; 50 nM T₄: $t_{14} = 2.55$, $p = 0.01$).

E₂ exposure

After exposure to E₂, avoidance responses to the amino acid mixture were measured with a linear trough-style maze, and results are presented in Figure 4.3. Exposure to E₂ had no effect on avoidance responses to the amino acid mixture. In all

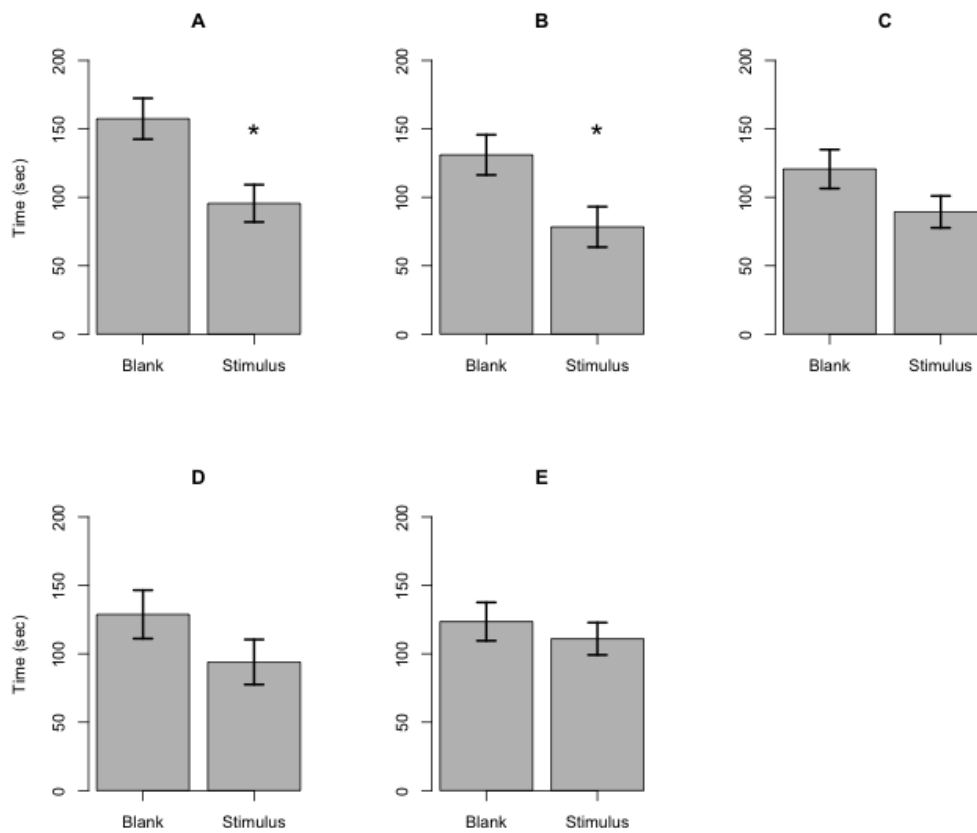


Figure 4.1 –

Time *Lithobates (Rana) catesbeiana* tadpoles spent in the stimulus (amino acid mixture) and blank (dechlorinated water) arms of the linear-style choice maze after exposure to (A) water blank (B) 800 nM NaOH (vehicle control), (C) 0.1 nM 3,5',3-triiodothyronine (T₃), (D) 1.0 nM T₃, or (E) 10 nM T₃. Error bars represent mean ± standard error. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 25 - 31$.

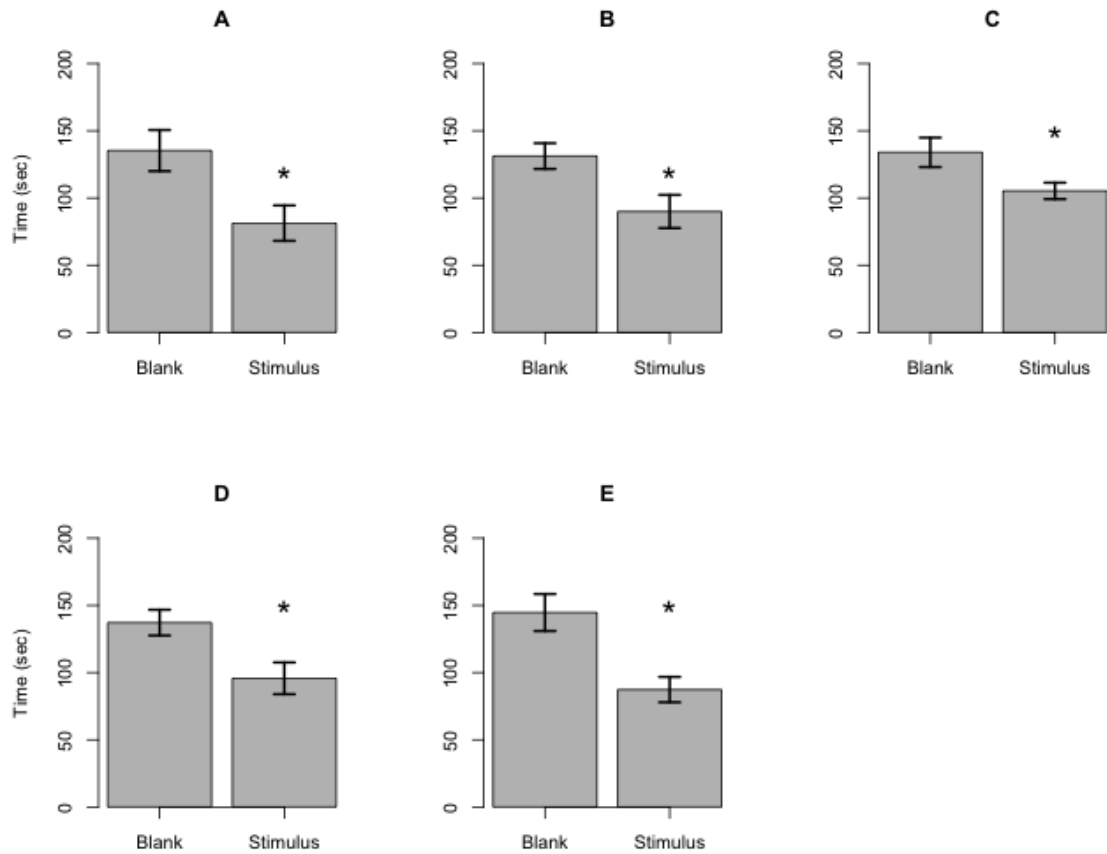


Figure 4.2 –

Time *Lithobates (Rana) catesbeiana* tadpoles spent in the stimulus (amino acid mixture) and blank (dechlorinated water) arms of the linear-style choice maze after exposure to (A) water blank (B) 800 nM NaOH (vehicle control), (C) 0.5 nM thyroxine (T₄), (D) 5.0 nM T₄, or (E) 50 nM T₄. Error bars represent mean ± standard error. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 14 - 16$.

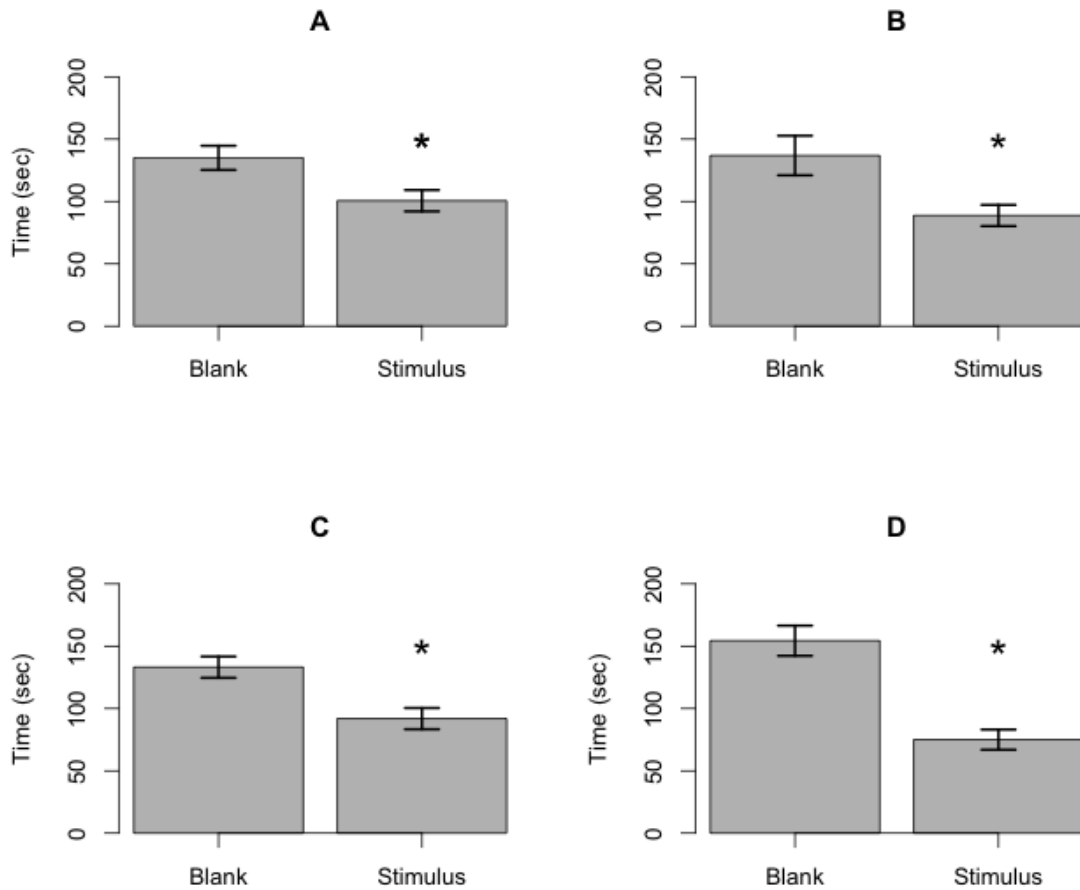


Figure 4.3 –
 Time *Lithobates (Rana) catesbeiana* tadpoles spent in the stimulus (amino acid mixture) and blank (dechlorinated water) arms of the linear-choice maze after exposure to (A) water blank (B) 0.1 nM 17 β - estradiol (E₂) (C) 1 nM E₂, or (D) 10 nM E₂. Error bars represent mean \pm standard error. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 16$.

treatment groups tadpoles spent significantly more time in the blank arm than the stimulus arm of the maze (water: $t_{15} = 2.15$, $p = 0.04$, $d = 0.5$; 0.1 M E₂: $t_{15} = 2.15$, $p = 0.04$, $d = 0.5$; 1.0 M E₂: $t_{15} = 3.83$, $p = 0.001$, $d = 0.9$; 10.0 M E₂: $t_{15} = 4.15$, $p = 0.0008$, $d = 1$). Interestingly, tadpoles exhibited increased aversion to the olfactory cue with exposure to increasing concentrations of E₂.

Discussion

Avoidance responses to an olfactory stimulus were significantly reduced after exposure to all concentrations of T₃ tested (Figure 4.1). Tadpoles were exposed to T₃ at concentrations comparable to circulating plasma T₃ concentrations in tadpoles at metamorphic climax (White and Nicoll 1981). Exposure to T₃ may have initiated precocious metamorphic changes on a cellular level in the olfactory epithelium. The observed reduction in olfactory-mediated avoidance responses after T₃ 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 (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 in *X. laevis* tadpoles OSN apoptosis increased with increasing developmental stage and peaked at the metamorphic climax, when TH concentrations are highest. The T₃ -induced inhibition of olfactory-mediated avoidance responses observed in the present study are further

supported by the results reported in Chapter 3. *Lithobates (Rana) catesbeiana* tadpoles at metamorphic climax exhibited reduced olfactory acuity to *Spirulina* extract when compared to prometamorphic tadpoles, as measured by electro-olfactography (EOG) and olfactory-mediated attraction responses (Chapter 3). Thus the reduced avoidance responses to the amino acid mixture reported in the present study may have been mediated by precocious metamorphic changes in the olfactory epithelium, including specialization of OSNs or the mass OSN apoptosis.

Other aspects of the anuran olfactory system, including olfactory axons, also undergo changes initiated by THs. For example, Burd (1991) reported that the number of axonal projections from OSNs to the OB increased with increasing developmental stage in *X. laevis* tadpoles. Furthermore axonal projections are also reorganized during metamorphosis so that they target different areas of the OB in the juvenile frog than they did in the tadpole (Higgs and Burd 2001). Therefore in the present study T₃ exposure may have influenced changes in the axonal projections from OSNs to the OB, and therefore with the signalling of olfactory responses to the OB.

Although no previous studies have identified a direct link between the olfactory system and THs in amphibians, a link has been established at both the cellular and functional level in fish. For example, Lema and Nevitt (2004) exposed *Onchorynchus kisutch* to T₃ to emulate a sensitive life-stage when fish undergo smoltification and physiologically prepare to imprint on natal streams. Lema and Nevitt (2004) reported an increase in olfactory epithelial cells and developing OSNs in the olfactory epithelium after exposure to T₃. Effects of TH on olfaction in fish from a functional perspective have also been reported. Morin et al. (1995) observed decreased responses to L-alanine in the olfactory bulb after exposure to T₄ in juvenile *Salmo salar*, as measured by

electroencephalogram. Additionally, increased thyroid gland activity has been associated with increased olfactory learning capability in *S. salar* undergoing smoltification (Morin et al. 1989). Therefore, given extent that THs can affect the olfactory system in fish, it is likely that THs can also influence the olfactory system in tadpoles.

The discrepancy between the effects of T₃ and T₄ (Figures 4.1 and 4.2) on olfactory-mediated responses observed in the present study was unexpected. Traditionally T₄ is considered to be a precursor for the more biologically active T₃. Deiodinase enzymes (D1, D2, and D3) convert T₄ to T₃ locally at target tissues (reviewed in 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 (reviewed in Tata 2006). However, one form of deiodinase enzyme, D3, converts both T₄ and T₃ to TH derivatives, which renders them biologically inactive. 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 tailfin, are rich in D3, and depleted in D1 and D2 during premetamorphosis to keep THs from binding too early and maintain metamorphic timing (reviewed in Forrest et al. 2002; Maher et al. 2016). In the present study, it is possible the combination of D1, D2, and D3 present in the olfactory epithelia of tadpoles exposed to T₄ inhibited the conversion of T₄ to T₃, or perhaps even resulted in the conversion of T₄ to an inactive form. However, results reported in a recent study by Maher et al. (2016) further complicate the T₄ exposure results observed in the present study. Maher et al. (2016) reported that T₄ is biologically active in premetamorphic *L. catsebeiana* tadpoles, even in tissues depleted in deiodinase enzymes. Thus T₄ might also be active on the olfactory epithelium even without conversion to T₃.

There is a clear difference in the stages of tadpoles used in the T₃ and T₄ exposures. Tadpoles exposed in the T₃ experiment were predominantly premetamorphic (median TK III; Table 4.1; White and Nicoll 1981), whereas tadpoles exposed in the T₄ experiment were in the early stages of prometamorphosis (median TK IIX; Table 4.1; White and Nicoll 1981). The different developmental stages used between exposure groups may have introduced a confounding variable because of the inherent differences in endogenous circulating THs between groups (White and Nicoll 1981). However, Degitz et al. (2005) previously concluded there is minimal difference in sensitivity to exogenous TH exposure between premetamorphic and prometamorphic *X. laevis* tadpoles, as determined by measurement of time to metamorphosis. Therefore the explanation for the contradicting effects of exogenous T₄ and T₃ exposure on olfactory-mediated behaviour in the present study remains unclear.

A secondary hypothesis for the reduced olfactory-mediated avoidance responses observed in the present study is that T₃ interacted with the olfactory epithelium and caused olfactory toxicity. Previous studies have reported that toxicants, including metals and pesticides, can inhibit olfactory acuity in fish (reviewed in Tierney et al. 2010). Many driving mechanisms behind the impairment have been identified including entry into the olfactory system, damaged OSNs, blockage of ion channels, and competitive binding to ORs (Scott and Sloman 2004; Tierney et al. 2010). Therefore, it is possible that T₃ disrupted the normal function of the larval olfactory system in one of the previously mentioned mechanisms. However, no previous studies have reported olfactory toxicity as a result of T₃ exposure.

Exposure to E₂ did not impair any olfactory-mediated avoidance responses (Figure 4.3). 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 *Rana pipiens* tadpoles exposed to E₂ or 17 α -ethinylestradiol (EE₂) respectively experienced delayed metamorphosis (Gray and Janssens 1990; Hogan et al. 2008). However from a molecular perspective there has been no direct action of estrogenic compounds on TRs reported. Therefore it is not surprising that in the present study E₂ exposure did not cause impairment of olfactory-mediated avoidance responses. Interestingly there is evidence that E₂ increased olfactory acuity to the olfactory stimulus. The magnitude and effect sizes of avoidance responses to the olfactory stimulus were dependent on the concentration of the E₂ exposure. For example, after exposure to dechlorinated water tadpoles spent 1.3 x more time in the blank arm than the stimulus arm (d = 0.5). Comparatively, after exposure to 10 nM E₂ tadpoles spend twice as much time in the blank arm than the stimulus arm of the maze (d = 1.0; Figure 4.3). Although there are no previous studies on the effects of estrogenic compounds on olfactory acuity or behaviour in tadpoles, effects on adult anurans have been reported. Elevated endogenous estrogen concentrations in female frogs stimulate egg production and are typical during the breeding season (reviewed in Gilbert 2000). Nakazawa et al. (2009) reported increased OSN counts in the olfactory epithelium, and increased olfactory acuity (as measured by EOG) in adult *Bufo japonicas* in the breeding season. In the present study, exposure to exogenous E₂ may have facilitated changes in the tadpole olfactory epithelium that are typical in adult frogs during the breeding season. However, further study is needed to establish a link between olfactory acuity and endogenous E₂ concentrations in anurans. Alternatively, E₂ could have influenced the central processing of olfactory information or

locomotion (reviewed in Scott and Sloman 2004), resulting in the change in chemosensory-mediated avoidance responses that were observed.

From the present study it is clear that exposure to exogenous T₃ caused impaired olfactory function in premetamorphic *L. catesbeiana* tadpoles, however this study is limited. Quantification of TRs mRNA expression in the olfactory epithelium would provide an indication of the direct action of THs on the olfactory epithelium. In closing, exogenous T₃ may have initiated precocious metamorphic changes in the olfactory system and resulted in reduced olfactory-mediated avoidance responses. However, exogenous T₄ and E₂ had no effect on avoidance responses to the olfactory stimulus. Consequently, there is potential for EDCs that mimic T₃ to impair olfactory-mediated behavioural responses in *L. catesbeiana* tadpoles.

CHAPTER 5: Effects of wastewater effluent and endocrine disrupting compounds on chemosensation in *Lithobates (Rana) catesbeiana* tadpoles

Introduction

Endocrine disrupting compounds (EDCs) are contaminants that are biologically active and have potential to disrupt the normal function of the endocrine system in vertebrates (Quanrud and Propper 2010). A multitude of everyday products including pharmaceuticals, personal care products, pesticides, plasticizers, and flame-retardants contain EDCs. After use, these products accumulate in wastewater and undergo treatment. Unfortunately, wastewater treatment processes are not equipped to fully remove EDCs, and therefore EDCs are persistent in treated effluent and discharged into receiving waters such as rivers and reservoirs. As a result, these compounds have been detected in effluent impacted rivers across the globe at concentrations as high as $\mu\text{g/L}$ (Kolpin et al. 2002).

Past EDC research has focused on their effects on the reproductive system (Jobling et al. 1998; Scott and Sloman 2004), but other effects of EDCs have been identified. For example, some EDCs are thyroid active and can interfere with the thyroid system (Crofton 2008). The thyroid hormone (TH), thyroxine (T_4), is secreted from the thyroid gland and transported to target tissues by transport proteins. Through deiodinase activity, T_4 is converted to the more active TH, tri-iodothyronine (T_3), and T_3 binds with TH receptors (TRs) $TR\alpha$ and $TR\beta$ to mediate gene responses (reviewed in Brown and Cai 2007; Grimaldi et al. 2013). The thyroid system can be interrupted along many levels including the synthesis or transport of THs, and the binding of receptors (reviewed in Jugan et al. 2010). Suspected thyroid hormone disruptors include polychlorinated biphenyls (PCBs), phthalates, bisphenol A, brominated flame-retardants and

perflourinated chemicals (reviewed in Brown et al. 2004). Proper function of the thyroid system is especially imperative in early life-stages in developing organisms, as it is involved with growth and differentiation of tissues (reviewed in Jugan et al. 2010).

For larval anurans, the normal functioning of the thyroid system is critical for their transformation into frogs because metamorphosis is primarily driven by THs (Tata 2006). Premetamorphic tadpoles have essentially no THs circulating endogenously. The thyroid gland begins secreting THs during prometamorphosis, which initiates some developmental changes in the body plan. At metamorphic climax, the maximal concentration of THs is reached and comprehensive changes occur in the body plan, which results in a terrestrial organism. Given that tadpoles are aquatic and undergo the TH-driven process of metamorphosis, they are excellent sentinels for studying the effects of wastewater EDCs on the thyroid system (Tata 2006). Previous studies have reported changes in TH-mediated gene expression profiles (Crump et al. 2002; Veldhoen et al. 2006), endogenous TH concentrations (Li et al., 2016), metamorphic timing (Veldhoen et al., 2006), and body morphology (Jelaso et al. 2002; Sowers et al. 2009) as a result of exposure to either individual EDCs or wastewater in a variety of tadpole species. The effects of TH on tadpole behaviour are mainly unstudied, however there is evidence for thyroid hormone disruption leading to changes in behaviour in fish (Zhou et al. 2000).

The chemosensory system in larval anurans is substantially remodelled during metamorphosis, which results in considerable cellular and morphological changes. Prior to metamorphosis the olfactory system is equipped for aquatic environments exclusively, but transitions to function in both aquatic and terrestrial environments in the adult frog (reviewed in Gascuel and Amano 2013). Like other metamorphic changes, the remodelling of the olfactory system is driven by endogenous THs. Consequently the

disruption of thyroid system on any level could result in changes in olfactory acuity in tadpoles. In aquatic environments chemosensation is an important sense that informs behavioural responses. Therefore, intact chemosensation is vital for locating food and avoiding predators (Lurling and Scheffer 2007).

The purpose of this study is to investigate the effects of EDCs and treated municipal wastewater effluent on the olfactory-mediated behaviour in premetamorphic *Lithobates (Rana) catesbeiana* tadpoles. Tadpoles were exposed to an EDC cocktail-spiked treated wastewater effluent from either Waterloo, ON, or Vancouver, BC. Olfactory-mediated avoidance responses to a chemosensory stimulus were measured with a linear-style choice maze. We also tested olfactory-mediated avoidance responses after exposure to the EDC cocktail itself. After behavioural tests tissues were collected for molecular analysis, however these results will not be presented or discussed here.

Materials and Methods

Experimental animals

Wild caught tadpoles were obtained from the University of Victoria, British Columbia. For wastewater exposures, tadpoles were housed at the Pacific Environmental Science Centre (PESC), North Vancouver, British Columbia in a covered outdoor facility. Tadpoles were brought indoors 48 hours prior to the start of the experiment, and housed at 19°C under a 16:8 h light: dark photoperiod. For the EDC cocktail exposure, tadpoles were housed at 15°C on a re-circulatory system in the Aquatic Research Facility at the University of Lethbridge, Alberta. Tadpoles were held on a light: dark 16:8 h photoperiod and fed *Spirulina* flakes *ad libitum* daily. Prior to running experiments tadpoles were

acclimated to 24°C for 24 hours. Tadpoles were staged according to the Taylor Kollros (TK) guide (Taylor and Kollros 1946; Appendix A).

Wastewater effluent preparation

Two types of treatment technologies were utilized to treat municipal wastewater: (1) anaerobic membrane bioreactors (AnBR) method at the University of Waterloo, Waterloo, Ontario (UWat) and (2) a biological nutrient removal (BNR) activated sludge method at the University of British Columbia, Vancouver, British Columbia (UBC). The AnMBR at UWat 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. The BNR at UBC was operated as described by Monti et al. (2006). Detailed descriptions of both wastewater treatment processes are provided in Appendix B. To generate wastewater effluent, a cocktail of known EDCs (Table 5.1) was prepared at the University of Victoria (UVic) and shipped to UWat and UBC. At each site, raw sewage was collected every other day from nearby sanitary sewers and spiked with either the EDC cocktail or a vehicle control comprised of methanol (0.0015%) and acetone (0.00014%). Two bench-top treatment plants were run in parallel at both UBC and UVic. At each site, one treatment plant processed the EDC cocktail-spiked sewage, and the other processed the vehicle control-spiked sewage. In each effluent type ammonia was monitored and is presented in Table 5.2. Effluent was collected over the course of approximately four days and stored at 4 °C before being shipped to PESC.

Table 5.1 –
 Concentrations of the endocrine disrupting compounds (EDCs) in the cocktail. All values represent µg/L. The common anthropogenic use for each EDC is provided.

Endocrine disrupting compounds	Concentration (µg/L)	Compound use	Reference
17α-ethynylestradiol (EE2)	0.006	synthetic hormone	(Gilbert 2000)
4-nonylphenol	22	detergent metabolite	(World Health Organisation 2012)
bis/Di (2-ethylhexyl) phthalate (DEHP)	40	plasticizer	(Kolpin et al. 2002)
butylparaben (butyl 4-hydroxybenzoate)	1	cosmetics	(World Health Organisation 2012)
caffeine	45	stimulant	(World Health Organisation 2012)
D4 (octamethylcyclotetrasiloxane)	5	cosmetics	(Kolpin et al. 2002)
D5 (decamethylcyclopentasiloxane)	5	cosmetics	(World Health Organisation 2012)
estrone	0.07	natural hormone	(World Health Organisation 2012)
ibuprofen	27	anti-inflammatory	(Kolpin et al. 2002; World Health Organisation 2012)
ketoconazole	0.02	antifungal	(Kolpin et al. 2002)
naproxen	15	anti-inflammatory	(Waye and Trudeau 2011)
thyroxine (T4)	0.1	natural hormone	(Melvin et al. 2014)
tonalide	1	cosmetics	(World Health Organisation 2012)
triclocarban	3	antibacterial	(Quanrud and Propper 2010)
triclosan	3	antimicrobial	(World Health Organisation 2012)

Table 5.2 –
 Measured and estimated ammonia concentrations of the University of Waterloo (UWat) and University of British Columbia (UBC) vehicle and endocrine disrupting compound (EDC) cocktail-spiked effluents. All measurements were taken in 100% effluent. Values represent mean \pm standard error (sample size).

Treatment	Ammonia (mg/L)
UWat effluent	
100 % vehicle-spiked	38.4 \pm 0.8 (7)
15 % vehicle-spiked*	5.8
7.5 % vehicle-spiked*	2.9
EDC cocktail-spiked	36.3 \pm 0.8 (7)
15 % EDC cocktail-spiked*	5.5
7.5 % EDC cocktail-spiked*	2.7
UBC effluent	
vehicle-spiked	0.1 \pm 0.0 (4)
50 % vehicle-spiked*	0.05
EDC cocktail-spiked	0.1 \pm 0.0 (4)
50 % EDC cocktail-spiked*	0.05

*estimated values

Wastewater exposures

For the UWat set, tadpoles (mean \pm 1 SE; total length 91.5 ± 0.1 mm; body mass 6.1 ± 0.3 g; median, range; TK 2.5, 0 – 12, n = 100) were exposed to one of (1) dechlorinated water, (2) 7.5% cocktail-spiked effluent, (3) 15% cocktail-spiked effluent, (3) 7.5% vehicle-spiked effluent, or (4) 15% vehicle-spiked effluent. For the UBC set, tadpoles (mean \pm 1 SE; total length 99.0 ± 0.1 mm; body mass 7.7 ± 0.2 g; median, range; TK 2, 3.4 – 11, n = 105) were exposed to one of (1) dechlorinated water, (2) 50% cocktail-spiked effluent, (3) 100% cocktail-spiked effluent, (4) 50% vehicle-spiked effluent, or (5) 100% vehicle spiked effluent. Tadpoles were exposed for 48 hours at 19°C at a ratio of 3.5 L per tadpole in aerated aquaria (3 – 4 tadpoles / aquaria). For UWat and UBC exposure sets, concentrations of effluent tested were determined with a range-finder test. Tadpoles were exposed to a geometric dilution series of effluent for 48 hours and mortality was measured. The highest concentration of effluent to cause no mortality was utilized in this study. Water quality parameters were measured daily and are reported in Table 5.3.

Effluent EDC analysis

For both exposure sets, water samples were collected from exposure aquaria on the first and last days of selected treatments. For UWat effluent exposures, 15% cocktail-spiked effluent, 15% vehicle-spiked effluent, and dechlorinated water samples were collected at the start and end of exposures. Additionally, 100% cocktail-spiked effluent and 100% vehicle-spiked effluent samples were also collected. For UBC effluent exposures, 100% cocktail-spiked effluent, 100% vehicle-spiked effluent, and well water samples were collected from exposure aquaria on the first and last days of exposures. All

Table 5.3 –
Water quality measurements for University of Waterloo (UWat), University of British
Columbia (UBC) wastewater, and endocrine disrupting compound (EDC) cocktail
exposures.

Parameter	UWat	UBC	Cocktail
Temperature* (°C)	15.0 ± 0.2 (60)	16.3 ± 0.3(60)	23.5 ± 0.2 (48)
Dissolved oxygen* (mg/L)	9.7 ± 0.1 (60)	9.5 ± 0.1(60)	7.2 ± 0.1 (48)
pH**	7.80, 7.08 – 8.48 (60)	8.08, 7.35 – 9.20 (60)	8.31, 7.40 – 8.5 (43)
Hardness* (mg/L as CaCO ₃)	88 ± 1 (7)	88 ± 1 (7)	176 ± 1 (10)
Alkalinity* (mg/L as CaCO ₃)	56.0 ± 1 (7)	56.0 ± 1 (7)	133 ± 1 (10)

* = mean ± standard error (sample size); ** = median, range (sample size).

samples were analyzed for the EDCs in the EDC cocktail (Table 5.4 and 5.5) using liquid chromatography at PESC.

Cocktail exposure

Tadpoles were exposed to either the EDC cocktail (Table 5.1), vehicle control (0.0015% methanol, 0.00014 % acetone), or dechlorinated water at 24°C for 48 hours. All exposures were conducted in aerated 15 L polypropylene buckets (Home Depot, North York, ON) at a ratio of 7.5 L per tadpole (2 tadpoles / bucket).

Behavioural experiments

After exposures to either UWat effluent, UBC effluent, or the EDC cocktail olfactory-mediated behavioural responses to 0.10 M, 0.15 M and 0.022 M (of L-alanine, L-serine, and glycine) amino acid mixture respectively were measured with the linear trough-style choice maze, as described in Chapter 3. Concentrations of the amino acid mixture used for each exposure were determined by preliminary experiments, which were completed at both PESC and the University of Lethbridge ARF. To prepare the amino acid mixtures, equal molarity L-alanine (USP grade, Sigma-Aldrich, Oakville, ON), L-serine (USP grade, VWR, Radnor, PA), and glycine (proteomics grade, AMRESCO, Cleveland, OH) were stirred into dechlorinated water. Amino acid mixtures were prepared fresh on the days of the behavioural experiments.

Subsequent to behavioural tests, tadpoles were euthanized in buffered tricaine methanesulfonate (1000 mg/L; TMS, Aqua Life, Syndel Laboratories, Nanaimo, BC, Canada). Tail fin, olfactory epithelium, olfactory bulb, eyes, and liver were excised and preserved in RNAlater solution (Ambion, Foster City, CA), to be analyzed with qPCR at the University of Victoria.

Statistical analyses

For behavioural experiments, tadpoles that did not leave the acclimation chamber throughout the test were removed from the analyses, as determined *a priori*. For each treatment, the average time spent in the stimulus and blank arm was calculated.

Parametric assumptions were tested using the Shapiro-Wilk normality test on the paired differences of time spent in the stimulus and blank arms of the maze. For data that satisfied parametric assumptions, mean time spent in the blank and stimulus 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). All analyses were completed using R version 3.2.2 (R Core Team 2015). Mean differences were considered to be significant when $p \leq 0.05$.

Results

UWat effluent exposure

Effluent analyses

During exposures it was noted that the EDC cocktail-spiked effluent smelled strongly of effluent, but the vehicle-spiked effluent did not. The results for the EDC analysis for the UWat effluent are presented in Table 5.4. Overall, most EDCs analyzed were below the detection limit, with some exceptions. Ibuprofen, naproxen, and caffeine, were detectable in 15% vehicle-spiked effluent and 15% EDC cocktail-spiked effluent treatments, as well as the 100% vehicle and 100% EDC cocktail-spiked effluent samples.

Table 5.4 –

Concentrations of endocrine disrupting compounds (EDCs) in University of Waterloo (UWat) treated wastewater effluent measured at the start and end of exposure periods. All values represent ng/mL. For Water, VL, VH, CL and CH values represent mean \pm standard error (n = 3). For 100% V and 100% C values represent measurement for one sample.

Endocrine disrupting compound	DL	Water – 0 hr	Water – 48 h	VH – 0 h	VH – 48 h	100 % V	CH – 0 h	CH – 48 h	100 % C
17 α -ethynylestradiol	0.05	0.149 \pm 0.032	0.195 \pm 0.010	< DL	< DL	< DL	< DL	< DL	< DL
17 β -estradiol	0.06	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
bis/Di (2-ethylhexyl) phthalate (DEHP)	0.06	1.123 \pm 0.174	1.410 \pm 0.394	0.914 \pm 0.223	7.687 \pm 5.567	5.975	1.346 \pm 0.017	1.789 \pm 0.217	1.202
caffeine	0.05	< DL	< DL	0.527 \pm 0.304	0.517 \pm 0.056	2.938	0.225 \pm 0.002	0.231 \pm 0.002	1.562
estrone	0.06	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
ibuprofen	0.05	< DL	< DL	1.335 \pm 0.2	1.292 \pm 0.097	7.949	5.585 \pm 0.034	5.177 \pm 0.276	40.087
ketoconazole	0.05	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
naproxen	0.05	< DL	< DL	0.727 \pm 0.092	0.720 \pm 0.079	3.497	1.862 \pm 0.052	1.854 \pm 0.033	10.204
nonylphenol		< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
thyroxine	0.05	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
tonalid	0.06	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
triiodothyronine	0.05	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
triclocarban	0.05	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL
triclosan	0.05	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< D

Short forms: VH = 15% vehicle-spiked effluent; 100% V = 100% vehicle-spiked effluent; CH = 15% cocktail spiked-effluent; 100% C = 100% cocktail-spiked effluent; DL = detection limit.

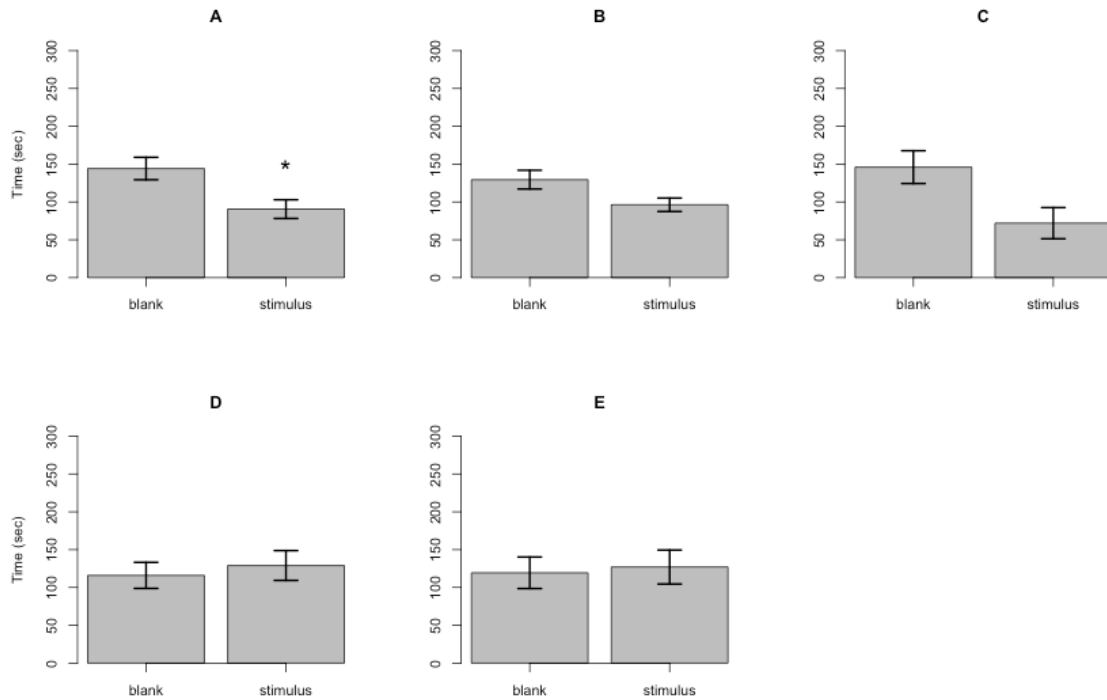


Figure 5.1 –

Time *Lithobates (Rana) catesbeiana* tadpoles spent in the blank (water) and stimulus (0.30 M amino acid mixture) arms of the linear-style choice maze after exposure to one of the University of Waterloo effluent treatments: (A) dechlorinated water, (B) 7.5% vehicle spiked-effluent, (C) 15% vehicle-spiked effluent, (D) 7.5% cocktail-spiked effluent, or (E) 15% cocktail-spiked effluent. Error bars represent mean \pm standard error. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 15 - 20$.

All treatments analyzed contained DEHP, with the highest concentration in 15% vehicle after 48 hours of exposure. Overall, the concentrations of EDCs observed were consistent through time. Interestingly, EE2 was measured in the dechlorinated water treatments, but not in any effluent treatments. All other EDCs analyzed were not detectable in any treatments.

Behaviour

Avoidance responses to the chemosensory stimulus are presented in Figure 5.1. Tadpoles exposed to dechlorinated water avoided the chemosensory stimulus and spent approximately 1.5 fold more time in the blank arm of the choice maze than the stimulus arm ($t_{18} = 2.09$, $p = 0.05$). Tadpoles exposed to 7.5 and 15% vehicle-spiked effluent also spent more time in the blank arm than the stimulus arm, however these differences were not significant (7.5% vehicle: $t_{18} = 1.89$, $p = 0.07$; 15% vehicle: $t_{14} = 1.96$, $p = 0.07$). Tadpoles exposed to 7.5 and 15% cocktail-spiked effluent spent a similar amount of time in the blank and stimulus arms of the maze (7.5% cocktail: $t_{19} = -0.37$, $p = 0.71$; 15% vehicle: $t_{16} = -0.19$, $p = 0.86$).

UBC effluent exposure

Effluent analyses

The results for the EDC analysis for the UBC effluent are presented in Table 5.5. Ketoconazole was the only EDC measured in all effluent treatments, however, it was only present in two samples in the 100% vehicle treatment. The concentration of ketoconazole was similar between all treatments. The detection of DEHP was inconsistent among treatments. Water samples taken at both time points and 100% vehicle and 100% cocktail.

Table 5.5 –

Concentrations of endocrine disrupting compounds (EDCs) measured in University of British Columbia (UBC) treated wastewater effluent measured at the start and end of exposure periods. Mean \pm standard error (n = 3) is provided where the EDC was detected in all replicates. A range or single value is given where the EDC was detected in < 3 replicates. All values represent ng/mL.

Endocrine disrupting compound	DL	Water – 0 hr	Water – 48 h	VH – 0 h	VH – 48 h	CH – 0 h	CH – 48 h
17 α -ethynylestradiol	0.05	< DL	< DL	< DL	< DL	< DL	< DL
17 β -estradiol	0.06	< DL	< DL	< DL	< DL	< DL	< DL
bis/Di (2-ethylhexyl) phthalate (DEHP)	0.06	0.928	0.581	1.46 – 1.735	< DL	1.866	< DL
caffeine	0.05	< DL	< DL	< DL	< DL	< DL	< DL
estrone	0.06	< DL	< DL	< DL	< DL	< DL	< DL
ibuprofen	0.05	< DL	< DL	< DL	< DL	< DL	< DL
ketoconazole	0.05	< DL	< DL	0.05 – 0.068	0.052 \pm 0.005	0.059 \pm 0.001	0.055 \pm 0.003
naproxen	0.05	< DL	< DL	< DL	< DL	< DL	< DL
nonylphenol	0.05	< DL	< DL	< DL	< DL	< DL	< DL
thyroxine	0.05	< DL	< DL	< DL	< DL	< DL	< DL
tonalid	0.06	< DL	< DL	< DL	< DL	< DL	< DL
tri-iodothyronine	0.05	< DL	< DL	< DL	< DL	< DL	< DL
triclocarban	0.05	< DL	< DL	< DL	< DL	< DL	< DL
triclosan	0.05	< DL	< DL	< DL	< DL	< DL	< DL

Short forms: VH = 100% vehicle-spiked effluent; CH = 100% cocktail-spiked effluent; DL = detection limit.

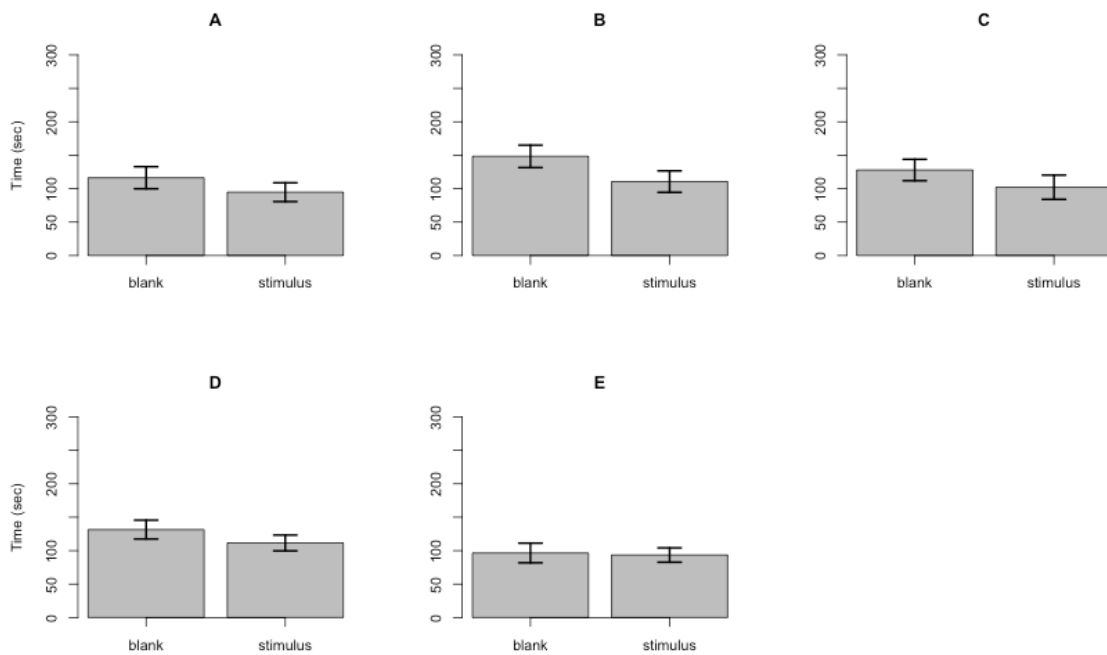


Figure 5.2 –

Time *Lithobates (Rana) catesbeiana* tadpoles spent in the blank (water) and stimulus (0.45 M amino acid mixture) arms of the linear-style choice maze after exposure to one of the University of British Columbia (UBC) effluent treatments: (A) dechlorinated water, (B) 50% vehicle-spiked effluent, (C) 100% vehicle-spiked effluent, (D) 50% cocktail-spiked effluent, or (E) 100% cocktail-spiked effluent. Error bars represent mean \pm standard error. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 17$

– 19.

treatments at the 0 hour time point contained DEHP, but not in all samples tested. No other analyzed EDC was detectable in any treatment.

Behaviour

Olfactory-mediated avoidance responses to the amino acid mixture were measured after exposure to the UBC effluent (Table 5.5), and are presented in Figure 5.2. There were no significant differences between time spent in the stimulus or blank arms for any treatments (water: $t_{18} = 0.79$, $p = 0.47$; 50% vehicle-spiked effluent: $t_{17} = 1.18$, $p = 0.25$; 100 % vehicle-spiked effluent: $t_{18} = 0.91$, $p = 0.37$; 50% EDC cocktail-spiked effluent: $t_{19} = 0.85$, $p = 0.40$; 100% cocktail-spiked effluent: $t_{19} = 0.21$, $p = 0.83$).

Cocktail exposure

Behaviour

Olfactory-mediated avoidance responses to the amino acid mixture were measured after exposure to the EDC cocktail (Table 5.1), and are presented in Figure 5.3. Tadpoles exposed to water and vehicle treatments spent significantly more time in the blank arm of the maze than the stimulus arm (water: $t_{21} = 2.9$, $p = 0.007$; vehicle: $t_{20} = 2.9$, $p = 0.008$). Tadpoles exposed to the EDC cocktail avoided the olfactory stimulus and spent approximately twice as much time in the blank arm than the stimulus arm of the maze ($t_{21} = 2.14$, $p = 0.05$).

Discussion

The present study tested the effects of treated wastewater from two wastewater treatment technologies, and a cocktail of EDCs on olfactory acuity in *L. catesbeiana*

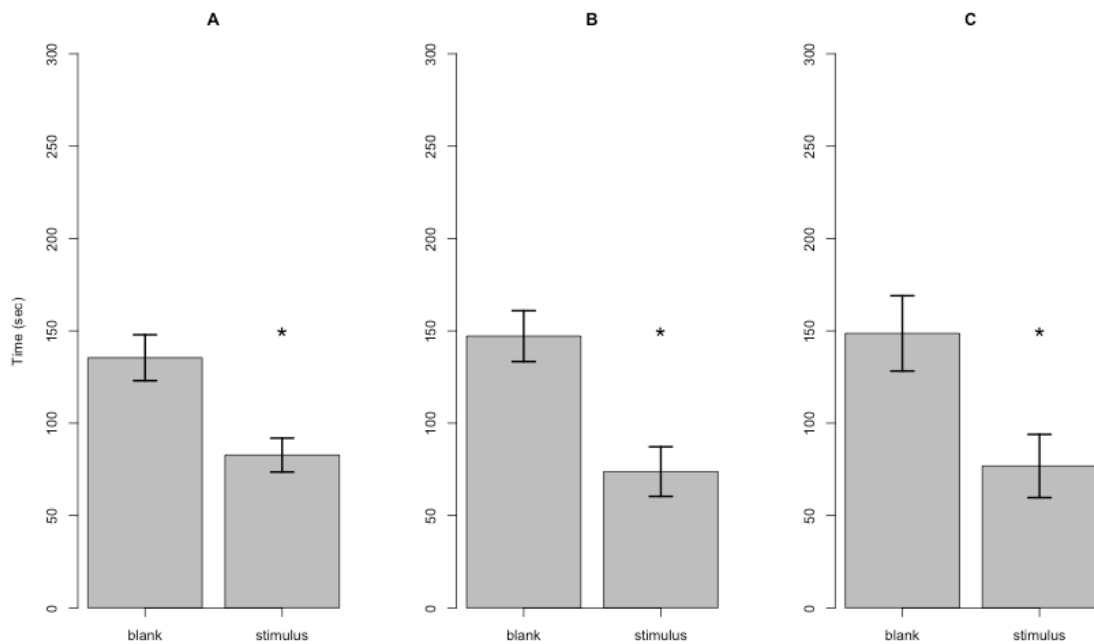


Figure 5.3 –

Time *Lithobates (Rana) catesbeiana* tadpoles spent in the blank (water) and stimulus (0.022 M each of L-alanine, L-serine, and glycine) arms of the linear-style choice maze after exposure to (A) dechlorinated water, (B) vehicle, or (C) endocrine disrupting compound (EDC) cocktail. Error bars represent mean \pm SE. An asterisk above a bar denotes a significant difference ($p \leq 0.05$), $n = 20 - 21$.

tadpoles. Unfortunately, the behavioural trials after exposure to UBC effluent were unsuccessful. As evident in Figure 5.2, tadpoles in the control treatments, water and vehicle- effluent, did not avoid the chemosensory stimulus. Therefore, these behavioural results will not be further discussed here.

Exposure to UWat treated wastewater effluent resulted in reduced olfactory-mediated avoidance responses to a chemosensory stimulus (Figure 5.1). Although tadpoles exposed to the vehicle-spiked effluent did not significantly avoid the chemosensory stimulus (Figure 5.1 B & C), it is evident these tests were trending towards significance, as tadpoles tended to spend more time in the blank arm than the cue arm. Tadpoles exposed to the cocktail-spiked effluents however, spent a similar amount of time in each arm (Figure 5.1 D & E). Though previous studies have reported effects of EDCs on behavioural endpoints including activity, startle response, and survivorship (Fraker and Smith 2004; Smith and Burgett 2005), there are a limited number of studies that investigate 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 our study however, it is unlikely poor water quality contributed to the reduced avoidance responses. Although the UWat cocktail-spiked effluent did smell strongly of sewage, behavioural trials were completed in clean dechlorinated water. Therefore, the reduced olfactory acuity observed in our study cannot be attributed to contaminant and stimulus interactions. Other studies have reported reduced behavioural or electrophysiological responses to chemosensory stimuli

after exposure to pesticides in fish (Moore and Waring 2001) and tadpoles (Ehram 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. Again, this experiment, and those previously mentioned, was 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 a 30 minutes in clean water. In the present study tadpoles were subjected to a 20-minute acclimation period in the behavioural 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 contributed to by thyroid disruption. As reported in Chapter 4, exposure of premetamorphic *L. catesbeiana* tadpoles to exogenous T₃ resulted in a comparable decrease in avoidance responses to the same amino acid mixture. Given the similarity in reduced avoidance responses between exposures, the persistent EDCs in the effluent in our study may have been agonistically T₃ active. As discussed in Chapter 4, the T₃ agonistic action of persistent EDCs may have contributed to early metamorphic changes in the olfactory system. In the olfactory system the olfactory epithelium is located inside the olfactory chamber and comes into contact with the aquatic environment. Olfactory sensory neurons (OSNs) are bipolar neurons that are housed in the olfactory epithelium and detect chemosensory stimuli (reviewed in Manzini and Schild 2010). Premetamorphic tadpoles have essentially no endogenous THs in circulation, and at this time there are no metamorphic changes in the olfactory epithelium.

However, during the metamorphic climax, when TH concentrations peak, the majority of the olfactory epithelium is remodelled. Almost all OSNs undergo apoptosis during the metamorphic climax and new olfactory neurons suited for aquatic and terrestrial environments will replace larval neurons (Dittrich et al. 2016). In this study, apoptosis of OSNs may have been initiated in the olfactory epithelium because of the T₃ agonistic action of persistent EDCs in the effluent. This hypothesis is further supported by the reduced olfactory-mediated behavioural and neurophysiological responses to chemosensory stimuli observed at the metamorphic climax, when compared to earlier developmental stages in *L. catesbeiana* (Chapter 3). Furthermore, Veldhoen et al. (2006) and Crump et al. (2002) reported early onset of metamorphosis after exposure to EDCs, which demonstrated the possible TH agonistic effects of EDCs. Therefore, the mechanism driving the reduced avoidance responses in this study could be partly developmental.

However, behaviour is not influenced by olfaction alone, but also by multiple hormone and sensory systems (reviewed in 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 in *O. tshawytscha* (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 UWat effluent. Therefore, the effects of UWat effluent exposure on the olfactory system were likely not resultant solely of TH disruption, but also by other pathways.

Wastewater treatment at the UWat was effective at removing most of the added EDCs from the UWat effluent (Table 5.4). However, even after treatment, some EDCs were detected in the UWat exposure effluent (Table 5.4). Caffeine, DEHP, ibuprofen, and naproxen are commonly found in treated effluents (reviewed in Snyder et al. 2008) and persisted in all vehicle and cocktail-spiked exposure treatments (Table 5.4). Of the EDCs persistent in the UWat treated effluent, there is evidence that DEHP, ibuprofen, and naproxen can be TH active. With respect to DEHP, there is no current literature on its effects on amphibian or fish behaviour, 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 (Jia et al. 2016), 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, Veldhoen et al. (2014) demonstrated ibuprofen can have agonistic effects on THs in *L. catesbeiana* tadpoles, as measured by the mRNA profile in the liver. But, given that both vehicle and cocktail-spiked effluents contained the same persistent EDCs, and olfactory function was not impaired in vehicle-spiked treatments, it is unlikely any of these EDCs contributed to the olfactory impairment in cocktail-spiked treatments. Tadpoles exposed to the EDC cocktail (Table 5.1) alone were subjected to higher concentrations of DEHP, ibuprofen, and naproxen than in the treated effluent (Table 5.4) and did not exhibit decreased avoidance responses to the chemosensory stimulus (Figure 5.5). Therefore, we can conclude that spiking the wastewater influent with the EDC cocktail did not contribute to the thyroid disrupting properties of the effluent, as measured by olfactory-mediated behaviour.

There is a discrepancy in olfactory-mediated behavioural responses after exposure to EDC cocktail-spiked UWat effluent and EDC cocktail. Although UWat effluent impaired olfactory-mediated avoidance responses (Figure 5.1), the EDC cocktail itself did not (Figure 5.3). The EDC cocktail spiked-UWat effluent treatments appear to have caused more extensive disruption of the olfactory-mediated avoidance responses than the EDC cocktail itself. This discrepancy may be a consequence of mixture effects. Although analysis of the effluent revealed undetectable to very low concentrations of the EDCs analyzed, there may have been other EDCs present and able to contribute to the effect on olfactory function. 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 NSAIDs inhibited tactile function in *L. peronii* tadpoles at lower concentrations than when tadpoles were exposed to individual NSAIDs (Melvin et al. 2014). Therefore, in our study the EDCs present in treated effluent may have acted synergistically to disrupt olfactory-mediated behavioural responses.

Overall, concentrations of persistent EDCs measured in the UWat treated effluent (Table 5.4) in our study were above global real-world concentrations. Particularly, concentrations of caffeine, DEHP, ibuprofen, and naproxen in the 100% effluents were higher than real-world effluent concentrations (Brun et al. 2006; Lishman et al. 2006; Ryu et al. 2011). When compared to concentrations measured in effluent impacted streams in

the USA, caffeine, ibuprofen, and naproxen concentrations in the 15% vehicle and effluent treatments of this study were also higher than real-world concentrations (Kolpin et al. 2002; Boyd et al. 2003). Conversely, concentrations of DEHP in the 15% vehicle and effluent treatments were comparable to DEHP concentrations measured in effluent receiving water (Jackson and Sutton 2008). The magnitude of effluent dilution in receiving waters varies depending on river size and flow, and the level of urbanization in the area (reviewed in Snyder et al. 2008), but previous studies have reported dilution factors of 13 – 26 (Kasprzyk-Hordern et al. 2009), or even as low as two (Sumpter 2005). Therefore, 7.5% effluent treatments (equivalent to a dilution factor of 13) are more representative of real-world effluent receiving streams than the 15% effluent treatments (equivalent to a dilution factor of 6). Given that even with comparable dilution factors, the concentrations of EDCs were higher than those measured in receiving streams, it is unlikely tadpoles in the environment would encounter EDCs at the concentrations tested in this study.

To better understand the possible causes of the effects of effluent exposures on chemosensory-mediated avoidance responses, future studies should routinely monitor ammonia, nitrates, and nitrites directly in exposure waters. Although ammonia was high in 100 % vehicle and EDC cocktail-spiked UWat effluents, after dilution, treatment ammonia concentrations were likely within British Columbia water quality guidelines (Meays 2009; Table 5.2). However, negative effects of chronic ammonia (≥ 0.6 mg/L) exposure on survival, growth, and development in *Rana clamitans* have been reported (Jofre and Karasov 1998). Other studies have also reported negative effects of nitrate exposures on behavioural endpoints, morphology, and growth (reviewed in Rouse et al. 1999; Krishnamurthy et al. 2006) in various tadpole species, but often at elevated

concentrations. Therefore, nitrate and ammonia concentrations could contribute olfactory-mediated behavioural effects and should be closely monitored.

The lack of data on the molecular action of the EDC spiked UWat effluent and EDC cocktail limit our study. Analysis of additional TH-responsive endpoints such as plasma T₃ and T₄ concentrations, and expression of nuclear TH receptors TR α and TR β could establish a link between TH disruption and possible associated olfactory-mediated behavioural responses.

To conclude, exposure of premetamorphic *L. catesbeiana* tadpoles to EDC-spiked treated UWat effluent inhibited olfactory-mediated avoidance responses. Tadpoles exposed to the EDC cocktail did not reduce avoidance responses to the olfactory stimulus. Reduced olfactory-mediated avoidance responses after effluent exposure may have been contributed to by thyroid disruption from EDCs, but were likely resultant of disruption on multiple hormone and sensory systems. Mixture effects from the multitude of EDCs that may have been present in the wastewater effluent may be intensifying both TH disruption and other mechanisms of olfactory disruption, which contributed to the observed reduced olfactory-mediated avoidance responses. In closing, impaired olfactory function may have a profound effect on frog populations, as it may directly influences their ability to avoid predators.

CHAPTER 6: General conclusions

The present study addressed a variety of research questions regarding olfaction in *Lithobates (Rana) catesbeiana* tadpoles. The objectives of the present study were two-fold: first, to develop techniques to study tadpole olfactory acuity and study the ontogeny of olfactory acuity throughout metamorphosis; and second, to investigate the effects of thyroid hormones (THs), endocrine disrupting compounds (EDCs), and wastewater on olfactory-mediated avoidance responses.

To study olfactory acuity in *L. catesbeiana* tadpoles a neurophysiology technique was developed. Electro-olfactography (EOG) is commonly used with fish but was modified for use with *L. catesbeiana* tadpoles (Chapter 2). Consistent EOG responses to *Spirulina* extract and L-alanine were measured in tadpoles Taylor and Kollros (1946; TK) stages XII – XVIII. Although the development of the tadpole EOG methodology introduces a new technique from which to study tadpole olfaction, it is likely only suitable for use with *L. catesbeiana*. For tadpole EOG to be successful, olfactory pits must be exposed, yet intact, and electrodes must be placed at specific places on the olfactory epithelium. The large size of *L. catesbeiana* tadpoles, when compared to all other species, satisfies these EOG requirements and makes this technique possible. Therefore, it highly unlikely this EOG technique would be suitable with other tadpole species. The usefulness of tadpole EOG is also limited by the specific developmental stage requirements in order for the technique to be successful. As reported in Chapter 3, successful EOG measurements are only possible on *L. catesbeiana* tadpoles > TK IX. Considering the limitations associated with tadpole EOG, neurophysiological techniques such as patch-clamp and calcium imaging will continue to be used to study olfactory acuity in tadpoles.

A linear trough maze was used to study chemosensory-mediated behavioural responses in *L. catesbeiana* tadpoles. The linear trough maze has been previously used with fish (Dew et al. 2014), but was modified for use with *L. catesbeiana* tadpoles. In the present study both attraction and avoidance responses to *Spirulina* extract (Chapter 3) and an amino acid mixture (Chapter 3 – 5) respectively were measured. Considering the few developmental stage limitations (Chapter 3) and the simplicity of the assay, the linear trough maze is a useful behavioural test can have applications across many tadpole species.

The ontogeny of olfactory structures and associated acuity were characterized in *L. catesbeiana* tadpoles, as discussed in Chapter 3, and are summarized in Figure 6.1. Scanning electron micrographs indicated that olfactory structures developed gradually in premetamorphic *L. catesbeiana* tadpoles. Although olfactory epithelium development had not been previously studied in *L. catesbeiana*, similar findings were reported by Klein and Graziadei (1983) in premetamorphic *X. laevis* tadpoles. In the present study, as tadpoles approached metamorphic climax, reduced olfactory responses to *Spirulina* extract were reported, as measured by EOG and the linear trough maze (Chapter 3). The observed reduction in chemosensory acuity, were likely due to developmental changes in the olfactory epithelium. Decreased olfactory acuity to chemosensory stimuli at metamorphic climax has also been previously reported in *X. laevis* tadpoles as a result developmental changes in the olfactory epithelium, such as increased selectivity of olfactory sensory neurons (OSNs) and mass OSN apoptosis (Manzini and Schild 2004; Dittrich et al. 2016). Although data on the development of *L. catesbeiana* olfactory system during metamorphosis are limited, the olfactory system of *X. laevis* tadpoles has

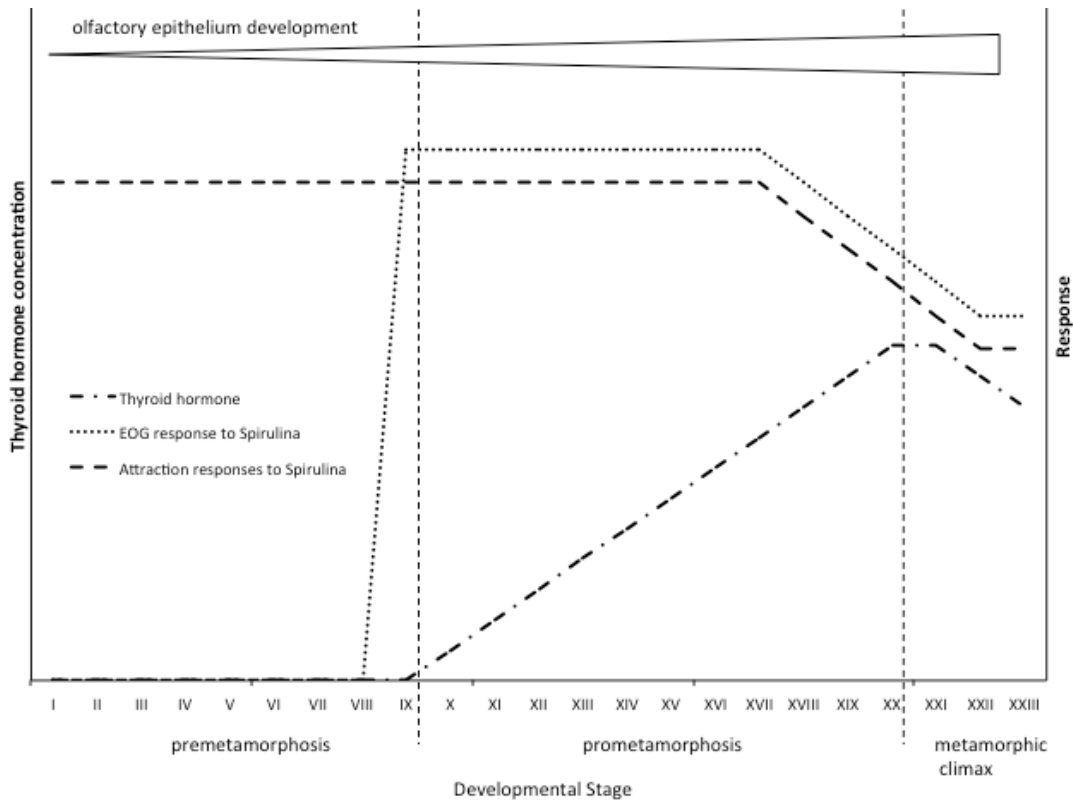


Figure 6.1 –

Representation of the ontogenetic changes to olfactory sensory structures and associated acuity in *Lithobates (Rana) catesbeiana* tadpoles, based on data collected in the present study. Endogenous thyroid hormone (TH) concentrations begin to rise in prometamorphosis and peak at metamorphic climax. Olfactory sensory structures are underdeveloped in premetamorphic tadpoles and continue to develop gradually throughout metamorphosis. Electro-olfactography (EOG) responses to *Spirulina* extract are measurable in tadpoles > Taylor and Kollros (1946; TK) stage IX. *Spirulina* extract elicits the greatest EOG responses during prometamorphosis, but EOG responses diminish at the metamorphic climax. Reduced attraction responses to *Spirulina* extract at metamorphic climax were also observed.

been well documented and is overall considered well conserved across anuran species (Gascuel and Amano 2013). Therefore OSN apoptosis and increased selectivity of OSNs may also be the mechanisms behind the change in olfactory acuity observed in the present study. However, the life histories of *L. catesbeiana* and *X. laevis* are very different. Adult *X. laevis* tadpoles remain predominantly aquatic but adult *L. catesbeiana* emerge onto land (reviewed in McDiarmid and Altig 1999). Hence, there are likely differences in olfactory systems at a cellular level between *X. laevis* tadpoles and other anuran species, including *L. catesbeiana*. In contrast, life histories among *L. catesbeiana* and most other anurans are comparable (reviewed in McDiarmid and Altig 1999), and therefore the results reported in the present study may apply to other tadpole species.

To investigate the effects of THs on olfactory-mediated behaviour, premetamorphic *L. catesbeiana* tadpoles were exposed to one of 3,5',3-triiodothyronine (T₃), thyroxine (T₄), or 17 β -estradiol (E₂) and their chemosensory-mediated avoidance responses were measured (Chapter 4). Exposure to T₃ resulted in reduced chemosensory-mediated avoidance responses. Considering the change in chemosensory acuity observed at later developmental stages (Chapter 3), T₃ exposures might have initiated precocious metamorphic changes in the olfactory system. In contrast, T₄ exposures had no effect on chemosensory-mediated avoidance responses. The discrepancy between effects of T₃ and T₄ exposure was surprising and may have been a result of tadpoles from different developmental stages used (Chapter 4). To gain a better understanding of the effects of exogenous thyroid hormone exposure on the anuran olfactory system, future study could include T₃ and T₄ exposures using similarly staged tadpoles.

To study the possible thyroid activity of EDCs in treated wastewater effluent, tadpoles were exposed to two different treated municipal effluents. Exposure to treated municipal wastewater effluent from Waterloo, ON (UWat; Chapter 5) resulted in reduced avoidance responses. Similar reduced chemosensory-mediated avoidance effects were observed after both T₃ (Chapter 4) and UWat effluent exposure, and therefore the mechanism behind these effects may have been similar. Endocrine disrupting compounds in the UWat effluent may have acted synergistically to mimic T₃, resulting in precocious metamorphic changes in the olfactory system. However, given the variable composition of effluent, some compounds in effluent may have caused reduced chemosensory-mediated avoidance responses through damaging OSNs, inducing chemosensory fatigue, or acting on other hormone and sensory systems. Therefore the mechanism behind the reduced chemosensory-mediated behavioural responses caused by effluent exposure is unclear. Furthermore, in the present study effluent exposures were above environmentally relevant effluent concentrations. Therefore, in order to gain a better understanding on the potential effects of wastewater effluent exposure on behaviour in *L. catesbeiana* tadpoles, future studies should use more realistic exposure scenarios.

Further study the effects of exogenous TH exposure on olfaction, and the possible thyroid disrupting properties of wastewater, should incorporate additional TH-responsive endpoints. Unfortunately, in the present study a clear link between exogenous THs and olfactory acuity or wastewater effluent exposure and TH disruption was not established. Anchoring behavioural responses to 1) physiological endpoints such as plasma T₃, T₄, and thyrotropin (TSH) concentrations, and 2) molecular markers including thyroid receptors (TRs) TR α and TR β , could establish a clear link between the thyroid system and the olfactory system. Furthermore, measuring multiple TH-responsive endpoints could

inform the development of an adverse outcome pathway (AOP) (Ankley et al. 2010). An AOP for TH disruption in *L. catesbeiana* tadpoles would be a valuable tool to monitor the efficacy of wastewater treatment plants at removing EDCs, and to identify thyroid activity in effluent. Lastly, intact olfaction is necessary for the survival of *L. catesbeiana* tadpoles and directly influences their ability to locate food and avoid predators. Therefore chemosensory-mediated behavioural effects of TH disruption are ecologically relevant endpoints that can be indicative of the potential effects of EDCs in wastewater effluent receiving streams.

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












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Appendix A: Tadpole staging guide

TK Stage	Gosner Stage	Image	Stage Criteria
LIMB BUD (26 – 30)			
I	26		length < 0.5 x diameter
II	27		length ≥ 0.5 x diameter
III	28		length ≥ 1 x diameter
IV	29		length ≥ 1.5 x diameter
V	30		length = 2 x diameter
TOE DEVELOPMENT (31 – 37)			
VI	31		paddle, no toe development
VII	32		indentation between 4-5
VIII	33		2nd indentation between 3-4
IX	34		3rd indentation between 2-3
X	35		4th indentation between 1-2
XI	36		toes 3-5 separated
XII	37		all toes separated
XIII	38		metatarsal tubercle apparent
XIV	39		leg 1.0-1.4 cm (based upon ~6 g tadpoles); subarticular tubercles
XV	40		leg 1.5-1.9 cm (based upon ~6 g tadpoles)
XVI	40		leg 2.0-2.9 cm (based upon ~6 g tadpoles); cloacal tail piece present.
XVII	40		leg > 3.0cm (based upon ~6 g tadpoles); cloacal tail piece present.
XVIII	41		loss of cloacal tail piece
XIX	41		clearing of forelimb skin window
XX	42		emergence of forelimb; mouth anterior to nostril

TK Stage	Gosner Stage	Image	Stage Criteria
XXI	43		mouth angle midway between nostril and anterior margin of the eye, tail longer than extended hindlimb
XXII	44		mouth angle middle of eye, tail shorter than extended hindlimb
XXIII	45		mouth angle reach posterior margin of eyeball
XXIV	45		tail stub < 1cm
XXV			tail gone = froglet (no black stub)

*Provided by Dr. Caren Helbing, Department of Biochemistry and Microbiology, University of Victoria.

**Based on Gosner (1960).

Appendix B: Wastewater treatment configurations

University of Waterloo wastewater treatment

AnMBR Configuration

Two lab scale submerged AnMBR's that each had a total volume of 5 L (working volume of 4 L) and equipped with ZeeWeed hollow fibre PVDF membranes (nominal pore size of 0.04 μm and surface area of 0.047 m^2) were employed. An automated Stenner peristaltic pump was used to transfer raw wastewater from a continuously mixed feed tank to the bioreactor while waste biomass (WAS) was manually wasted from the bottom of the bioreactor. Permeate was drawn from the membrane cartridge using another Stenner peristaltic pump. Both the feed pump and the permeate pump were programmed and automated using a Light-O-Rama controller unit to achieve the desired operating conditions. A pressure transducer connected to a Track-It Data Logger was attached to the permeation line to measure transmembrane pressure (TMP). The membrane was operated in a relaxed mode with 8 minutes of permeation followed by 2 minutes of relaxation. Gas sparging was employed to control membrane fouling using a Cole-Parmer peristaltic pump, that recycled gas from the headspace to a sparger located the bottom of the membrane module. Figure 1 displays the schematic of the bioreactors.

Operational Conditions

The two AnMBR's were maintained at room temperature (22 – 24 °C) and operated in parallel throughout the experiment. The feed was untreated municipal sewage collected directly from the City of Waterloo sanitary sewers. Collection was done once every two days and it was stored at 15 °C to prevent biodegradation.

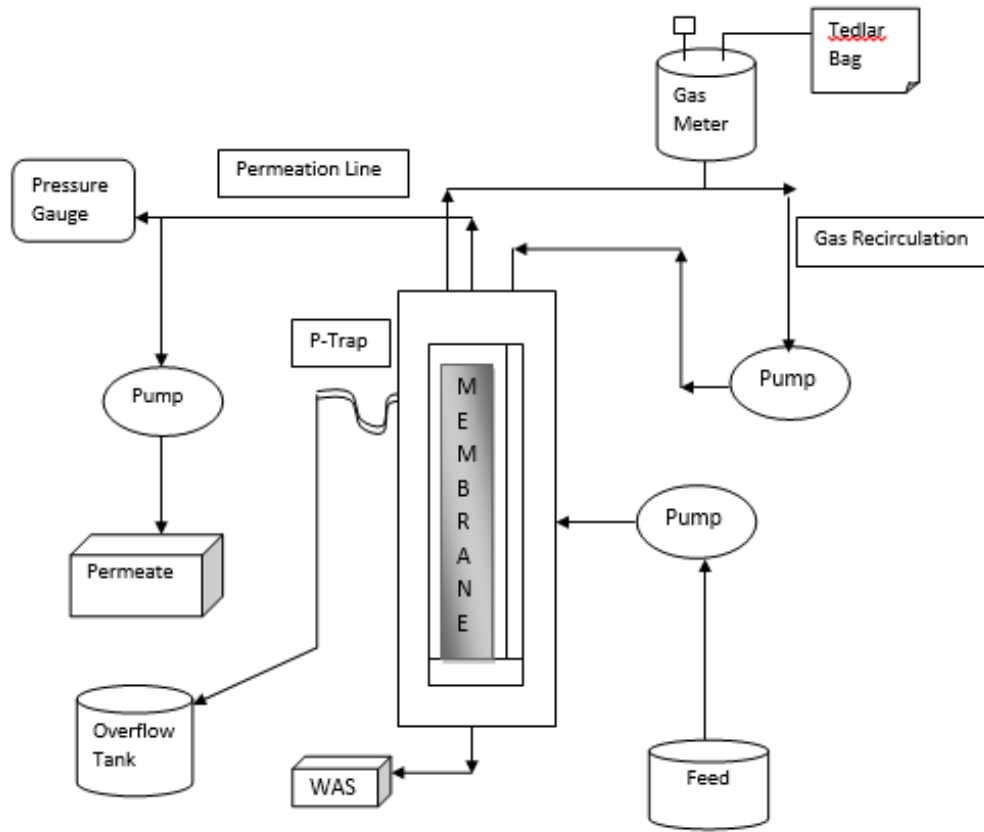


Figure 1 -
Schematic of the lab scale AnMBR

The bioreactors in this study were operated at a constant flux of 8.86 litres per m²/hour (LMH_ while TMP was monitored. A volume of 10 L of permeate was generated from each AnMBR per day yielding an hydraulic retention time (HRT) of 9.5 hours. A Solids Retention Time (SRT) of 30 days was employed to provide sufficient duration for biological activities and to prevent biomass washout and hence a sludge wasting rate of 0.13 L/d was employed.

Provided by: Dr. Wayne J. Parker, Department of Civil and Environmental Engineering,
University of Waterloo, Waterloo, ON.

University of British Columbia wastewater treatment MEBPR Reactor Layout and configuration

The biological phosphorus and nitrogen removal wastewater treatment pilot plant at the University of British Columbia has been operated and maintained by the Department of Civil Engineering for a number of years. The pilot plant was used for conducting the proposed research project. It consists of two parallel trains of the University of Cape Town (UCT) type wastewater treatment process. Currently, both the trains are operated as a membrane enhanced biological phosphorus removal processes (MEBPR) by using ZeeWeed ultrafiltration membrane units (GE Water and Process Technologies - ZENON Membrane Solutions, Oakville, Ontario) placed in the aeration tanks. ZeeWeed is a hollow fiber membrane assembled in a shell-less module that is immersed directly in the mixed liquor. The membrane module has a surface area of about 12.2 m² and a nominal pore size of 0.04 micrometers. Filtration is from the outside-in under a negative pressure applied to the permeate side. The hollow fibers are continuously scoured by coarse bubble aeration. Both treatment trains are identical in process configuration and operation.

Raw sewage from a nearby UBC main sewer is pumped into three storage tanks at the pilot plant to maintain a constant head. The sewage is kept well mixed in the tanks using mixers to avoid suspended solids settling. The raw sewage is then pumped to a primary clarifier. Overflow from the primary clarifier (primary effluent) serves as the influent to both biological treatment trains. The influent enters the anaerobic reactors and overflows by gravity to the anoxic and then the aerobic reactors.

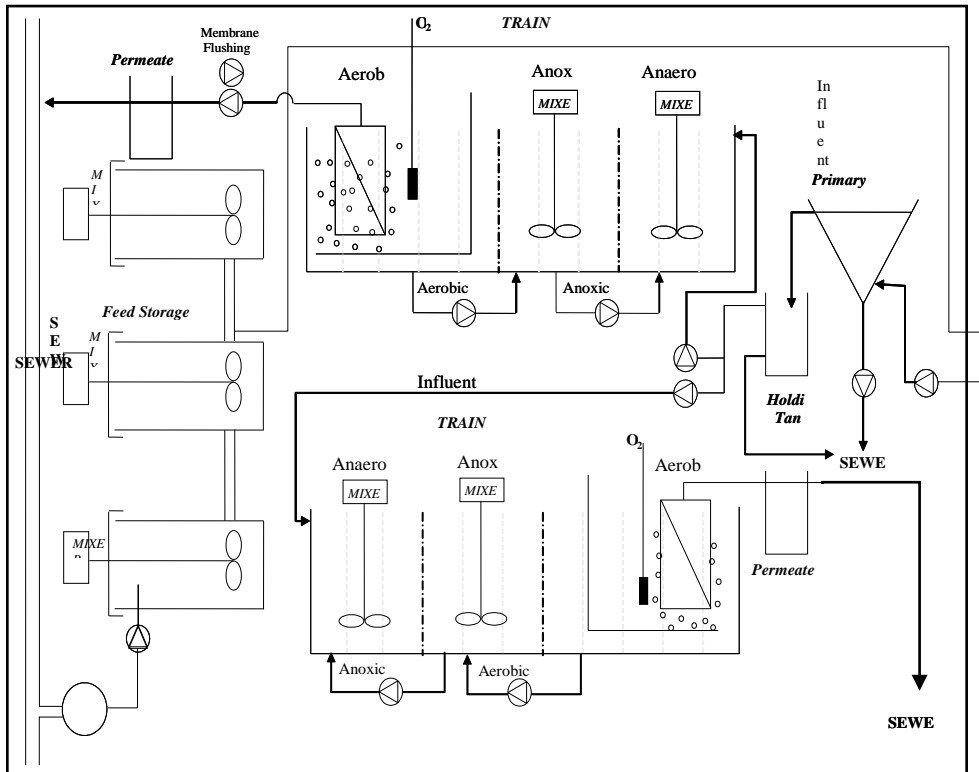


Figure 1 –
Schematic of parallel MEBPR trains

For the MEBPR process, the air needed in the aeration stage is introduced into the reactor at the membrane module to create the required agitation to reduce membrane fouling. Membrane filtered effluent exits the system after the aerobic stage. Mixed liquor is recycled from the aerobic reactor to the anoxic reactor. A recycle stream also leaves the anoxic reactor to the anaerobic reactor. Process reactor volumes are given in Table 1. More details about the UBC wastewater treatment pilot plant process description are given by Monti *et al.* (2006).

Provided by: Dr. Eric Hall, Department of Civil Engineering, University of British Columbia, Vancouver, BC

Table 1 -
Design specifications of the biological zones of the UBC wastewater treatment
pilot plant.

Biological	Train A	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