5-2009

The effects of wastewater effluent on a fish and amphibian species

Anthony Sowers
Clemson University, asowers@clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations

Part of the Animal Sciences Commons

Recommended Citation
https://tigerprints.clemson.edu/all_dissertations/355

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
THE EFFECTS OF WASTEWATER EFFLUENT ON
A FISH AND AMPHIBIAN
SPECIES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

by
Anthony D. Sowers
May 2009

Accepted by:
Dr. Stephen Klaine, Committee Chair
Dr. Cindy Lee
Dr. Aaron Roberts
Dr. William Baldwin
Dr. Michelle Boone
ABSTRACT

Wastewater effluents have been shown to contain a variety of anthropogenic compounds, many of which have endocrine-disrupting properties. While multiple laboratory studies have shown the effects of such compounds on an individual basis at elevated concentrations, little research has attempted to characterize the effects of exposure to environmentally relevant mixtures of endocrine disruptors. The current study examined the effects of long-term exposure to graded concentrations of wastewater effluent on the fathead minnow, *Pimephales promelas*, and the northern leopard frog, *Rana pipiens*. Fathead minnows were exposed from the larval stage through sexual maturity, while northern leopard frogs were exposed as eggs and grown through metamorphosis. An F1 generation of fathead minnows was cultured in control water to test for transgenerational effects of the parental exposure to wastewater effluent. A third experiment examined the effect of triclosan, an antimicrobial compound commonly found in wastewater effluent, on the growth and development of the pickerel frog, *Rana palustris*. A decrease in the expression of secondary sex characteristics and an increase in the gonadosomatic index of male fathead minnows exposed to wastewater effluent in the parent generation were observed. Accelerated reproductive activity and an increase in the expression of male secondary sex characteristics were observed in the F1 generation whose parent generation was cultured in wastewater treatments. Juvenile male northern leopard frogs exhibited an increased incidence of individuals with testicular oocytes. Leopard frogs cultured in the two highest concentrations of wastewater effluent took significantly longer to reach metamorphosis than both the
control and the lowest wastewater concentration treatment. Exposure to triclosan did not significantly affect the growth or development of the pickerel frog. The results of this study suggest that long-term exposure to wastewater effluent can interfere with the sexual development of the fathead minnow and can elicit responses in subsequent generations. Sexual development of male leopard frogs and the metamorphic process in leopard frogs is particularly sensitive to wastewater exposure.
ACKNOWLEDGEMENTS

I would like to take this opportunity to thank everyone who has contributed to the completion of this dissertation. My committee members as well as my labmates were all instrumental in my success as a PhD candidate. My achievements would not have been possible without the continued support of my family. I would also like to acknowledge the United States Environmental Protection Agency for funding me as a research assistant.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>CHAPTER 1: Literature review of endocrine disruptors in wastewater effluent and their biological effects on fish and amphibians</td>
<td></td>
</tr>
<tr>
<td>I. Endocrine disruptors in wastewater effluents</td>
<td>1</td>
</tr>
<tr>
<td>II. Fish reproductive endocrinology</td>
<td>4</td>
</tr>
<tr>
<td>III. Effects of estrogenic EDCs on fish</td>
<td>6</td>
</tr>
<tr>
<td>IV. Amphibian developmental endocrinology</td>
<td>8</td>
</tr>
<tr>
<td>V. Effects of estrogenic EDCs on amphibians</td>
<td>11</td>
</tr>
<tr>
<td>VI. References</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER 2: The effects of wastewater effluent on the fathead minnow, <em>Pimephales promelas</em></td>
<td>19</td>
</tr>
<tr>
<td>I. Abstract</td>
<td>19</td>
</tr>
<tr>
<td>II. Introduction</td>
<td>20</td>
</tr>
<tr>
<td>III. Materials &amp; methods</td>
<td>22</td>
</tr>
<tr>
<td>IV. Results</td>
<td>28</td>
</tr>
<tr>
<td>V. Discussion</td>
<td>32</td>
</tr>
<tr>
<td>VI. References</td>
<td>40</td>
</tr>
</tbody>
</table>
# CHAPTER 3: The effects of wastewater effluent on the northern leopard frog, *Rana pipiens*

I. Abstract .......................................................... 53  
II. Introduction ................................................... 54  
III. Materials & methods ...................................... 56  
IV. Results .......................................................... 61  
V. Discussion ..................................................... 66  
VI. References .................................................... 73  

# CHAPTER 4: Long-term exposure of the pickerel frog, *Rana palustris*, to triclosan

I. Abstract .......................................................... 82  
II. Introduction ................................................... 82  
III. Material & methods ........................................ 84  
IV. Results .......................................................... 87  
V. Discussion ..................................................... 88  
VI. References .................................................... 92  

CONCLUSION ......................................................... 96
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Endocrine-disrupting compounds measured in Mauldin Road Wastewater Treatment Plant effluent</td>
</tr>
<tr>
<td>2.2</td>
<td>Estradiol equivalency factors in wastewater effluent</td>
</tr>
<tr>
<td>2.3</td>
<td>Length, weight, condition factor, GSI, and HSI of fathead minnows exposed to wastewater effluent</td>
</tr>
<tr>
<td>2.4</td>
<td>Cumulative egg production of fathead minnows exposed to effluent</td>
</tr>
<tr>
<td>3.1</td>
<td>Endocrine-disrupting compounds measured in Mauldin Road Wastewater Treatment Plant effluent</td>
</tr>
<tr>
<td>3.2</td>
<td>Estradiol equivalency factors in wastewater effluent</td>
</tr>
<tr>
<td>3.3</td>
<td>Survival, HSI, length, weight, and swim speed of northern leopard frogs exposed to wastewater effluent</td>
</tr>
<tr>
<td>4.1</td>
<td>Survival, HSI, length, hind limb length, and weight of pickerel frogs exposed to triclosan</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Outline of isolated breeding experiment</td>
<td>47</td>
</tr>
<tr>
<td>2.2</td>
<td>Percent survival of fathead minnows exposed to wastewater effluent</td>
<td>48</td>
</tr>
<tr>
<td>2.3</td>
<td>Secondary sex characteristics of male fathead minnows</td>
<td>49</td>
</tr>
<tr>
<td>2.4</td>
<td>Histological sections of fathead minnow gonads</td>
<td>50</td>
</tr>
<tr>
<td>2.5</td>
<td>Secondary sex characteristics of male fathead minnows in an F1 generation</td>
<td>51</td>
</tr>
<tr>
<td>2.6</td>
<td>Testis from fathead minnows displaying gonadal abnormalities</td>
<td>52</td>
</tr>
<tr>
<td>3.1</td>
<td>Time to metamorphosis for the northern leopard frog and the percentage of</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>individuals reaching metamorphosis by the end of the experiment</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Histological sections of northern leopard frog gonads</td>
<td>80</td>
</tr>
<tr>
<td>3.3</td>
<td>Histological sections of northern leopard frog thyroid glands</td>
<td>81</td>
</tr>
<tr>
<td>4.1</td>
<td>Time to forelimb emergence for the pickerel frog and the percentage of</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>individuals at forelimb emergence by the end of the experiment</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1: Literature review of endocrine disruptors in wastewater effluent and their biological effects on fish and amphibians

I. Endocrine disruptors in wastewater effluents:

Municipal wastewater effluents around the world have been shown to contain measurable concentrations of pharmaceuticals and personal care products (PPCPs) and natural and synthetic hormones (Nakada et al. 2004, Nakada et al. 2006, Snyder 2008). A large number of these compounds act as endocrine disruptors (EDC) and interfere with endocrine system-mediated functions acting as either agonists or antagonists of endogenous hormones. A survey conducted throughout the United States between 1999 and 2000 by the US Geological Survey found the presence of organic wastewater-derived contaminants, many of which are known EDCs, in 80% of the 139 surface waters sampled (Kolpin et al. 2002).

Aquatic systems in arid regions that rely on wastewater effluents as a dominant portion of their flow and/or volume are vulnerable to elevated EDC concentrations when compared to systems with significant dilution effects. During dry periods, the flow in the Trinity River below the Dallas/Ft. Worth metroplex in Texas is comprised of >90% wastewater effluent (Fono et al. 2006). Boulder Creek, a tributary of the South Platte River, receives wastewater effluent discharge from the city of Boulder. During low-flow conditions, >75% of Boulder Creek’s flow is comprised of effluent. A large increase in the estrogenic activity of Boulder Creek is positively correlated with increased effluent concentrations (Vajda et al. 2008).
The presence of estrogenic/anti-androgenic compounds in wastewater effluents is a major area of concern and has been studied extensively (Byrns 2001, Murk et al. 2002, Nakada et al. 2004, Servos et al. 2005). Estrogenic/anti-androgenic compounds of interest include the natural estrogen, 17-β estradiol (E2); the synthetic estrogen originating in oral contraceptives, ethynylestradiol (EE2); bisphenol-A (BPA), which is a component of polycarbonate plastics; and a class of industrial compounds known as the alkylphenols, which include 4-nonylphenol (NP) and 4-tert-octylphenol (OP). E2 and EE2 are the most potent estrogen receptor agonists of the aforementioned compounds, while BPA, NP, and OP have estrogen receptor affinities that are orders of magnitude weaker (Rutishauser et al. 2004). The steroid hormones testosterone, androstenedione, progesterone, and dihydrotestosterone are also commonly found in wastewater effluents and may elicit androgenic and/or anti-estrogenic responses (Kirk et al. 2002). The occurrence of steroid hormones in wastewater effluent is generally in the parts per trillion (ng/L) range, while the industrial compounds may reach the parts per billion (ug/L) range making them relevant to study despite their very low estrogen receptor affinity.

In an effort to compare the estrogenic activity of wastewater effluents from different wastewater treatment plants, estradiol equivalency factors (EEQs) have been used. An EEQ is determined either by an in vivo assay comparing the magnitude of an estrogen-dependent response of organisms treated with known concentrations of E2 with the same response of organisms exposed to a solution of unknown estrogenicity or by multiplying measured concentrations of estrogenic compounds by established estrogenic potencies. Tilton et al. (2002) determined an EEQ by exposing male channel catfish to
known concentrations of E2 and measuring plasma vtg following exposure. An E2
collection-vtg response curve was generated and used to determine the EEQ of
effluent from two wastewater treatment plants by measuring vtg concentrations in male
channel catfish exposed to the effluents. Another method of determining EEQs is
through the use of a recombinant yeast estrogen screen. In this method, a human
estrogen receptor is transfected into yeast cells along with a reporter gene. Activity of the
translated product of the reporter gene can then be quantified. A response curve of E2
consentations to reporter gene activity can be established and used to quantify the EEQ
of other compounds. Rutishauer et al. (2004) used this method to determine the EEQs of
EE2, NP, BPA, OP, and whole effluent from two wastewater treatment plants.

A wide range of EEQ values have been reported for wastewater effluents. Tilton
et al. (2002) measured effluent EEQ values ranging from 21 to 47 ng/L from two
wastewater treatment plants in Mississippi. Effluent from a wastewater treatment plant in
northern Italy had an EEQ that ranged from 0.28-2.40 ng/L (Schiliro et al. 2004). Two
wastewater treatment plants in Switzerland displayed EEQs generally in a range between
1 and 10 ng/L, although at one sampling event an EEQ well above 10 ng/L was measured
(Rutishauser et al. 2004). A wastewater treatment plant in Minnesota displayed a mean
EEQ of 44.0 ± 0.9 ng/L (Martinovic et al. 2007). Effluent from five wastewater
treatment plants in Australia displayed EEQs generally ranging from <1 to 14.8 ng/L,
with one sample reaching a maximum EEQ of 67.8 ng/L (Tan et al. 2007). Based on the
aforementioned data, EEQs of municipal wastewater effluent can range from below 1 to
greater than 50 ng/L.
Despite their ubiquitous occurrence, the removal of EDCs is not targeted through traditional wastewater treatment processes. Recent research has focused on assessing the potential for municipal wastewater treatment plants to degrade EDCs through a variety of alternative processes. The use of ozone and/or advanced oxidative processes has proven to be a viable option for the degradation of EDCs, but has not seen widespread application (Ikehata et al. 2008, Snyder 2008). Further studies on the risks associated with EDCs in wastewater effluent are needed to warrant the implementation of treatment processes targeting EDC removal.

II. Fish reproductive endocrinology:

The sexual maturation and reproductive activity of teleost fish is controlled by the hypothalamus-pituitary-gonadal (HPG) axis of the endocrine system. Gonadotropic-releasing hormone (GnRH) secreted by the hypothalamus stimulates the anterior portion of the pituitary gland to produce the gonadotropic hormones (GtHs) (Moyle and Cech 2004). GtHs, which include luteinizing hormone (LH) and follicle stimulating hormone (FSH), initiate and regulate gonadal differentiation and development (Swanson et al. 2003).

In addition to regulating gonadal development, GtHs also stimulate the gonads to produce androgenic and estrogenic steroid hormones. Androgens, including testosterone and 11-ketotestosterone, produced by gonadotropic-stimulated testis regulate the spermatogenic process and lead to the development of mature spermatozoa. Estradiol (E2), produced in the ovary as a result of GtH exposure, stimulates the liver to produce
vitellogenin, an egg yolk precursor. Vitellogenin circulates back to the ovary and accumulates in developing oocytes prior to egg maturation (Moyle and Cech 2004). Circulating levels of steroid hormones serve as negative feedback messengers to the hypothalamus and pituitary to inhibit GtH production.

Steroid hormones also drive breeding behavior and the development of secondary sexual characteristics (Moyle and Cech 2004). The fathead minnow, *Pimephales promelas*, provides a model of such breeding behavior and secondary sexual characteristic expression controlled by the HPG axis. Male fathead minnows establish and guard nest sites, attract female mates, and guard eggs until hatching (Unger 1983). Secondary sexual characteristics in male fathead minnows include the presence of nuptial tubercles on the heads of reproductively active males (Jensen et al. 2001). The presence of a dorsal fat pad, vertical banding coloration, and a dorsal fin dot are also secondary sexual characteristics under androgenic control and are associated with reproductively active males (Ankley et al. 2001). A prominent secondary sexual characteristic under estrogenic control in female fathead minnows is the presence of an ovipositor (Parrott and Blunt 2005). The ovipositor is an egg-laying structure responsible for the oviposition of mature eggs prior to fertilization. The appearance of secondary sexual characteristics coincides with the initiation of reproductive activity and is an excellent marker for the successful endocrine-driven maturation of a fathead minnow.
III. Effects of estrogenic EDCs on fish:

Secondary sex characteristics

Markers that display endocrine function on a morphological level are useful in examining EDC effects on fish. The prominence of secondary sexual characteristics is a morphological marker of androgenic and estrogenic function. Female fathead minnows from a laboratory study displayed increased ovipositor size when exposed to EE2 concentrations ranging from 3.5-23 ng/L. Male fathead minnows, *Pimephales promelas*, from the same study displayed a reduction in secondary sexual characteristics after exposure to an environmentally relevant concentration (0.96 ng/L) of EE2 (Parrott and Blunt 2005). In another laboratory study, exposure to environmentally relevant concentrations of alkylphenolethoxylates reduced male fathead minnow nuptial tubercle and dorsal fat pad prominence (Bistodeau et al. 2006). Both of the aforementioned studies observed decreased reproductive success that correlated with alterations in secondary sexual characteristics.

Gonadal development

The histological examination of gonadal tissue is also widely used to assess the developmental effects of EDC exposure. Intersex fish, defined as individuals containing both male and female gonadal tissue, have been found downstream from wastewater effluent discharge sites (Jobling et al. 1998, Woodling et al. 2006, Vajda et al. 2008). Kidd et al. (2007) observed male *P. promelas* with immature oocytes residing within normal testicular tissue following exposure to 5-6 ng/L EE2. Laboratory exposure
studies have also revealed a correlation between exposure to estrogenic EDCs and increased intersex incidence (Blazquez et al. 1998, Seki et al. 2002, Orn et al. 2003).

**Sex ratios**

Skewed sex ratios and complete sex reversal in fish populations as a result of EDC exposure is an area of concern. A female biased sex ratio of white suckers was observed in Colorado below a wastewater effluent discharge site over two sampling seasons (Vajda et al. 2008). The percentage of males at this site (17-21%) was half of the percentage of males (36-46%) found at an upstream site. Lange et al. (2009) observed a complete feminization of wild roach, *Rutilus rutilus*, following long-term exposure to 4 ng/L EE2. Parrott and Blunt (2005) also observed a complete feminization of *P. promelas* following exposure to concentrations of EE2 ≥ 3.5 ng/L. In support of Parrott and Blunt’s findings, Lange et al. (2001) observed a strongly female-biased sex ratio in *P. promelas* exposed to 4 ng/L EE2. Female biased sex ratios in zebrafish exposed to 1 ng/L EE2 and complete feminization in zebrafish exposed to 2 ng/L EE2 have been observed (Orn et al. 2003).

**Reproductive activity**

The ability of fish to successfully reproduce in the presence of EDCs has been examined in the literature. Parrott and Blunt (2005) observed a decrease in egg fertilization rate of *P. promelas* at concentrations of EE2 as low as 0.32 ng/L. Bistodeau et al. (2006) exposed *P. promelas* to mixtures of alkylphenols and observed a reduction in the ability of male fish to acquire and maintain a nest site for reproduction.
Studies correlating effects of EDCs on individual organisms with effects at the population level are relatively rare. One such study utilized a variety of endpoints including male vitellogenin (vtg) production, gonadal development, and population viability when examining the effects of EE2 on a fathead minnow population over a 7-year period (Kidd et al. 2007). A near extinction of the species coincided with an increase in male vtg production, an increase in male intersex gonads, and an altered female oogenesis. This study provides a rare example of how common organism-level endpoints can infer effects at higher levels of organization.

IV. Amphibian developmental endocrinology:

Sexual development in amphibians is controlled by a system that parallels that which was previously described for fish. The hypothalamus releases GnRH which in turn stimulates the pituitary to release the GtHs LH and FSH. The GtHs subsequently stimulate the testi and ovaries to produce the sex steroids testosterone and 17β-estradiol and to develop into mature sex organs. Circulating levels of these sex steroids work as a negative feedback mechanism thus preventing release of GnRH from the hypothalamus and GtHs from the pituitary (Kloas et al. 2006).

In addition to sexual development, metamorphosis is a dramatic developmental process under control of the endocrine system that most amphibians undergo during their transition from an aquatic larval stage to a terrestrial juvenile stage. The metamorphic process can be divided into three stages (Etkin 1932). Premetamorphosis is a period of growth and development of larval structures. Prometamorphosis is a period of continued
growth that includes limb development and minor metamorphic processes. Metamorphic climax describes a period of intense morphological restructuring. During metamorphic climax, larval characteristics are lost. Some events that mark metamorphic climax are the emergence of the forelimbs, complete tail resorption, and a regression of gill structures.

The primary drivers behind the metamorphic process are the thyroid hormones triiodothyronine (T3) and thyroxine (T4) (Duellman and Trueb 1986). Thyroid hormone levels are low during premetamorphosis, but reach a maximum during late prometamorphosis leading into metamorphic climax. T4 is a relatively-inactive form of thyroid hormone. It is produced and released into systemic circulation by the thyroid gland where it associates with serum carrier proteins. Once reaching target tissues, T4 is transported across cell membranes where it is subjected to deiodinase activity. Deiodinase enzymes remove an iodine atom from the tyrosyl ring of T4 converting it to the more biologically active T3 (Degitz et al. 2005). T3 then binds to nuclear receptors and initiates gene transcription that ultimately leads to the dramatic remodeling that occurs during metamorphosis (Norris 1997). The release of thyroid hormone from the thyroid gland is stimulated by circulating thyroid-stimulating hormone (TSH) (Dodd and Dodd 1976). TSH is produced and released by the pituitary gland. In a traditional mammalian system, TSH release is thought to be stimulated by thyrotropin releasing hormone (TRH) produced by the hypothalamus. However, in larval amphibians the production and release of TSH is thought to be controlled by release of corticotropin releasing factor (CRF) from the hypothalamus (Denver 1996).
The concentrations of thyroid hormones in circulation are controlled by several mechanisms. Circulating levels of thyroid hormones exert negative feedback on the hypothalamus and pituitary to stop the release of additional thyroid hormone into circulation. In addition, metabolism of thyroid hormones occurs in the liver where a class of enzymes known as the glucuronidases (UDGPTs) create thyroid hormone conjugates that are subsequently excreted (Crofton 2008).

V. Effects of estrogenic EDCs on amphibians:

Sex ratio

Exposure to estrogenic compounds has produced significant results in several amphibian species. *Xenopus tropicalis* and *Rana temporaria* exposed to environmentally relevant concentrations of EE2 exhibited female-biased sex ratios (Pettersson and Berg 2007). The susceptibility of amphibians to sex reversal following estrogenic exposure varies among different species. Mackenzie et al. (2003) observed female-biased sex ratios in *R. pipiens* following exposure to EE2, but did not observe an altered sex ratio in *R. sylvatica* exposed to the same concentrations of EE2. Gyllenhammar et al. (2009) also observed a female-biased sex ratio in *X. tropicalis* at a concentration of EE2 as low as 1.8 ng/L. Hogan et al. (2008) also observed female-biased sex ratios in *R. pipiens* following early-life exposure to elevated concentrations of EE2. The weak estrogen mimics NP and BPA have also been shown to induce a higher percentage of female *X. laevis* (Mosconi et al. 2002).
Gonadal development

In addition to complete sex reversal resulting in skewed sex ratios, the potential for estrogenic compounds to induce an intersex condition, defined as an individual having both male and female gonadal tissue, has been shown. Park and Kidd (2005) observed intersex gonads in both *R. serpertinialis* and *R. clamitans* following exposure to environmentally relevant concentrations of EE2 that ranged from 2.9 to 9.1 ng/L. Intersex gonads were also observed in *X. laevis* exposed to E2 concentrations \( \geq 1 \text{ ug/L} \) (Hu et al. 2008). Hogan et al. (2008) found that *R. pipiens* tadpoles exposed to EE2 both chronically and early in development exhibited higher incidences of intersex gonads at metamorphosis than were observed in both control individuals and tadpoles exposed in mid to late development. Mackenzie et al. (2003) also observed an increased intersex condition in *R. pipiens* exposed as tadpoles to E2, EE2, and NP.

Metamorphosis

The ability of sex steroids to delay the metamorphic process has also been observed; suggesting that exposure to estrogenic compounds may increase the time to reach metamorphosis in amphibians. The time to reach metamorphosis is an important aspect of an amphibian’s life history and has been linked to the size, survival, and reproductive output of adult amphibians (Wilbur and Collins 1973, Smith 1987, Semlitsch et al. 1988, Berven 1990). Leatherland et al. (1985) measured reduced T3 and T4 levels in rainbow trout and observed morphological changes in thyroid glands of E2-exposed fish. Gray and Janssens (1990) reported that both testosterone and E2 inhibited
T3-induced metamorphosis in *X. laevis*. *R. catesbeiana* tadpoles exhibited a decrease in the rate of cranial transformation and tail resorption, both of which are characteristics of metamorphic development (Christensen et al. 2005). T3-induced metamorphosis in *R. rugosa* was inhibited by exposure to BPA and several BPA analogues, while these same compounds inhibited thyroid-receptor (TR) mediated gene expression *in X. laevis* tadpoles, suggesting the compounds have the ability to prevent T3 binding to TR, which would subsequently explain the inhibition of metamorphosis in *R. rugosa* (Goto et al. 2006). Exposure to EE2 chronically and during mid-metamorphosis significantly increased the time for *R. pipiens* to reach metamorphosis (Hogan et al. 2008).

The mechanisms by which sex steroids affect the metamorphic process are not well elucidated, but previous research has identified several possibilities. Jacobs et al. (1988) observed an increase in plasma levels of T4 in both *R. ridibunda* and *R. esculenta* following injection of luteinizing hormone-releasing hormone (LHRH). LHRH is responsible for stimulating the pituitary to release gonadotropins which in turn stimulate sexual development. The results of Jacobs et al. (1988) suggest that the thyroid and gonadal systems may interact in ways previously unknown. In support of this idea, Denver et al. (1988) observed an increase in the secretion of TSH from pituitary glands of adult *R. pipiens* following exposure to both corticotropin-releasing hormone (CRH) and gonadotropin releasing hormone (GnRH). These findings also suggest that the thyroid system is under the control of a variety of hormones.
Exposure of amphibians to exogenous estrogenic compounds may inhibit stimulation of the thyroid gland, and thus lead to slower metamorphic development. An increase in the circulating levels of estrogenic compounds in an amphibian would exert negative feedback on the hypothalamus-pituitary-gonadal axis. This negative feedback could prevent additional stimulation by preventing the release of both GnRH and the gonadotropins LH and FSH. As evidenced by the aforementioned research a lack of GnRH release would subsequently prevent additional release of TSH, leading to a reduction in thyroid hormone release and a slower metamorphic development. While such cross-regulation of the thyroid and gonadal systems has been noted, the exact mechanisms are still poorly understood.

As evidenced by the previously discussed studies, a great deal of research has examined the effects of EDCs on the growth, development, and metamorphosis of amphibian species. While many of the studied compounds are considered to be wastewater-derived, no studies have attempted to characterize the effects of a whole wastewater effluent on these same endpoints. Examining the effects of such a mixture will add considerably to the existing knowledge base and will provide an example of the effects of an environmentally relevant complex on amphibian health.
References

and evaluation of a short-term reproduction test with the fathead minnow  

Berven, K.A. 1990. Factors affecting population fluctuations in larval and adult stages  

Bistodeau, T.J., Barber, L.B., Bartell, S.E., Cediel, R.A., Grove, K.J., Klaustermeier, J.,  
Woodard, J.C., Lee, K.E., Schoenfuss, H.L.  2006. Larval exposure to  
environmentally relevant mixtures of alkylphenolethoxylates reduces reproductive  

effects of early exposure to estradiol-17 beta and 17 alpha-ethynylestradiol on the  
gonads of the gonochoristic teleost *Dicentrarchus labrax*. Fish Physiol. Biochem.  
18,37-47.

Byrns, G. 2001. The fate of xenobiotic organic compounds in wastewater treatment  
plants. Water Res. 35,2523-2533.

Christensen, J.R., Richardson, J.S., Bishop, C.A., Pauli, B., Elliot, J.  2005. Effects of  
nonylphenol on rates of tail resorption and metamorphosis in *Rana catesbeiana*  

Androl.  31,209-223.

Progress towards development of an amphibian-based thyroid screening assay  
using *Xenopus laevis*. Organismal and thyroidal responses to the model  
compounds 6- propylthiouracil, methimazole, and thyroxine. Toxicol. Sci.  
87,353-364.

Denver, R.J.  1988. Several hypothalamic peptides stimulate *in vitro* thyrotropin  

Denver, R.J.  1996. Neuroendocrine control of amphibian metamorphosis. In  
Metamorphosis: Postembryonic Reprogramming of Gene Expression in  


Chapter 2: The effects of wastewater effluent on the fathead minnow, *Pimephales promelas*

I. Abstract

Municipal wastewater effluents have been shown to contain a variety of anthropogenic compounds, many of which are known to display estrogenic properties. While multiple laboratory studies have shown the effects of such compounds on an individual basis at elevated concentrations, little research has attempted to characterize the effects of exposure to environmentally relevant mixtures of estrogenic compounds. The current study examined the effects of long-term exposure to graded concentrations (0, 50, 100%) of wastewater effluent on the fathead minnow, *Pimephales promelas*. The F1 generation were cultured in control water to test for transgenerational effects from the parental exposure to wastewater effluent. Total estrogenic activity in the wastewater was determined to be approximately 1.5 ng/L 17β-estradiol. Survival, growth, and reproduction in the parent generation were not affected by exposure to the wastewater treatments. An increase in the gonadosomatic index and a reduction in the expression of secondary sex characteristics in male fathead minnows exposed to 100% wastewater in the parent generation were observed. Conversely, the expression of secondary sex characteristics was greater in males from the F1 generation of wastewater-exposed parents. Additionally, a positive correlation between parental exposure to wastewater and the onset of reproductive activity in the F1 generation was observed. Results of this study suggest that exposure to wastewater effluent did not pose a significant threat to the
successful growth, development, and reproduction of the fathead minnow. Early onset of reproductive activity observed in the F1 generation of wastewater-exposed parents suggests that exposure to estrogenic compounds in a parent generation can elicit effects in subsequent generations and should be studied further.

II. Introduction

Endocrine disrupting compounds (EDCs) are defined as exogenous substances that disrupt endocrine system-mediated physiological functions. One of the major pathways for EDCs to enter the aquatic environment is through municipal wastewater effluents. Effluents around the world have been shown to contain measurable concentrations of pharmaceuticals, personal care products and natural and synthetic hormones that are capable of acting as EDCs (Nakada et al. 2004, Nakada et al. 2006, Snyder 2008). A survey conducted in the United States between 1999 and 2000 by the United States Geological Survey found the presence of organic wastewater-derived contaminants, many of which are known EDCs, in 80% of 139 surface waters (Kolpin et al. 2002).

The presence of estrogenic compounds in wastewater effluents is a potential risk to aquatic ecosystems and has been studied extensively (Byrns 2001, Murk et al. 2002, Nakada et al. 2004, Servos et al. 2005). Estrogenic compounds of interest include the natural estrogen, 17-β estradiol (E2); the synthetic oral contraceptive-derived estrogen, ethynylestradiol (EE2); bisphenol-A (BPA), which is a component of polycarbonate plastics; and a class of industrial compounds known as the alkylphenols, which include
nonylphenol (NP) and octylphenol (OP). The estrogen receptor (ER) affinities of E2 and EE2 are orders of magnitude higher than those of BPA, NP, and OP (Rutishauser et al. 2004), although in some wastewater effluents such industrial compounds may reach concentrations in the parts per billion range making them relevant to study as endocrine disruptors despite their low ER affinities.

In addition to studying the occurrence of estrogenic EDCs, a great deal of research has focused on their effects on aquatic organisms. The developmental and reproductive status of EDC-exposed fish species has been studied in detail. Altered secondary sexual characteristics as well as a decrease in reproductive success have been observed in fathead minnows exposed to EE2 (Parrott and Blunt 2005) and alkylphenolethoxylates (Bistodeau et al. 2006). Intersex fish, defined as individuals containing both male and female gonadal tissue, have been sampled downstream from wastewater effluent discharge sites (Jobling et al. 1998, Woodling et al. 2006, Vajda et al. 2008). Laboratory dosing studies have also revealed correlations between exposure to estrogenic EDCs and an increased intersex incidence (Blazquez et al. 1998, Seki et al. 2002, Orn et al. 2003). Kidd et al. (2007) observed a near extinction of a fathead minnow population over a seven-year period in an experimental lake after exposure to an environmentally relevant concentration of EE2. This near extinction coincided with an increase in male vitellogenin production, an increase in male intersex gonads, and an altered female oogenesis.
While the effects of EDC exposure to fish have been studied thoroughly, the existing knowledge base has a few limitations. Much of the work characterizing the effects of EDC exposure on wild caught fish lacks data regarding the exposure history of the sampled organisms. In addition, much of the laboratory EDC work has either been conducted at concentrations greater than those found in the environment or has not adequately addressed the issue of complex mixture exposures that are likely to be found in the environment. Also, not much is known about the responses of F1 generation fish after exposure to the parent generation. The goal of this study was to characterize the responses of laboratory-cultured fathead minnows, *Pimephales promelas*, to wastewater effluent exposure and subsequently examine the development of fish in the F1 generation of an exposed parental generation.

**III. Material & methods**

*Solution preparation*

Reconstituted moderately-hard water (nominal hardness and alkalinity of 80 and 60 mg/L as CaCO₃, respectively) was made from 18 mega-ohm water and reagent grade salts. Final wastewater effluent was collected after chlorination and before reaching the outfall at the Mauldin Road Wastewater Treatment Plant in Greenville, South Carolina, USA and transported to the Clemson University Institute of Environmental Toxicology (CU-ENTOX) in Pendleton, South Carolina, USA. The effluent was diluted with moderately hard water to produce treatments of 0, 50, and 100% wastewater.
Mauldin Road Wastewater Treatment Plant

The Mauldin Road Wastewater Treatment Plant in Greenville, South Carolina, USA, has a drainage area of 66 square miles with a maximum daily flow of 45 million gallons per day (MGD). Approximately 37,000 residences comprise the service area. The average daily plant flow is 16.5 MGD, of which 2.5 MGD comes from industrial sources (Dr. Stephen Graef, personal communication).

Experimental setup

Nine-hundred 24-36hr old larval fathead minnows were taken from an in-house culture at CU-ENTOX, divided into groups of 100, and stocked into glass dishes containing 2 L of test solution. Each treatment was replicated three times and each dish contained 100 organisms. Larval fish were fed brine shrimp twice daily. Test solutions were renewed bi-weekly with fresh wastewater effluent collected on the day of the renewal. Partial renewals were conducted daily using excess effluent from the most recent collection event. Test organisms were maintained in a climate controlled test room at CU-ENTOX on a 16:8 light:dark cycle and were continuously aerated. Water temperature, pH, and dissolved oxygen ranged from 22.32-22.42 °C, 7.80-8.07, and 7.21-7.32 mg/L, respectively.

On day 7 of the experiment, 10 organisms were sampled from each replicate and frozen at -80°C for later analysis. On day 24 of the experiment, test organisms were moved to 30L glass aquaria containing 10 L of test solution and the food source was changed to Tetramin™ commercial fish food. As the test organisms grew, test solution
volumes were gradually increased to a maximum of 20 L. On day 68, replicates were thinned to 25 fish apiece and two breeding tiles were placed in each aquarium. Mortality up to day 68 was recorded. Egg production was quantified until the end of the experiment. On day 145, male secondary sexual characteristics (SSCs) were assessed following the methods of Parrott et al. (2003). Banding strength (0-3 points) and the presence of nuptial tubercles, dorsal fat pad, and dorsal fin dot (1 point each) were assessed for the two males with the most prominent SSCs in each tank to create a subjective index score ranging from 0-6. At this time, four males and eight females were removed from each tank and placed in separate 30L aquaria for an isolated breeding experiment. Males displaying SSCs and females displaying distinct ovipositors were selected. The four males and eight females that were selected from each tank were divided into two groups, each containing two males and four females. One group was stocked in a breeding tank containing the same test solution in which they had previously been exposed. The other group was placed in a test solution that differed from their previous exposure and went as follows: fish from the control treatment were placed in 100% effluent, while fish from the 100% and 50% effluent treatments were placed in control solutions (Figure 2.1). Organisms were allowed to acclimate to the breeding tanks for 48hrs after which two breeding tiles were placed in each tank. Over the next 14 days, tiles were checked for eggs three times daily. Egg production was recorded as the total number of eggs laid per female over the 14 days. A subset of breeding tiles were removed from the breeding tanks and placed in separate containers containing the appropriate test solution for 96hrs to examine fertilization success. At the conclusion of
the breeding experiment, all adult organisms were euthanized. Total lengths and weights were measured and used to calculate a condition factor \[\frac{\text{weight}}{\text{length}^3} \times 10^5\]. Ovipositor size in females was measured. Organisms were sexed internally and gonads were weighed to calculate a gonadosomatic index (GSI) \[\frac{\text{gonad weight}}{\text{total weight}} \times 100\] before being fixed in 10% neutral-buffered formalin for histological analysis. Livers were weighed to determine a hepatosomatic index (HSI) \[\frac{\text{liver weight}}{\text{total weight}} \times 100\]. A small number of individuals (six in the control treatment, three each in 50 and 100% treatment) exhibited poorly developed gonads and were determined to be quiescent males due to the lack of an ovipositor. These individuals were not used in the analysis of growth and development endpoints. Fish that spawned in the test solution that differed from their original treatment were not used for HSI or GSI analysis.

During the final 48hrs of the isolated breeding experiment, eggs from all tanks were collected, pooled by treatment, and allowed to hatch in moderately-hard water. At 10-11 days post-hatch, F1 generation larval organisms from each treatment were split into four replicates, each containing 50 organisms, and placed in plastic containers of moderately-hard water. F1 treatment groups were labeled to correspond with the parent breeding group that produced them (i.e. 0M, 0W, etc.) On day 93 of the F1 generation experiment, all tanks were thinned to 30 organisms. On day 95 of the F1 experiment, two breeding tiles were placed in each tank. Tiles were checked for the presence of eggs three times daily. On day 125 of the F1 generation experiment, the test was ended. SSCs of the two males with the most prominent SSCs in each tank were scored as described
above. Gonads were removed from the highest SSC scoring male in each tank and fixed as described above for histological analysis.

Histological analysis

Fixed gonads were dehydrated using a graded series of ethanol concentrations (50, 75, 90, 100%), cleared with xylene, and embedded in resin (Immunobed, Polysciences, Warrington, PA) prior to histological examination. Male gonads were sectioned at a thickness of 1.5 µm at increments of 30 µm over the entire tissue and were observed for the presence of normal spermatogenic development, defined as the presence of spermatogonium, spermatocytes, and mature spermatozoa. The presence of testicular oocytes, defined as oocytes residing within normal testicular tissue, was also noted. Female gonads were sectioned at a thickness of 1.5 µm at increments of 30 µm until a minimum of 10 sections were obtained and were observed for the presence of normal oocyte development, defined as tissue containing oocyte developmental stages that range from early stage oogonia to late stage vitellogenic oocytes.

EDC analysis

Over the course of the experiment, effluent brought into the laboratory for treatment solution renewal was subsampled and analyzed for the presence of a suite of natural and synthetic hormones and alkylphenolic compounds. Thirteen times during the parental exposure period, a 2.5L sample of whole effluent was collected in an amber bottle, sealed with no headspace, placed on ice, and shipped overnight to a laboratory at the United States Environmental Protection Agency’s Office of Research and
Development in Cincinnati, OH, where it was processed for the detection of steroid hormones within 24 hrs of collection. Twelve times during the parental exposure period, a 2.5L sample of whole effluent was collected in an amber bottle, the effluent pH was set to 2 using concentrated sulfuric acid, and the sample was shipped to a laboratory at the United States Environmental Protection Agency’s Office of Research and Development in Chicago, IL, where it was processed for the detection of a suite of alkyphenolic compounds. An estradiol equivalency (EEQ) factor was calculated by multiplying measured chemical concentrations by experimentally determined (Rutishauser et al. 2004) estrogenic potencies (relative to 17β-estradiol) to characterize the estrogenic activity of the effluent.

Statistical analysis

All statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC). Levene’s test was used to examine the assumption of variance homogeneity. A nonparametric Wilcoxon rank-sum test was used to examine treatment differences \( p < 0.05 \) in SSC scores. A nonparametric Kruskal-Wallis test examined differences in ovipositor length in the parent generation and percent survival in the F1 generation. A chi-square analysis tested whether sex ratios in each treatment differed from an expected 1:1 ratio. A Fisher’s exact test examined the relationship between reproductive activity in the F1 generation and parental exposure to wastewater. For all other analyses, one-way analysis of variance (ANOVA) tests were conducted to examine differences \( p < 0.05 \) between treatment means. Fisher’s LSD post hoc test was used to test for treatment
differences when a significant effect was detected by ANOVA. Proportion data were
arcsine square root transformed prior to analysis by ANOVA.

IV. Results

EDC analysis

EDC analysis is summarized in Table 2.1. Testosterone, androstenedione, E1,
and EE2 were detected at all sampling events, while all other compounds of interest were
detected periodically. The calculated EEQs for each sampling event are summarized in
Table 2.2.

Survival-parent generation

Percent survival (Figure 2.2) through exposure day 68 was significantly greater ($p$
= 0.031, $F = 6.52, df = 2,6$) in the 50% treatment than the 100% treatment; but neither the
50 nor 100% treatment was significantly different from the control. Percent survival, of
the 25 individuals remaining in each tank after day 68, at the end of the test did not differ
between any treatment ($p = 0.752, F = 0.30, df = 2,6$).

Growth measurements-parent generation

No differences in female length ($p = 0.170, F = 2.42, df = 2,6$), weight ($p = 0.223,$
$F = 1.95, df = 2,6$), GSI ($p = 0.814, F = 0.21, df = 2,6$), HSI ($p = 0.291, F = 1.53, df =$
$2,6$), or condition factor ($p = 0.870, F = 0.14, df = 2,6$) (Table 2.3) and no differences in
male length ($p = 0.289, F = 1.54, df = 2,6$), weight ($p = 0.359, F = 1.22, df = 2,6$), HSI ($p$
$= 0.308, F = 1.44, df = 2,6$), or condition factor ($p = 0.427, F = 0.99, df = 2,6$) (Table 3)
were detected between any treatments. GSI of males from the 100% treatment was significantly greater ($p = 0.032$, $F = 6.46$, $df = 2,6$) than the control treatment, but did not differ from the 50% treatment. No difference in GSI was detected between the 50% and control treatment.

**Secondary sex characteristics-parent generation**

SSC scores for males were significantly higher in the control treatment than the 100% treatment ($p = 0.036$, $Z = 1.80$) (Figure 2.3). No differences in SSC scores were detected between the 50% treatment and the control or 100% treatment. Ovipositor length treatment means ranged from $2.06 \pm 0.05$ mm in the 100% treatment to $2.42 \pm 0.07$ mm in the control treatment and did not differ significantly between any treatments ($p = 0.148$, $df = 2$).

**Sex distribution-parent generation**

The percentage of females per treatment increased in a dose-dependent manner as wastewater concentration increased, but did not differ significantly between treatments ($p = 0.404$, $F = 1.06$, $df = 2,6$). All treatment means were skewed toward a female bias. The control treatment contained $59.8 \pm 15.8$% females, while the 50 and 100% treatments contained $68.9 \pm 16.2$% and $74.8 \pm 9.8$% females, respectively. When all organisms were pooled within each treatment and analyzed by chi-square analysis, both the 50 and 100% treatment sex ratios differed significantly ($p = 0.006$ and $p <.001$, respectively) from the expected 1:1 ratio of males:females. The control treatment did not differ significantly from the expected ratio ($p = 0.099$).
Egg production-parent generation

Cumulative egg production (Table 2.4), up to test day 145, increased as wastewater concentration increased, but did not differ significantly between treatments \((p = 0.1581, F = 2.55, df = 2,6)\). Egg production, in terms of the number of eggs produced per female, during the isolated reproduction test (Table 2.4) did not differ between treatments \((p = 0.552, F = 0.83, df = 5,11)\).

Gonadal histology-parent generation

A normal pattern of spermatogenesis (Figure 2.4A) was observed in all males from the control and 100% treatment. One male from the 100% treatment contained only one gonad, a testis on the right side of the body cavity. Upon histological examination, this individual contained testicular oocytes ranging from early stage oocytes to later stage vitellogenic oocytes residing within normal testicular tissue (Figure 2.4B). No other occurrences of testicular oocytes were noted. Examination of the ovaries (Figure 2.4C) revealed no observable differences between the control and 100% treatment. All observed ovaries contained oocytes ranging from early stage oogonia to late stage vitellogenic oocytes.

Survival-F1 generation

Percent survival of individuals in the F1 generation did not differ significantly between any treatments \((p = 0.078, df = 5)\). Treatment percent survival means ranged
from 91.3-98.6% and did not follow a dose-dependent pattern based on parental exposure.

*Secondary sex characteristics-F1 generation*

SSC scores for males in the F1 generation of the 0M treatment were significantly lower than the scores for the 100W ($p = 0.030$, $Z = -1.89$), 100M ($p = 0.0210$, $Z = -2.03$), and 50W ($p = 0.015$, $Z = -2.18$) treatments, but did not differ from the 50M ($p = 0.073$, $Z = -1.45$) and 0W ($p = 0.500$, $Z = 0.00$) treatments (Figure 2.5).

*Reproductive activity-F1 generation*

Significant relationships between parental exposure and F1 generation reproductive activity were observed. Fisher’s exact test revealed no difference in the likelihood of reproductive activity occurring in the 0M and 0W treatments. For all other comparisons, treatments were pooled based on parental exposure (0W and 0M, 50W and 50M, and 100W and 100M tanks were pooled together to form three treatment groups with eight replicates per group) prior to analysis. Parental exposure to the 50 ($p = 0.041$) and 100% ($p = 0.041$) treatments was correlated with an increased distribution of reproductively active tanks in the corresponding F1 generations when compared to the F1 generation of control parents.

*Gonadal histology-F1 generation*

All observed males from the 0M, 100M, and 100W treatments displayed normal patterns of spermatogenesis. Two males from the 0W treatment displayed early stage
testicular oocytes (Figure 2.6A) residing in normal testicular tissue, while a third male from the 0W treatment contained immature testis (Figure 2.6B) lacking mature spermatozoa. The final individual examined in the 0W treatment displayed normal testicular tissue.

V. Discussion

The estrogenic activity, measured as an EEQ value (ng/L), of the wastewater effluent used in this study was within values previously reported for other municipal wastewater effluents. Schiliro et al. (2004) tested final effluent from a wastewater treatment plant in Northern Italy and observed an EEQ that ranged from 0.28-2.40 ng/L, while final effluent from two wastewater treatment plants in Switzerland displayed EEQs generally in a range between 1 and 10 ng/L, although one sample at one of the treatment plants displayed an EEQ well above 10 ng/L (Rutishauser et al. 2004). Higher EEQ values were reported by Tilton et al. (2002) who calculated EEQ values ranging from 21 to 147 ng/L from two wastewater treatment plants in Mississippi; effluent from a wastewater treatment plant in Minnesota displayed a mean EEQ of 44.0 ± 0.9 ng/L (Martinovic et al. 2007). Effluent from five wastewater treatment plants in Australia displayed EEQs generally ranging from <1 to 14.8 ng/L, with one sample reaching a maximum EEQ of 67.8 ng/L (Tan et al. 2007). The effluent used in the current study exhibited a mean EEQ of 1.5 ng/L, which is on the low end of the range of EEQ values based on the aforementioned studies. Previous research has shown that EEQ values have the potential to reach levels much higher than those observed in this study. The results obtained from the current study should be viewed as being representative of long-term
exposure to an environmentally relevant mixture containing very low levels of estrogenic compounds.

The reproductive activity, survival, and growth of fathead minnows in the parent generation were not adversely affected by exposure to the 50% and 100% wastewater treatments. This was evidenced by the lack of effects in egg production both cumulatively and in the isolated breeding experiment, as well as the lack of treatment differences in the survival, size, and condition factors of both male and female fathead minnows. These findings support previous research by Schoenfuss et al. (2002) who found no significant effects of wastewater effluent on the frequency of male spawning behavior or sperm production in goldfish. Barber et al. (2007) also observed no differences in the size of male fathead minnows exposed to wastewater effluent over a period of 28 days.

The sexual development of fish exposed to wastewater effluents and estrogenic compounds is of great concern because of its implications on populations. Intersex fish, containing both male and female gonadal tissue, have been observed downstream from wastewater treatment discharge sites (Woodling et al. 2006, Vajda et al. 2008, Jobling et al. 1998). Kidd et al. (2007) observed male fathead minnows with testicular oocytes in an experimental lake system dosed with EE2. In the present study, only one individual in the parent generation contained testicular oocytes, suggesting that long-term exposure to wastewater effluent did not significantly impact male gonadal development. It should be noted that a female-biased sex ratio was observed in the 50 and 100% treatments when
individuals were pooled within their treatments. This finding suggests that sex reversal may have occurred in the wastewater treatments. The lone individual in the 100% treatment exhibiting testicular oocytes was unique in comparison to testicular oocytes observed in previous studies. This individual only contained one developed gonad that was partially composed of later stage vitellogenic oocytes. Generally, testicular oocytes have been observed to be early stage primary oocytes. The individual in the current study appeared to be a phenotypic male, but possessed a gonadal status that suggested this individual may have been in a transition state between the development of primarily male or female gonadal tissue.

In contrast to the single case of testicular oocytes in the parent generation, three of the four males observed from the F1 generation 0W treatment displayed gonadal abnormalities. Two of these individuals displayed testicular oocytes, while the third individual was immature and had not began producing mature spermatozoa. These results suggest that early life exposure to estrogenic compounds may impact male sexual development later in life. The individuals from the 0W treatment were only exposed to wastewater as developing embryos inside the females and for no more than 12 hours as fertilized eggs on breeding tiles, yet developmental effects were observed over 120 days later. All males sampled from the 100W treatment displayed a normal pattern of spermatogenesis and no gonadal abnormalities despite also being exposed as developing embryos and briefly as fertilized eggs, suggesting that male offspring from the wastewater-exposed parents were estrogen-resistant to some degree.
Exposure to estrogenic compounds and wastewater effluent has also been shown to reduce GSI values in male fathead minnows. The observed increase in the GSI of males in the 100% treatment in this study conflicts with the aforementioned reports, but may be attributed in part to the estrogenic activity of the test solutions. Kidd et al. (2007) exposed fathead minnows to concentrations of EE2 ranging from 5-6 ng/L, while Hemming et al. (2001) measured EE2 concentrations ranging from 200-900 ng/L during three out of four sampling events at an experimental site receiving wastewater effluent. Both aforementioned studies detected reduced male GSIs. The maximum concentration of 0.5 ng/L EE2 measured in the current study and the mean EEQ of 1.5 ± 2.1 ng/L measured over the course of the study support the assertion that the estrogenic activity in this study was lower than that at which reduced GSI values were observed in the two aforementioned studies. Concentrations of estrogenic compounds in this study may have been below a threshold level required to inhibit male gonadal growth. Parrott and Blunt (2005) observed no effect on male fathead minnow GSI at nominal EE2 concentrations of 0.32 and 0.96 ng/L, which are more comparable to the total EEQ concentrations observed in the present study. Barber et al. (2007) observed an increased GSI in male fathead minnows exposed to wastewater effluent and attributed this observation to the presence of increased nutrient sources in the wastewater allowing for more energy to be allocated for gonadal growth. Though not statistically significant, a dose-dependent increase in length and weight was observed in the current study in both males and females, supporting the hypothesis that the wastewater may have provided nutrient sources. An algal biofilm was consistently observed growing on the sides and bottoms of the
wastewater treatment tanks despite daily cleaning. It can be assumed that the test fish would be able to utilize such a biofilm as an additional food source, which in turn would provide additional energy that could be utilized for gonadal development.

As an alternative explanation, a compensatory mechanism may be responsible for the increased GSIs observed in the 100% treatment males. Ankley et al. (2007) observed increased GSIs in male fathead minnows following exposure to ketoconazole, a fungicide. Ketoconazole inhibited testosterone production \textit{ex vivo} by fathead minnow testis tissue but did not alter plasma concentrations of testosterone in male fathead minnows following 21 days of exposure. It was hypothesized that an increased gonadotropin release stimulated the testis in an effort to increase testosterone production and maintain plasma concentrations. This increased stimulation would lead to increased GSI values. Although plasma testosterone and gonadotropin levels were not measured in the current study, it can be hypothesized that the increased GSI observed in males from the 100% treatment was a compensatory response to estrogenic exposure.

The reduced SSC scores observed in males in the 100% wastewater treatment were consistent with previous findings where fathead minnows were exposed to estrogenic compounds (Hemming et al. 2001, Lange et al. 2001, Martinovic et al. 2007, Parrott and Blunt 2005). However, Barber et al. (2007) observed an increase in SSC scores of males exposed to wastewater effluent, although their scoring index differed from the index used in this study. While in the present study, vertical banding strength was weaker in the 100% wastewater-exposed fish, banding strength was not assessed by
Barber et al. (2007), suggesting that this parameter may be a more sensitive marker of estrogenic exposure. Reduced SSC scores in this study did not correlate with reproductive inhibition.

In addition to the previously discussed gonadal abnormalities observed in the F1 generation, several other findings in the F1 generation experiment warrant discussion. The increased SSC scores observed in F1 generation males from wastewater-exposed parents coupled with the increased reproductive activity in the F1 generation of wastewater-exposed parents suggests that male organisms in these treatments underwent accelerated sexual development. Male fathead minnows in the 15 yr old culture at CU-ENTOX have always developed to a point where SSCs were strongly expressed and reproductive activity occurred. This suggests that male offspring from the control parents were not exhibiting inhibited development. Had the test been conducted for a longer time-period, the F1 generation from control parents would have reached sexual maturity with strongly expressed SSCs and a high degree of reproductive activity.

The mechanism responsible for the accelerated development of F1 males from wastewater-exposed parents is unknown, but a possible explanation is that sexual selection favored males in the parent generation who were resistant to exposure to estrogenic compounds/wastewater effluent. A transgenerational resistance to estrogenic effects would explain the observed lack of testicular oocytes in the F1 generation from the 100W treatment. The males who were able to establish dominance within the reproductive hierarchy in the wastewater treatments in the parent generation had to
overcome pressure from both male competitors and the estrogenic activity of the test solution, while dominant males in the control only had to out-compete other males within the exposure tank. In the absence of exogenous estrogenic compounds, the accelerated development of male offspring from dominant males in the wastewater treatments may have been the result of selection of males with enhanced sexual development traits. Coe et al. (2008) observed a shift in parentage success of male zebrafish following exposure to EE2, supporting the idea that estrogenic compounds may play a role in sexual selection, although in the Coe et al. (2008) study dominant male parentage success was suppressed in response to EE2 while insubordinate male success increased. The concentration of EE2 in the Coe et al. (2008) study may have been too high (10 ng/L) for any dominant male to overcome. Concentrations of $\text{EE2} \geq 3.5 \text{ ng/L}$ have been shown to completely feminize fathead minnows (Parrott and Blunt 2005, Lange et al. 2001). An alternative explanation for the accelerated sexual development and reproductive activity of F1 males from wastewater-exposed parents is that a compensatory response, as was discussed previously, to estrogenic exposure in wastewater-exposed males from the parent generation was passed to the male offspring. Though not measured in this study, an upregulation of gonadotropic activity in the F1 offspring could have led to an accelerated sexual development.

Very little research has examined the transgenerational effects of parental exposure to estrogenic compounds in fish models, most likely due to the extensive time and resource requirements. One such study (Lange et al. 2001) examined the effects of continuous exposure to EE2 on a parent and F1 generation (up to 28 days post-hatch) of
fathead minnows based on U.S. EPA methodology (1982). While such studies following the EPA guidelines for fathead minnow life cycle studies are useful in examining the effects of long-term and multigenerational exposure to contaminants, the continuous exposure scenario over both generations makes it difficult to elucidate transgenerational effects that are independent of immediate contaminant exposure. In addition, the abbreviated test length in the F1 generation provided no insight into the reproductive capabilities of the test organisms in the F1 generation. The present study avoided this shortcoming by following sexual development of the F1 generation. Hence, the accelerated sexual development of the F1 males from wastewater-exposed parents would not have been detected following the EPA guidelines.

In the present study, wastewater effluent did not pose a significant threat to the survival, growth, and reproduction of a fathead minnow parent generation, despite a reduction in secondary sexual characteristics of 100% wastewater-exposed males. Males in the F1 generation exhibited an accelerated sexual development, although the underlying mechanism for this observation remains unknown. Future work is needed to better understand the mechanistic basis of effects of low-level environmentally relevant estrogenic exposure on the reproductive endocrinology of fish species and how such effects may impact subsequent generations.
VI. References


Table 2.1. Minimum, maximum, and mean concentrations of targeted compounds in wastewater effluent collected from the Mauldin Road Wastewater Treatment Plant in Greenville, SC. E2-relative potency describes the estrogenic activity of each compound relative to 17β-estradiol. n = number of sampling events, nd = non-detect, na = not available

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Minimum (ng/L)</th>
<th>Maximum (ng/L)</th>
<th>Mean+SD (ng/L)</th>
<th>E2-relative potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrotestosterone</td>
<td>13</td>
<td>nd</td>
<td>33.2</td>
<td>2.8±9.2</td>
<td>na</td>
</tr>
<tr>
<td>Testosterone</td>
<td>13</td>
<td>0.8</td>
<td>5.2</td>
<td>1.6±1.3</td>
<td>na</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>13</td>
<td>2.4</td>
<td>5.6</td>
<td>3.0±1.2</td>
<td>na</td>
</tr>
<tr>
<td>Progesterone</td>
<td>13</td>
<td>nd</td>
<td>2.6</td>
<td>0.2±0.7</td>
<td>na</td>
</tr>
<tr>
<td>E1</td>
<td>13</td>
<td>0.4</td>
<td>15.9</td>
<td>2.1±4.2</td>
<td>0.38</td>
</tr>
<tr>
<td>E2</td>
<td>13</td>
<td>nd</td>
<td>2.6</td>
<td>0.5±0.7</td>
<td>1</td>
</tr>
<tr>
<td>EE2</td>
<td>13</td>
<td>0.1</td>
<td>0.5</td>
<td>0.2±0.1</td>
<td>1.19</td>
</tr>
<tr>
<td>E3</td>
<td>13</td>
<td>nd</td>
<td>nd</td>
<td>na</td>
<td>2.4x10⁻³</td>
</tr>
<tr>
<td>NP</td>
<td>11</td>
<td>nd</td>
<td>430</td>
<td>71±159</td>
<td>2.5x10⁻⁵</td>
</tr>
<tr>
<td>OP</td>
<td>11</td>
<td>nd</td>
<td>41</td>
<td>4±12</td>
<td>7.8x10⁻⁶</td>
</tr>
<tr>
<td>NP1E</td>
<td>11</td>
<td>nd</td>
<td>3200</td>
<td>754±1003</td>
<td>na</td>
</tr>
<tr>
<td>NP2E</td>
<td>11</td>
<td>nd</td>
<td>2300</td>
<td>391±872</td>
<td>na</td>
</tr>
<tr>
<td>BPA</td>
<td>11</td>
<td>nd</td>
<td>270</td>
<td>83±116</td>
<td>1.1x10⁻⁴</td>
</tr>
</tbody>
</table>

*taken from Rutishauser et al. (2004)
<table>
<thead>
<tr>
<th>Sampling event</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEQ (ng/L)</td>
<td>6.6</td>
<td>3.7</td>
<td>1.2</td>
<td>0.8</td>
<td>0.6</td>
<td>0.7</td>
<td>0.3</td>
<td>0.5</td>
<td>1.2</td>
<td>1.0</td>
<td>0.6</td>
<td>1.2</td>
<td>0.4</td>
<td>1.5 ± 2.1</td>
</tr>
</tbody>
</table>

Table 2.2. Estradiol equivalency factors (EEQs) determined for each sampling event to describe the estrogenic activity of the final wastewater effluent used to create treatment solutions.
<table>
<thead>
<tr>
<th></th>
<th>Length (mm)</th>
<th>Weight (g)</th>
<th>Condition factor</th>
<th>GSI</th>
<th>HSI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50.44 ± 1.26</td>
<td>1.91 ± 0.19</td>
<td>1.46 ± 0.05(23)</td>
<td>1.66 ± 0.24^a(8)</td>
<td>3.05 ± 0.30(10)</td>
</tr>
<tr>
<td>50%</td>
<td>51.76 ± 2.99</td>
<td>2.02 ± 0.29</td>
<td>1.45 ± 0.06(21)</td>
<td>1.86 ± 0.10^ab(9)</td>
<td>3.26 ± 0.36(9)</td>
</tr>
<tr>
<td>100%</td>
<td>53.21 ± 0.83</td>
<td>2.17 ± 0.06</td>
<td>1.44 ± 0.01(14)</td>
<td>2.29 ± 0.28b(10)</td>
<td>3.85 ± 0.93(10)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44.71 ± 1.14</td>
<td>1.21 ± 0.13</td>
<td>1.35 ± 0.07(43)</td>
<td>16.69 ± 1.68(10)</td>
<td>3.39 ± 1.13(10)</td>
</tr>
<tr>
<td>50%</td>
<td>45.50 ± 1.27</td>
<td>1.24 ± 0.05</td>
<td>1.32 ± 0.09(47)</td>
<td>15.59 ± 1.67(10)</td>
<td>4.07 ± 0.40(9)</td>
</tr>
<tr>
<td>100%</td>
<td>46.71 ± 0.94</td>
<td>1.38 ± 0.13</td>
<td>1.29 ± 0.04(51)</td>
<td>15.94 ± 2.76(13)</td>
<td>2.98 ± 0.60(13)</td>
</tr>
</tbody>
</table>

Table 2.3. Total length, weight, condition factor, gonadosomatic index (GSI), and hepatosomatic index (HSI) of male and female fathead minnows sampled at test termination. Each treatment consisted of three replicate tanks. The total number of organisms assessed in each treatment for each endpoint is listed in parenthesis. Differing letters denote significant differences between treatments (ANOVA, p < 0.05). Data are presented as (mean ± SD)
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cumulative egg production (eggs/tank)</th>
<th>Isolated reproductive test (eggs/female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>534 ± 440</td>
<td>365 ± 226</td>
</tr>
<tr>
<td>50%</td>
<td>1283 ± 641</td>
<td>222 ± 124</td>
</tr>
<tr>
<td>100%</td>
<td>1874 ± 993</td>
<td>1249 ± 124</td>
</tr>
<tr>
<td>100%M</td>
<td>365 ± 226</td>
<td>277 ± 88</td>
</tr>
<tr>
<td>100%W</td>
<td>222 ± 124</td>
<td>258 ± 189</td>
</tr>
</tbody>
</table>

Table 2.4. Cumulative egg production (mean ± SD) measured as the number of eggs produced per tank up to exposure day 145. Isolated reproduction experiment egg production (mean ± SD) measured as the number of eggs produced per female over a 14 day period.
Figure 2.1. General outline of isolated reproduction experiment. The treatment nomenclature designation describes the treatments that were created after individuals were removed from original exposure tanks and placed in reproduction media. F1 generation data is referenced to the treatments created during the isolated reproduction experiment.
Figure 2.2. A) Percent survival (mean ± SD) of fathead minnows (3 replicate tanks per treatment each containing 90 individuals) on exposure day 68. B) Percent survival (mean ± SD) of fathead minnows (3 replicate tanks per treatment each containing 25 individuals) from exposure day 68 until the end of the experiment. Differing letters denote significant differences between treatments (ANOVA, p < 0.05).
Figure 2.3. Secondary sex characteristic scores for male fathead minnows (mean ± SD) (2 males assessed per tank) in the parent generation. Asterisk* denotes a significant difference from the control (0% treatment) (Wilcoxon rank sum test, p < 0.05).
Figure 2.4. A) Testis from a control male displaying a normal pattern of spermatogenesis. B) Testis from a male in the 100% treatment displaying testicular oocytes (OC) developing within normal testicular tissue (T). C) Ovary from a control female displaying a normal variation in oocyte development.
Figure 2.5. Secondary sex characteristic scores for male fathead minnows (mean ± SD) (2 males assessed per tank) in the F1 generation. Asterisk* denotes a significant difference from the 0M treatment (Wilcoxon rank sum test, p < 0.05).
Figure 2.6. A) Testis from a 0W treatment male displaying testicular oocytes (OC) residing within normal testicular tissue (T). B) Immature testis from a 0W treatment male lacking mature spermatozoa.
Chapter 3: The effects of wastewater effluent on the northern leopard frog, *Rana pipiens*

I. Abstract

Wastewater effluents are complex mixtures containing a variety of anthropogenic compounds, many of which are known endocrine disruptors. In order to characterize the developmental and behavioral effects of such a complex mixture, northern leopard frogs, *Rana pipiens*, were exposed to a range of concentrations (0, 10, 50, 100%) of municipal wastewater effluent from the egg stage through metamorphosis. The estrogenic activity of the effluent was quantified by the calculation of an estradiol (E2) equivalency factor (EEQ) and was determined to be equivalent to $1.724 \pm 2.103$ ng/L E2. Individuals from the 50 and 100% wastewater treatments took significantly longer to reach metamorphosis than individuals in the 0 and 10% treatments. An increased incidence of male testicular oocytes was observed in the 50 and 100% treatments when compared to the control treatment. Morphological changes in the thyroid glands of 100% wastewater-treated individuals were also noted. No effects of wastewater exposure on growth, sex ratio, swim speed, startle response, or female gonadal development were observed. These results suggest that municipal wastewater effluent can alter the timing of the metamorphic process and impact male sexual development in *R. pipiens*.
II. Introduction

Wastewater effluents around the world may contain measurable amounts of a variety of pharmaceuticals, personal care products, industrial compounds and natural and synthetic hormones and serve as a major pathway for the aforementioned classes of compounds to enter the aquatic environment (Nakada et al. 2004, Nakada et al. 2006, Snyder 2008). A survey conducted throughout the United States between 1999 and 2000 by the United States Geological Survey found organic wastewater-derived contaminants in 80% of the 139 surface waters sampled (Kolpin et al. 2002).

Wastewater contaminants, including pharmaceuticals and industrial chemicals, are capable of interfering with endocrine system-mediated physiological functions in a variety of species. Amphibians have been used as models to study the effects of such endocrine-disrupting compounds (EDCs). The ability of EDCs to alter development was characterized by Pettersson and Berg (2007) who observed female biased sex ratios in *Xenopus tropicalis* and *Rana temporaria* after exposure to the synthetic estrogen ethynylestradiol (EE2). Park and Kidd (2005) observed reduced hatching success in *R. clamitans* and increased gonadal intersex in *R. septentrionalis* following exposure to EE2. In addition, gonadal abnormalities in amphibian species following exposure to EDCs have been noted in multiple studies (Mackenzie et al. 2003, Tsai et al. 2003, Hogan et al. 2008).

Wastewater-derived EDCs also interfere with metamorphic processes. Goto et al. (2006) found that bisphenol-A (BPA), a component of polycarbonate plastics, suppressed
T₃-induced tail regression in *R. rugosa* and inhibited metamorphosis in *X. tropicalis*, suggesting that BPA interferes with thyroid-dependent processes. Hogan et al. (2008) observed a delay in the time to reach metamorphosis in *R. pipiens* tadpoles exposed to EE2, suggesting that estrogenic compounds may interfere with thyroid system homeostasis. Thyroid-disrupting EDCs are of particular interest in amphibian models due to the reliance of amphibians on thyroid hormones to drive the metamorphic process.

In addition to developmental considerations, the potential for wastewater-derived contaminants to affect behavior must also be considered. Acetaminophen and triclosan, two common wastewater constituents, have been shown to affect activity levels of *Bufo americanus* and *X. laevis* tadpoles (Smith and Burgett 2005, Fraker and Smith 2005). High concentrations of triclosan have also been shown to decrease the activity level and startle response of *R. pipiens* tadpoles (Fraker and Smith 2004). The behavioral effects of other pharmaceutical and personal care products on amphibian species have been largely unstudied. The potential for the psychoactive properties of many such products to cause behavioral effects warrants further study.

Much of the aforementioned research has been conducted in laboratory settings using single compound exposures. While such studies are useful in determining the effects of individual compounds, these studies do not represent real-world exposure scenarios where wildlife may be exposed to complex mixtures containing a variety of anthropogenic compounds. The present study examined the effects of such a complex mixture by using an actual municipal wastewater final effluent to create treatment
solutions. The goal of this study was to characterize the behavioral and developmental effects of long-term exposure to a municipal wastewater effluent on the northern leopard frog, *R. pipiens*.

III. Materials & methods

*Solution preparation*

Reconstituted moderately hard water (nominal hardness and alkalinity of 80 and 60 mg/L as CaCO$_3$, respectively) was used as control medium and was produced in the laboratory using 18 mega-ohm water and reagent grade salts (Weber 1993). Final municipal wastewater effluent comprised of 92% residential and 8% industrial input was collected biweekly from the Mauldin Road Wastewater Treatment Plant in Greenville, SC, brought directly to the Clemson University Institute of Environmental Toxicology (CU-ENTOX) and diluted with the control medium to produce 10%, 50%, and 100% effluent treatment solutions. Measured hardness and alkalinity of the effluent was 83.33 ± 8.8 mg/L and 97.72 ± 11.4 mg/L as CaCO$_3$, respectively.

*Mauldin Road Wastewater Treatment Plant*

The Mauldin Road Wastewater Treatment Plant in Greenville, South Carolina, USA, has a drainage area of 66 square miles with a maximum daily flow of 45 million gallons per day (MGD). Approximately 37,000 residences comprise the service area. The average daily plant flow is 16.5 MGD, of which 2.5 MGD comes from industrial sources (Dr. Stephen Graef, personal communication).
Experimental setup

Seven clutches of *R. pipiens*, eggs were purchased from the Carolina Biological Supply Company (Burlington, NC). Eggs from all clutches were pooled and then divided evenly into four aquaria containing a thin layer of 0% (control), 10%, 50%, or 100% wastewater treatment solutions. Upon becoming free swimming larvae (Gosner stage 25) (Gosner 1960), individuals were divided into groups of 40 and stocked in separate aquaria containing 20 L of the appropriate treatment solution, creating four replicate aquaria per treatment, each containing 40 individuals. Treatments were completely renewed twice a week, with daily partial renewals to maintain water quality. Effluent from the most recent collection event was kept in the laboratory and used during partial treatment renewals. Tadpoles were maintained in a climate controlled test room at CU-ENTOX on a 16:8 light:dark cycle and were continuously aerated. Treatment mean water temperature, dissolved oxygen, and pH ranged from 20.7-21.2 °C, 7.61-7.89 mg/L, and 7.89-7.96, respectively. Tadpoles were fed a 1:1 mixture of commercial fish food:commercial amphibian food. The test was terminated after 111 days of exposure.

EDC Analysis

Over the course of the experiment, effluent brought into the laboratory for treatment solution renewal was subsampled and analyzed for the presence of a suite of natural and synthetic hormones and alkylphenolic compounds. Nine times during the exposure period, a 2.5L sample of whole effluent was collected in an amber bottle, sealed with no headspace, placed on ice, and shipped overnight to a laboratory at the United

57
States Environmental Protection Agency’s Office of Research and Development in Cincinnati, OH, where it was processed for the detection of steroid hormones within 24hrs of collection. Six times during the exposure period, a 2.5L sample of whole effluent was collected in an amber bottle, the effluent pH was set to 2 using concentrated sulfuric acid, and the sample was shipped to a laboratory at the United States Environmental Protection Agency’s Office of Research and Development in Chicago, IL, where it was processed for the detection of nonylphenol (NP), nonylphenol monoethoxylate (NP1E), nonylphenol diethoxylate (NP2E), bisphenol-A (BPA), and octylphenol (OP). An estradiol equivalency (EEQ) factor was calculated by multiplying measured chemical concentrations by experimentally determined (Rutishauser et al. 2004) estrogenic potencies (relative to 17β-estradiol) to characterize the estrogenic activity of the effluent.

Growth and development measurements

Upon reaching forelimb emergence (Gosner stage 42) tadpoles were removed from the exposure aquaria and placed in inclined aquaria containing a wet (appropriate treatment solution) and dry phase. Tadpoles were maintained in the inclined aquaria until completing metamorphosis (tail length ≤ 2 mm). The time in days to reach metamorphosis was recorded. Upon reaching metamorphosis, individuals were euthanized, sexed internally, and sampled for snout-vent length (SVL), total body weight, and liver weight for the calculation of a hepato-somatic index (HSI) (ratio of liver weight to total body weight * 100). Heads (removed caudal to the eyes) and gonads (removed as
a complex with the kidneys) were fixed in 10% neutral-buffered formalin solution for histological examination.

**Histological preparation and analysis**

Fixed heads and gonadal tissues were dehydrated using a graded series of ethanol concentrations (50, 75, 90, 100%), cleared with xylene, and embedded in resin (Immunobed, Polysciences, Warrington, PA). Heads from the control and 100% treatments were sectioned at 3 um until a minimum of 5 sections containing intact thyroid gland tissue per individual were obtained. Male gonads in the control, 50 and 100% treatments were sectioned entirely with 1.5um sections being taken at increments of 25 um and were assessed for the presence of testicular oocytes, defined as immature oocytes residing within testicular tissue. Female gonads from the control and 100% treatments were sectioned at 1.5 um until 10 sections were obtained at increments of 30 um and examined for the presence of normal ovarian characteristics. Slides were stained with dilute azure II and basic fuchsin solutions.

Thyroid gland morphology was assessed according to guidelines laid forth in an EPA guidance document on amphibian thyroid histology (Grim 2007). Thyroid gland hypertrophy/atrophy, follicular cell hypertrophy, defined as an increase in the size of cells surrounding the follicles, and follicular hyperplasia, defined as an increase in the number of cells surrounding the follicles, were assessed on a 0-3 severity scale (0 = not remarkable, 1 = mild, 2 = moderate, 3 = severe). Thyroid glands from five control
individuals (minimum of one per replicate) and six 100% wastewater-treated individuals (minimum of one per replicate) were assessed.

Startle response

On exposure days 20 and 30, startle response experiments were conducted following methods modified from Fraker and Smith (2004). Five tadpoles were removed from each exposure aquaria and placed in plastic cups containing 350 mL of treatment solution taken from the exposure aquaria. The tadpoles were allowed to acclimate to the new container over a period of approximately three minutes. Following the acclimation period, a sharp tap was applied to the upper rim of the cup using a plastic rod. The number of tadpoles that exhibited a startle response (defined as any abrupt movement in response to the tapping stimulus) in each container was recorded. The number of tadpoles responding in both startle response experiments were averaged within each replicate and entered as one value per replicate for statistical analysis.

Swim speed

On day 22 of the exposure period, a swim speed experiment was conducted following methods modified from Jung and Jagoe (1995). Individually, tadpoles were removed from the exposure aquaria and placed in a glass swim channel containing treatment solution taken from the exposure aquaria. Over a period of approximately one minute, the tadpole was chased back and forth over the length of the swim channel using the bulb end of a plastic pipet while being timed with a stopwatch. Swimming activity and the stopwatch were recorded in the same frame using a digital video recorder (DCR-
DVD403, Sony®). Videos were observed in slow motion and the maximum swim burst was determined for each individual on a cm/s basis. Three tadpoles were assessed per replicate and their average maximum swim burst was calculated to achieve one swim speed value per replicate for statistical analysis.

**Statistical analysis**

All statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC). A nonparametric Wilcoxon rank-sum test was used to examine differences in thyroid gland severity scores. Chi-square analysis was used to test for a relationship between treatment and testicular oocyte occurrence. For all other analyses, one-way analysis of variance (ANOVA) tests were conducted to examine differences between treatment means ($p < 0.05$). Levene’s test was used to examine the assumption of variance homogeneity. Fisher’s LSD post hoc test was used to test for treatment differences when a significant effect was detected by ANOVA. Proportion data (hepato-somatic index, percent survival, and percent female) were arcsine square root transformed prior to analysis by ANOVA. Analysis of covariance (ANCOVA) was conducted to test for the influence of density on the time to reach metamorphosis using percent survival as a surrogate for density.

**IV. Results**

*EDC analysis*

EDC water analysis (Table 3.1) revealed the presence of steroid hormones, hormone precursors, and industrial compounds. Dihydrotestosterone (DHT), a
biologically active metabolite of testosterone, was detected at a relatively high concentration (33.2 ng/L) at one sampling event, but was only detected during three of the remaining eight sampling events and never reached a concentration greater than 1.0 ng/L. The steroid hormone precursor progesterone was detected at one sampling event at a concentration of 2.6 ng/L, while the steroid hormone precursor androstenedione was detected at all sampling events. Testosterone, the synthetic estrogen 17α-ethynylestradiol (EE2), and estrone (E1) were detected at all sampling events, while the natural estrogen 17β-estradiol (E2) was detected during four of the nine sampling events at a maximum concentration of 2.6 ng/L. The estrogenic hormone estriol (E3) was not detected at any sampling event. Analysis for industrial compounds did not detect OP at any sampling event. BPA was detected at three sampling events with a maximum concentration of 270 ng/L, while NP was detected twice with a maximum concentration of 430 ng/L. NP1E was detected during all sampling events while NP2E was detected twice. EEQ values (Table 3.2) were calculated using only the measured concentrations of E1, E2, and EE2. The measured concentrations of the industrial compounds coupled with their low E2-relative potencies provided no significant contribution to the estrogenic activity of the effluent and, therefore, were not included in the EEQ calculations.

**Survival**

Significant differences in survival were detected between treatments (\( p = 0.044, F = 3.66, df = 3,12 \)) (Table 3.3). Survival in the 50 and 100% treatments was
significantly greater than the control treatment, but did not differ from the 10% treatment. No difference in survival was detected between the control and 10% treatment.

*Time to metamorphosis*

When analyzed by ANCOVA, survival had no significant effect on the time in days to reach metamorphosis for any treatment; therefore, survival was not used as a covariate in the model testing for differences in the time to reach metamorphosis. Time to metamorphosis was significantly greater in the 50 and 100% treatments than the control and 10% treatments \((p = 0.002, F = 9.28, df = 3,12)\) (Figure 1A). All surviving individuals in the control and 10% treatments had reached metamorphosis by day 111, while a small percentage (Figure 1B) of individuals in all four of the 50% treatment replicates and in three of the four 100% treatment replicates had not reached metamorphosis by the end of the test. Because the remaining un-metamorphosed individuals were not entered into the time to metamorphosis analysis, the time to metamorphosis data underestimates the true difference between the two higher wastewater treatments and the control and 10% wastewater treatment.

*Growth measurements*

Snout-vent length (SVL) and total body weight treatment means decreased as percent wastewater increased, although no significant differences were detected between any treatments \((p = 0.093, F = 2.69, df = 3,12\) and \(p = 0.265 F = 1.50, df = 3,12\) respectively) (Table 3.3). Hepato-somatic index (HSI) treatment means did not follow a
dose-dependent pattern and did not differ significantly ($p = 0.394, F = 1.08, df = 3,12$) (Table 3.3).

**Sex distribution**

The proportion of juvenile frogs that were determined to be female did not differ between treatments ($p = 0.577, F = 0.69, df = 3,12$). All treatments were slightly skewed toward a female-biased sex ratio with the 10% treatment containing the highest percentage of females at 63% and the 100% treatment containing the lowest percentage at 56%.

**Swim speed and startle response**

Maximum swim speed treatment means ranged from 15.86 cm/s in the 10% treatment to 18.51 cm/s in the 50% treatment and did not follow a dose-dependent pattern nor differ significantly between treatments ($p = 0.403, F = 1.06, df = 3,12$) (Table 3.1). The mean proportion of tadpoles exhibiting a startle response was greater than 90% in all treatments and did not differ significantly between treatments ($p = 0.517, F = 0.80, df = 3,12$).

**Histology**

Histological examination of the ovaries (Figure 3.2A) revealed no observable morphological differences. All ovaries primarily contained diplotene oocytes surrounding an ovarian cavity. Larger diplotene oocytes were close to the centers of the ovaries with smaller oocytes surrounding them. Small nests of oogonia were deposited
around the outer edge of each ovary within the germinal epithelium. Cortical alveoli were present in the zona granulosa of some diplotene oocytes in all observed ovaries.

Histological examination of male gonads (Figure 3.2B and 3.2C) revealed an increased incidence of males containing testicular oocytes in the 50 and 100% treatments. Males from the 100% treatment displayed a 64%, nine out of 14 individuals, incidence of testicular oocytes, while males from the 50% treatment displayed a 37.5%, three out of 8 individuals, incidence of testicular oocytes. Individuals from the control displayed a 25%, three out of 12 individuals, incidence of testicular oocytes. Chi-square analysis revealed a significantly greater occurrence of testicular oocytes in the 100% treatment relative to the control ($p = 0.045$). No differences in testicular oocyte occurrence were observed between the 100 and 50% treatments ($p = 0.512$) or the 50% and control treatment ($p = 0.251$). The greatest abundance of testicular oocytes was noted in the 100% treatment with three males containing greater than ten testicular oocytes. No individuals from either the control or the 50% treatment contained greater than five testicular oocytes.

Histological examination of the thyroid glands (Figure 3.3A and 3.3B) revealed observable morphological differences. No degree of thyroid gland atrophy/hypertrophy or follicular cell hypertrophy was evident in any of the individuals. A small degree of follicular cell hyperplasia was observed in four out of five control individuals and one out of six 100% wastewater-treated individuals. The mean severity score, derived entirely from follicular cell hyperplasia scores, for the control individuals was 0.8, which was
significantly greater ($p = 0.029$) than the mean severity score of 0.17 for the 100% wastewater-treated individuals. As a strictly qualitative observation, slight differences were observed between the follicular cell shape of control and 100% wastewater-treated individuals. All control individuals displayed predominantly cuboidal follicular cells, while the follicular cells in only two of the 100% wastewater-treated individuals were characterized as predominantly cuboidal. Two 100% wastewater-treated individuals displayed primarily squamous follicular cells, while the remaining two 100% wastewater-treated individuals displayed an approximately 1:1 ratio of cuboidal:squamous follicular cell shape.

V. Discussion

The calculated EEQ of the effluent used in this study is within the range of previous studies. Schiliro et al. (2004) tested final effluent from a wastewater treatment plant in Northern Italy and observed an EEQ that ranged from 0.28-2.40 ng/L. Final effluent from two wastewater treatment plants in Switzerland displayed EEQs generally in a range between 1 and 10 ng/L, although one sample at one of the treatment plants displayed an EEQ well above 10 ng/L (Rutishauser et al. 2004). In Minnesota, effluent from a wastewater treatment plant displayed a mean EEQ of $44.0 \pm 0.9$ ng/L (Martinovic et al. 2007). Effluent from five wastewater treatment plants in Australia displayed EEQs generally ranging from <1 to 14.8 ng/L, with one sample reaching a maximum EEQ of 67.8 ng/L (Tan et al. 2007). The aforementioned studies suggest that the estrogenic activity of the effluent used in this study is on the low end of the range expected for a
final wastewater effluent and provide evidence that EEQs have the potential to reach levels much higher than those observed in this study.

The high cumulative mortality (approximately 40%) in the control treatment is consistent with previous findings in control treatments when *R. pipiens* was used as a model for long-term developmental testing in the laboratory (Orton et al. 2006, Harris et al. 2000, MacKenzie et al. 2003). In the present study, the greater survival in the 50 and 100% treatments versus the control treatment suggests that some aspect of the wastewater effluent provided protection against mortality. A possible hypothesis is that the effluent supplied nutrient sources (organic matter, bacteria, etc.) that were utilized by the treated tadpoles during development. The consistent growth of a biofilm on the sides and bottoms of the 50 and 100% treatment tanks was observed and also suggests the presence of additional nutrient sources not residing in the control solution. An alternative explanation for the greater survival in the higher wastewater treatments would be the expected presence of trace levels of antibiotic/antimicrobial compounds in the effluent (Kolpin et al. 2002). Such compounds could potentially provide protection against disease/infection that may otherwise increase mortality.

Beyond overt mortality, exogenous compounds pose a threat to the long-term survival of amphibian species through the inhibition or acceleration of the metamorphic process. The timing of the metamorphic process is a crucial factor in determining the size, survival, and reproductive output of mature amphibians (Wilbur and Collins 1973, Smith 1987, Semlitsch et al. 1988, Berven 1990). Because the metamorphic process in
amphibians is controlled primarily by the thyroid system (Duellman and Trueb 1986), any alteration in the timing of metamorphosis suggests a disruption of thyroid system homeostasis. In this study, an increase in the time to reach metamorphosis for the two highest wastewater concentrations was observed. A possible explanation for this increased time to metamorphosis might be that exposure to wastewater effluent caused an upregulation of biotransformation enzymes in an effort to metabolize the xenobiotics present in the effluent. Although poorly studied in amphibians, a subset of these enzymes, glucuronidases (UGTs), might reduce circulating levels of thyroid hormones through biliary elimination (Visser et al. 1993, Hood and Klaassen 2000, 2003).

In the present study, an increased metabolic clearance of THs would have disrupted the negative feedback mechanism of circulating THs on the hypothalamus-pituitary-thyroid (HPT) axis. Without negative feedback, the thyroid gland would have been continually stimulated by thyroid-stimulating hormone (TSH) being released from the pituitary. Such excessive stimulation would be expected to result in an increased thyroid gland size, increased degree of follicular cell hyperplasia, and/or increased degree of follicular cell hypertrophy (Tietge et al. 2005, Capen 1994). None of the aforementioned effects were observed in this study during histological examination of the thyroid glands of 100% wastewater-treated individuals, suggesting that such a mechanism was not responsible for the observed increase in the time to reach metamorphosis. In fact, the reduced severity score and increased prevalence of squamous-shaped follicular cells in the 100% wastewater-treated individuals suggests a reduction in thyroid gland stimulation, which would not be consistent with a hypothesis.
based on an increased metabolic clearance of THs. Additionally, the lack of differences in the HSIs of control and treated individuals supports the assertion that hepatic biotransformation activities were not significantly impacted by wastewater exposure.

A second hypothesis to explain the delayed metamorphosis would be that steroid hormones and/or hormone mimics in the effluent served to modulate thyroid system function. Evidence exists suggesting that steroid hormones may disrupt thyroid-dependent metamorphosis (reviewed in Hayes et al. 1997). Gray and Janssens (1990) observed that testosterone and 17β-estradiol inhibited T₃ –induced metamorphosis in X. laevis tadpoles, while Hogan et al. (2008) observed a delay in the time for R. pipiens tadpoles to reach metamorphosis when exposed to EE2 during either mid-metamorphosis or over the entire larval period. The mechanisms underlying such observations have not been clearly elucidated, although previous research has identified possible mechanistic pathways. MacLatchy et al. (1986) and Leatherland (1985) both observed that rainbow trout (Salmo gairdneri) injected with E2 had lowered T₃ and T₄ plasma levels. Jacobs et al. (1988) observed elevated T₄ plasma levels in R. ridibunda and R. esculenta after injection with synthetic luteinizing hormone-releasing hormone. Denver (1988) also presented similar evidence by showing an increase in thyroid-stimulating hormone (TSH) secretion after exposure to synthetic mammalian gonadotropin-releasing hormone (GnRH) in vitro using pituitaries from adult male R. pipiens. The aforementioned studies suggest that sex steroids have the potential to exert some control over the hypothalamic-pituitary-thyroid axis. Because the release of GnRH is controlled by circulating levels of sex steroids, an increase in the concentration of estrogenic compounds would lead to a
reduction in GnRH release through a negative feedback mechanism. Such a reduction in GnRH could prevent additional stimulation of the pituitary and lead to a reduced secretion of TSH, subsequently causing a reduction in the release of thyroid hormones. The lack of thyroid gland stimulation by TSH could explain the altered follicular cell shape that was observed in the 100% wastewater-treated individuals in this study.

Another important aspect of amphibian development is sexual differentiation. An increased incidence of male frogs containing testicular oocytes in response to larval exposure to estrogenic compounds has been observed in prior studies (Mackenzie et al. 2003, Pettersson and Berg 2007, Hogan et al. 2008). Female-biased sex ratios were also observed in the aforementioned studies following exposure to estrogenic compounds, suggesting that amphibian species are susceptible to sex reversal resulting in genotypic males becoming phenotypic females. In this study, the increased incidence of wastewater-exposed individuals containing testicular oocytes is consistent with previous observations, but the lack of significantly greater female-biased sex ratios in the wastewater treatments does not support the findings of other researchers. An explanation for this observation is that the aforementioned studies used a range of estrogenic compound concentrations that achieved much higher levels than those measured in this study. Pettersson and Berg (2007) observed differences in sex ratios of X. laevis at a measured concentration of approximately 18 ng/L EE2, but observed no differences at 1.8 ng/L EE2, which was much closer to the concentrations measured in this study. Additionally, Mackenzie et al. (2003) observed female-biased sex ratios of R. pipiens at minimum E2 and EE2 concentrations of 1 ug/L, which were still orders of magnitude
greater than the highest measured concentrations in this study. Results of the present study suggest that at low environmentally-relevant concentrations of estrogenic compounds, altered sex ratios are not a sensitive marker of estrogenic exposure. In this study, the incidence of male testicular oocytes was proven to be a better indicator for exposure to very low concentrations of estrogenic compounds.

In addition to examining developmental effects in amphibians after exposure to wastewater effluent, behavioral effects must also be considered. A widespread occurrence of pharmaceutical and personal care products (PPCPs) has been observed in surface waters (Kolpin et al. 2002). Many of these compounds are designed to alter biochemical pathways, subsequently leading to changes in mood and behavior. The effects of such compounds on wildlife exposed inadvertently through wastewater effluent discharges are not fully understood. Fraker and Smith (2004, 2005) observed a decrease in X. laevis tadpole activity following exposure to triclosan, a common antimicrobial compound, and acetaminophen, a common analgesic, both of which are frequently detected in wastewater effluents, while R. pipiens tadpoles displayed reduced activity after exposure to triclosan and an increased startle response after exposure to caffeine, but were not affected by acetaminophen. Using hybrid striped bass (Morone saxtilis X M. chrysops), Gaworecki and Klaine (2008) observed a dose-dependent increase in the time to catch live prey during and after exposure to fluoxetine, the active ingredient in Prozac™, although test concentrations were well above environmental levels. In the present study behavioral effects were quantified through the use of a swim speed and startle response tests. No significant effects of effluent exposure on either endpoint were
observed suggesting that the tested effluent did not pose a significant risk to behavior. Although PPCPs were not quantified, the nature of the test solution (actual wastewater effluent) would suggest their presence.

In conclusion, larval exposure to final municipal wastewater effluent delayed metamorphosis, altered thyroid gland morphology, and altered male gonadal development in *R. pipiens* juveniles. While such observations have been documented previously, this study utilized an environmentally relevant mixture and provided an important link between laboratory dosing studies and observations obtained through field experimentation. The results of this study suggest that exposure to very low environmentally relevant concentrations of estrogenic compounds poses a significant risk to the successful development and metamorphosis of *R. pipiens*. The timing of amphibian metamorphosis has been shown to be an important factor in determining the size, survival, and reproductive output of mature amphibians, therefore xenobiotic compounds that alter the timing of the process may also adversely affect these factors (Wilbur and Collins 1973, Smith 1987, Semlitsch et al. 1988, Berven 1990). Further research efforts should also examine the consequences of altered gonadal development beyond the juvenile stage and into reproductive maturity to determine the risk that wastewater effluents pose to successful reproduction of ranid frog populations following early-life exposure.
VI. References


Table 3.1. Minimum, maximum, and mean ± SD concentrations and E2-relative potencies of target analytes in final wastewater effluent collected from the Mauldin Road Wastewater Treatment Plant (n = number of sampling events for each compound).

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Minimum (ng/L)</th>
<th>Maximum (ng/L)</th>
<th>Mean±SD (ng/L)</th>
<th>E2-relative potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dihydrotestosterone</td>
<td>9</td>
<td>nd</td>
<td>33.2</td>
<td>3.98±10.97</td>
<td>na</td>
</tr>
<tr>
<td>Testosterone</td>
<td>9</td>
<td>0.8</td>
<td>5.2</td>
<td>1.82±1.36</td>
<td>na</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>9</td>
<td>2.4</td>
<td>5.6</td>
<td>3.56±0.97</td>
<td>na</td>
</tr>
<tr>
<td>Progesterone</td>
<td>9</td>
<td>nd</td>
<td>2.6</td>
<td>0.29±0.87</td>
<td>na</td>
</tr>
<tr>
<td>E1</td>
<td>9</td>
<td>0.4</td>
<td>15.9</td>
<td>2.74±4.98</td>
<td>0.38</td>
</tr>
<tr>
<td>E2</td>
<td>9</td>
<td>nd</td>
<td>2.6</td>
<td>0.42±0.85</td>
<td>1</td>
</tr>
<tr>
<td>EE2</td>
<td>9</td>
<td>0.1</td>
<td>0.5</td>
<td>0.21±0.13</td>
<td>1.19</td>
</tr>
<tr>
<td>E3</td>
<td>9</td>
<td>nd</td>
<td>nd</td>
<td>na</td>
<td>2.4x10⁻³</td>
</tr>
<tr>
<td>NP</td>
<td>6</td>
<td>nd</td>
<td>430</td>
<td>130±57</td>
<td>2.5x10⁻⁵</td>
</tr>
<tr>
<td>OP</td>
<td>6</td>
<td>nd</td>
<td>nd</td>
<td>na</td>
<td>7.8x10⁻⁶</td>
</tr>
<tr>
<td>NP1E</td>
<td>6</td>
<td>380</td>
<td>3200</td>
<td>1297±1092</td>
<td>na</td>
</tr>
<tr>
<td>NP2E</td>
<td>6</td>
<td>nd</td>
<td>2300</td>
<td>717±1114</td>
<td>na</td>
</tr>
<tr>
<td>BPA</td>
<td>6</td>
<td>nd</td>
<td>270</td>
<td>117±32</td>
<td>1.1x10⁻⁴</td>
</tr>
</tbody>
</table>

*taken from Rutishauser et al. (2004)

Table 3.1. Minimum, maximum, and mean ± SD concentrations and E2-relative potencies of target analytes in final wastewater effluent collected from the Mauldin Road Wastewater Treatment Plant (n = number of sampling events for each compound).

Table 3.2. Estradiol equivalents (EEQ) of whole effluent samples taken over the course of the experiment calculated from measured concentrations of E1, E2, and EE2.

<table>
<thead>
<tr>
<th>Sampling event</th>
<th>EEQ (ng/L)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.614</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.728</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.188</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.758</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.561</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.679</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.325</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.488</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.171</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Estradiol equivalents (EEQ) of whole effluent samples taken over the course of the experiment calculated from measured concentrations of E1, E2, and EE2.
Table 3.3. Percent survival of *R. pipiens* tadpoles and juveniles (n = 4 per treatment, 40 individuals per n). Hepato-somatic index (HSI), snout-vent length (SVL), and total body weight of juveniles sampled upon completing metamorphosis (n = 4 per treatment, 21-37 individuals per n) and swim speed of *R. pipiens* tadpoles (n = 4 per treatment, 3 individuals per n). Differing letters denote significant differences between treatment means (ANOVA, p < 0.05).

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control</th>
<th>10%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (%)</td>
<td>60.38±1.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.44±1.18&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>78.75±7.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77.00±11.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HSI</td>
<td>3.12±0.22</td>
<td>3.18±0.23</td>
<td>2.97±0.08</td>
<td>3.06±0.09</td>
</tr>
<tr>
<td>SVL (mm)</td>
<td>33.95±0.30</td>
<td>33.64±1.10</td>
<td>33.39±0.22</td>
<td>32.29±1.37</td>
</tr>
<tr>
<td>Total weight (g)</td>
<td>3.06±0.14</td>
<td>2.97±0.30</td>
<td>2.88±0.10</td>
<td>2.68±0.40</td>
</tr>
<tr>
<td>Swim speed (cm/s)</td>
<td>16.57±1.86</td>
<td>15.86±3.39</td>
<td>18.52±1.06</td>
<td>16.56±1.92</td>
</tr>
</tbody>
</table>
Figure 3.1.  

A) The time in days for *R. pipiens* tadpoles to reach complete metamorphosis.  

B) The percentage of surviving tadpoles that reached complete metamorphosis by exposure day 111.  
Differing letters denote significant differences between treatments (ANOVA, p < 0.05). Data is presented as mean ± SD.
Figure 3.2.  A) Ovarian tissue from a control *R. pipiens* female.  B) Testis from a control *R. pipiens* male.  C) Testis from a 100% wastewater-treated male containing distinct oocytes (OC) residing within normal testicular tissue (T).
Figure 3.3. A) Thyroid tissue from a control *R. pipiens* juvenile displaying cuboidal follicular cells and a mild degree of follicular cell hyperplasia (HP). B) Thyroid tissue from a 100% wastewater-treated individual displaying squamous follicular cells and no hyperplasia.
Chapter 4: Long-term exposure of the pickerel frog, *Rana palustris*, to triclosan

I. Abstract

Thyroid-disrupting compounds pose a significant threat to the successful growth and development of amphibians, due to amphibians’ reliance on thyroid hormones (THs) to drive the metamorphic process. This study investigated the effects of triclosan (TRC), an antibacterial agent and suspected thyroid disruptor, on the growth and development of the pickerel frog, *Rana palustris*. No differences in the time to reach forelimb emergence were observed between a control treatment and a range of TRC treatments (0.25-30 ug/L). Additionally, no differences were observed between treatments in the percentage of tadpoles reaching forelimb emergence after 105 days of exposure. No effects of TRC were observed on snout-vent length, weight, or thyroid gland morphology of juvenile frogs exposed throughout development. The aforementioned lack of effects suggests that triclosan does not significantly influence the developmental process in the pickerel frog.

II. Introduction

Triclosan (TRC) is a bisphenolic antimicrobial agent found in a variety of personal care products including, but not limited to, soaps, shampoos, deodorants, and toothpastes and is typically found at a concentration of 0.1-0.3% (w/w). A per capita daily usage rate has been estimated to range from 3-5mg/person/day [1]. Due to the nature of the personal care products containing TRC, it can be assumed that considerable amounts of the compound will enter into municipal wastewater systems and may enter the environment in wastewater effluents. A study conducted by the United States
Geological Survey detected TRC in more than half of the 139 surface waters they sampled across 30 states indicating a widespread prevalence of TRC in the aquatic environment (maximum level of 2.3 ug/L) [2].

The potential for TRC to act as an endocrine disrupting compound in aquatic systems has been briefly examined in the literature. Foran et al. [3] observed non-significant trends two months post-exposure of increased male sex ratios and increased male anal fin lengths after a 14-day larval exposure using Japanese medaka, suggesting that TRC may be weakly androgenic. Ishibashi et al. [4] observed an increase in vitellogenin production in male medaka after exposure to 20 and 100 ug/L TRC and concluded that a metabolite of TRC may be weakly estrogenic. In addition to interfering with sex-related steroid hormone homeostasis, the potential for TRC to disrupt thyroid hormone (TH) homeostasis has been observed in a mammalian model. Oral administration of TRC to Long-Evans rats resulted in a dose-dependent decrease in serum total thyroxine (T4) levels [5]. The reduction in circulating levels of T4 was hypothesized to be a result of activation of the pregnane-X-receptor (PXR) [6] leading to an induction of biotransformation enzymes responsible for catabolizing THs. Amphibian species have not been shown to possess a form of the PXR, but the induction of biotransformation enzymes, specifically glucuronidases, which catabolize THs, is an area of concern for amphibian species because THs are the primary agents responsible for driving the metamorphic process in amphibians. The timing of the metamorphic process is an important factor in determining the size, survival, and reproductive output of mature amphibians [7, 8,9,10] and, therefore, underlines the importance of understanding the
effects of xenobiotics that interfere with metamorphosis-related and/or thyroid-mediated processes.

The present work tested the hypothesis that aqueous exposure to TRC would delay the metamorphic process in the pickerel frog, *Rana palustris*, presumably by inducing biotransformation reactions, leading to an increased metabolism of THs and a longer time to reach metamorphic climax.

III. Materials & methods

*Solution preparation*

Reconstituted moderately-hard water with a nominal hardness and alkalinity of 80 and 60 mg/L as CaCO₃, respectively, was created in the laboratory and used as the control test solution. Appropriate volumes of stock solution were added to the reconstituted moderately-hard water to form each treatment solution. A 3mg/L stock solution of triiodothyronine (T3) (Sigma-Aldrich, St. Louis, MO) was made by dissolving 3 mg of T3 in 15 mL of 50mM sodium hydroxide and then diluting to 1 L. TRC (TRC) (Irgasan, Sigma-Aldrich, St. Louis, MO) stock solutions were made by dissolving 100 mg of TRC in 10 mL of 50mM sodium hydroxide followed by either a 100 fold dilution, used for the two highest TRC concentrations, or a 1000 fold dilution, used for the two lowest TRC concentrations.
Experimental setup

Two clutches of pickerel frog, *Rana palustris*, eggs were purchased from the Charles Sullivan Co. (Nashville, TN). Eggs were pooled and maintained in a thin layer of moderately-hard water. Upon becoming free swimming larvae (Gosner stage 25) [11] individuals were separated into groups of three and stocked in 750mL glass jars containing moderately-hard water. Each treatment consisted of nine replicate 750mL jars containing three tadpoles apiece. Exposures began 14 days after stocking larvae.

Experimental solutions consisted of a negative control containing moderately-hard water, a 3ug/L T3 positive control for thyroid hormone, a range of TRC treatments (0.25, 1.0, 10.0, and 30.0ug/L), and a combined 3ug/L T3 and 30ug/L TRC treatment. On three separate occasions during the experiment, a random sample of one replicate from each TRC treatment was taken at 0 and 24 hrs and analyzed to confirm the nominal TRC concentrations using a magnetic particle-based ELISA kit (Abraxis Kits, Warminster, PA) (Table 1). Treatments were renewed every 48hrs. Tadpoles were maintained in a climate controlled test room on a 16:8 light:dark cycle and were continuously aerated. Treatment mean water temperature, dissolved oxygen, and pH ranged from 21.54-21.61°C, 7.44-7.69 mg/L, and 7.78-7.87 respectively. Tadpoles were fed a 1:1 mixture of commercial fish food:rabbit chow. On day 43 of the test, all tadpoles were transferred from 750 mL to 3 L of test solution for the remainder of the experiment. The test was terminated after 105 days of exposure.
**Biological measurements**

Individuals were removed from exposure containers and euthanized upon reaching forelimb emergence (Gosner stage 42) and the time to reach this stage was recorded. Tadpoles alive at the end of the test that had not reached stage 42 were euthanized. A subset of stage 42 tadpoles from each exposure container were moved to inclined aquaria containing the appropriate treatment solution and allowed to reach complete metamorphosis (tail length ≤ 2 mm). Complete metamorphs were designated as juvenile frogs. Juveniles were used for the measurement of snout-vent length (SVL), weight, hepato-somatic index (HSI) (ratio of liver weight to total body weight), hind limb length (HLL), and thyroid gland histological examination.

**Histological preparation and analysis**

Heads from newly metamorphosed individuals were fixed in 10% neutral-buffered formalin overnight. Tissues were then dehydrated using a graded series of ethanol concentrations (50, 75, 90, 100%), cleared with xylene, and embedded in resin (Immunobed, Polysciences, Warrington, PA). Heads were sectioned at 3 um until a minimum of 10 sections containing intact thyroid gland tissue per individual were obtained. Slides were stained with dilute azure II and basic fuchsin solutions. Thyroid gland morphology was assessed according to guidelines laid forth in an EPA guidance document on amphibian thyroid histology [12]. Thyroid gland hypertrophy/atrophy, follicular hypertrophy, defined as an increase in the size of cells surrounding the follicles, and follicular hyperplasia, defined as an increase in the number of cells surrounding the
follicles, were assessed relative to controls and graded on a 0-3 severity scale (0 = not remarkable, 1 = mild, 2 = moderate, 3 = severe). Five individuals, each taken from a different replicate, were assessed per treatment.

Statistical analysis

All statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) tests were conducted to examine differences between treatment means ($p < 0.05$). Levene’s test was used to examine the assumption of variance homogeneity. Tukey’s post hoc test was used to test for treatment differences when a significant effect was detected by ANOVA. Proportion data (hepato-somatic index, percent survival, and percentage of individuals reaching FLE by day 105) were arcsine square root transformed prior to analysis by ANOVA. A Welch’s ANOVA (used to correct for heterogeneous variances when data transformation was unsuccessful) examined differences in arcsine square root transformed percent survival treatment means.

IV. Results

The T3 and T3 + TRC treatments displayed 100% mortality with mean times of death of 8.15 and 8.19 days post-exposure, respectively. The time to death did not differ between the two treatments. Tadpoles in both treatments displayed weight loss and a shortening of the tail, both of which are characteristics of the initiation of metamorphosis. No differences in survival ($p = 0.278, F = 1.32, df = 4$), SVL ($p = 0.637, F = 0.64, df = 4$), HLL ($p = 0.572, F = 0.74, df = 4$), or weight ($p = 0.661, F = 0.61, df = 4$) were
observed between any treatments (Table 4.1). HSI was significantly higher in the 0.25µg/L treatment than in the 10.0µg/L treatment \( (p = 0.041, F = 2.79, df = 4) \), but did not differ between any other treatments. Thyroid gland histological examination revealed no gradable differences in thyroid gland morphology between the control and any TRC treatments; therefore each treated individual received a severity score of 0. The time in days to reach forelimb emergence (Figure 4.1A) \( (p = 0.902, F = 0.26, df = 4) \) and the percentage of individuals per experimental unit reaching forelimb emergence by day 105 (Figure 4.1B) \( (p = 0.212, F = 1.53, df = 4) \) did not differ between treatments.

V. Discussion

The hypothesis being tested in this study relied upon the assumption that \( R. \) palustris possesses biotransformation systems that could be induced by TRC exposure, although such systems have been poorly studied in North American ranid frog models. As mentioned earlier, rats exposed to TRC displayed a decrease in circulating levels of T4 which was hypothesized to be a result of PXR activation leading to increased biotransformation and clearance of TRC [5], based on evidence that TRC activated the PXR in a human hepatocell line [6]. The closest relative (residing in the same subfamily) of the PXR that has been observed in an amphibian model is a nuclear receptor known as the benzoate-X-receptor (BXR) which has been identified in \( X. \) laevis, although the BXR is not as promiscuous as the PXR and has not been determined to function as a xenobiotic receptor [13,14,15,16]. In order to better understand the thyroid disrupting potential of xenobiotics, particularly compounds that induce biotransformation
reactions, to North American ranid frogs, a better understanding of the xenobiotic metabolic system of frogs must be achieved. This increased understanding will allow researchers to better extrapolate potential effects between species.

The 100% mortality with no difference in the time to death for the T3 and the T3 + TRC treatments suggests that TRC did not induce biotransformation reactions that would have offered protection against premature T3-induced metamorphosis through increased T3 metabolism, although the possibility exists that the concentration of T3 used was high enough to mask any TRC-mediated effects. TRC exposure alone did not pose a threat to the survival of \textit{R. palustris} at the concentrations tested, despite the highest concentration (30 ug/L) being well above expected environmental levels. This finding is consistent with previous research showing no effect of exposure of up to 23 ug/L TRC on the survival of \textit{Rana pipiens} tadpoles [17]. Reduced survival of \textit{R. pipiens} tadpoles has been observed at a concentration of 230 ug/L TRC [17], although this concentration did not adversely affect survival in \textit{Xenopus laevis} tadpoles [18].

The lack of differences observed in the morphology of the thyroid gland suggests that the gland itself was not directly impacted. Had my hypothesis been true, the increased metabolic clearance of THs would have disrupted the negative feedback mechanisms of circulating THs on the hypothalamus-pituitary-thyroid (HPT) axis. Such a lack of negative feedback would result in continual stimulation of the thyroid gland by thyroid-stimulating hormone (TSH) being released from the pituitary. This excessive stimulation would be expected to result in an increased thyroid gland size, increased
degree of follicular cell hyperplasia, and/or increased degree of follicular cell hypertrophy [19,20].

Although a difference in HSI was observed between the 0.25 and 10.0ug/L treatments, HSI in neither treatment differed from the control. I attribute the aforementioned difference to variability in sampling efficiency and do not believe the observed effect warrants further discussion. The lack of significant differences between treatments in SVL, weight, HLL, the time to reach forelimb emergence and the percentage of individuals per experimental unit reaching forelimb emergence by exposure day 105 suggest that TRC does not pose a threat to the successful development and metamorphosis of *R. palustris*, although previous researchers have found evidence that TRC does interact with the thyroid system in amphibians. Veldhoen et al. [21] observed a significant decrease in *Rana catesbeiana* tadpole body weight, which is associated with the progression of metamorphosis, following 4 days of exposure to a nominal TRC concentration of 30 ug/L and an increase in the abundance of thyroid receptor α mRNA in the brains of *R. catesbeiana* tadpoles following 6 days of exposure to a nominal TRC concentration of 0.3 ug/L. In addition to the effects observed after exposure to TRC alone, altered thyroid hormone-associated gene expression and increased T3-induced development was observed in *R. catesbeiana* tadpoles, as well as altered thyroid hormone-associated gene expression in a *X. laevis* cell line after co-treatment with T3 and TRC. The unrealistic exposure scenario (co-treatment with T3 and TRC) and the acute exposure period in the Veldhoen et al. study make it difficult to interpret how the observed molecular effects will extrapolate to higher levels of organization. Our study
attempted to fill this void by examining the effects of TRC on thyroid gland morphology and whole-individual developmental endpoints after long-term exposure. Given that no effects were observed in our morphological and developmental endpoints, we conclude that long-term exposure to TRC will not produce significant developmental effects.
VI. References


Table 4.1. The 0hr and 24hr mean measured TRC concentrations per treatment (n=3) and percent survival, hepatosomatic index (HSI), snout-vent length (SVL), hind limb length (HLL), and total weight presented as means ± SD of *Rana palustris* tadpoles (survival) and juvenile frogs (HSI, SVL, HLL, weight) exposed to a range of triclosan concentrations (n=7-9 per treatment, 1-3 individuals per n). Differing letters denote significant differences between treatment means.

* Data were arcsine square root transformed before undergoing statistical analysis.
Fig. 4.1.  A) The time in days, after exposures were initiated, for *Rana palustris* tadpoles to reach forelimb emergence (FLE).  B) The percentage of surviving tadpoles that reached FLE by exposure Day 105.  Data is presented as mean ± SD.
CONCLUSION

A low estrogenic activity, relative to values found in the literature for wastewater treatment plant effluent, was measured in wastewater effluent collected from the Mauldin Road Wastewater Treatment Plant in Greenville, SC. This low activity, measured as estradiol equivalents, still produced significant developmental effects in both the fathead minnow, *Pimephales promelas*, and the northern leopard frog, *Rana pipiens*. These findings suggest that an environmentally relevant exposure to wastewater effluent can produce measurable effects following long-term exposure. The primary chemical substituents that accounted for the estrogenic activity were estradiol, ethynlestradiol, and estrone. Industrial chemicals, including nonylphenol, octylphenol, and bisphenol-A, were not present at concentrations high enough to contribute to the estrogenic activity of the effluent, although the possibility does exist that these compounds may bioconcentrate and eventually reach effective levels within the organisms despite the very low aqueous concentrations. Future work should examine body tissue residues of such compounds to determine if significant bioconcentration is occurring. The aqueous concentrations may underestimate the toxicity of such compounds if they are bioconcentrating and reaching active sites at higher concentrations than predicted by aqueous concentrations.

Incidences of developmental impairment were noted in male gonads of both species, though the frequency of impairment was observed to be much higher in *R. pipiens* than *P. promelas*, suggesting that male amphibians exposed to estrogenic compounds are particularly susceptible to altered gonadal development. The results of
the *R. pipiens* experiment suggest that exposure to low levels of estrogenic endocrine-disrupting compounds can significantly impact male sexual development. The latent effects of larval endocrine disruptor exposure on adult amphibians were not examined in this experiment, but warrant further study. Future work should attempt to examine whether or not testicular oocytes in juvenile male frogs will lead to impaired reproduction at sexual maturity.

In addition to altered sexual development, *R. pipiens* displayed a longer time to reach metamorphosis during exposure to high concentrations of effluent. The timing of metamorphosis is a vital factor determining the size, survival, and reproductive success of amphibian populations. Any extrinsic factor that alters this process poses a serious threat to the success of amphibian populations. There appeared to be a link between estrogenic exposure and thyroid function in this study and was evidenced by altered thyroid gland morphology and an altered timing of metamorphosis. Future work should try to better elucidate the mechanisms behind this apparent relationship. Using a controlled dosing experiment with a known concentration of an estrogenic compound would allow for the determination of an observable effects level concentration. Additionally, a laboratory dosing experiment could be replicated to confirm that the observed effects are repeatable. Measuring circulating levels of thyroid stimulating hormones as well as gonadotropic releasing hormones would also provide insight into relationships between the thyroid and gonadal systems.
Male *P. promelas* displayed a reduction in the expression of secondary sex characteristics, though reproductive success did not appear to be impacted. A very small incidence of testicular oocytes was observed which suggests that sexual development of male fatheads was not significantly impaired, although a female-biased sex ratio was also observed following effluent exposure. It is possible that some males experienced sex reversal leading to the skewed sex ratio. Assuming this hypothesis is correct; one may argue that male fish were more sensitive to effluent exposure than male frogs. An alteration of sex distribution within a population could significantly reduce reproductive success.

The onset of reproductive activity and male sexual development in the F1 generation of an effluent-exposed parent generation occurred at an accelerated rate when compared with control offspring, suggesting that exposure to endocrine disruptors can elicit responses in a transgenerational manner. Exposure to a parent generation was shown to alter reproductive activity in an F1 generation, though the mechanisms behind such an effect should be studied further. Future work should examine the mechanistic basis behind the observed effects. Measuring circulating levels of gonadotropic hormones, as well as levels of gonadotropic releasing hormones in both the parent and F1 generation males would provide insight into the endocrine responses of fish exposed to estrogenic compounds. Such endpoints could provide insight on the molecular level into the possibility of male fish mounting a compensatory response to estrogen exposure. Such a compensatory response would explain the increased relative gonad size that was observed in wastewater-treated males. Circulating hormone levels in the parent and F1
generation would also examine transgenerational patterns of sexual development. Repeating this experiment using a laboratory dosing strategy with both continuous and pulsed exposures would test the repeatability of the observed responses.

The occurrence of testicular oocytes in the F1 generation following transient early-life exposure to wastewater effluent suggests that acute exposure to estrogenic compounds at an early stage can produce developmental effects at maturity. These findings suggest that periodic pulses of estrogenic compounds into the environment could threaten fish populations. A release of estrogenic compounds during critical periods of breeding activity could threaten an entire generation. This would be particularly important when dealing with fish species that only breed once, or a few times, each breeding season.

The findings from this study suggest that low levels of wastewater-derived compounds can produce measurable effects in fish and wildlife. These results should be taken into account when determining treatment guidelines and water quality standards as they relate to wastewater treatment. Advanced treatment operations such as ozonation and other mixed-oxidative processes have been shown to be effective in removing low levels of endocrine-disrupting compounds from wastewater. Before such processes will be implemented on a widespread basis, continued research examining the risk posed by wastewater effluents must be conducted. The benefits of advanced wastewater treatment will have to be shown to outweigh the costs of incorporating such treatments before municipalities and water authorities will adopt these methods.