Using Mosquitofish, *Gambusia affinis,* as Bioindicators for the presence of Endocrine Disrupting Compounds in Greenville's Wastewater Effluent

Allyson Middleton

A thesis submitted to the Department of Biology, East Carolina University, in partial fulfillment of the requirements for Biology Honors Thesis

Advisors: Dr. Joseph Luczkovich and Dr. Susan B. McRae
East Carolina University
Department of Biology

May , 2014

I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration, nor has it been submitted elsewhere as coursework for this or another degree.

Signed:_____ Date:

Allyson Middleton

Using Mosquitofish, *Gambusia affinis,* as Bioindicators for the presence of Endocrine Disrupting Compounds in Greenville's Wastewater Effluent

Allyson Middleton

Department of Biology, East Carolina University, Greenville N.C. 27858

ABSTRACT –

Endocrine disrupting compounds (EDCs) are compounds that arise from an assortment of manufactured sources (plastics, pharmaceuticals, pulp mill waste), which have the potential to interrupt hormone pathways by mimicking hormones naturally produced in the body. The secondary sex characteristics of mosquitofish *Gambusia affinis* respond to the presence of EDC's by becoming more masculinized or feminized, based on the type of compound and what it has the ability to mimic. Mosquitofish have proven to be model organisms to serve as bioindicators of whether EDCs are present in their environment due to their sexual dimorphism. Previous studies have shown the degree to which females develop masculinized features is directly proportional to the concentrations of androgen-mimicking compounds to which they are exposed. The most pronounced male secondary characteristic that was observed to be induced on female mosquitofish in previous studies was the development of a male gonopodium, which is an elongation of anal rays 3,4, and 5. The gonopodium is used by males to transfers sperm to females. This study seeks to answer the question of whether mosquitofish populations exhibit changes in the sex ratio and gonopodium of females in sewage effluent when compared with nearby reference locations and previous collections of mosquitofish made by Hildebrand (1932) in North Carolina prior

to the widespread use of EDC's. My results show that the sex ratio of mosquitofish was significantly different in Greenville utilities effluent water when compared with other locations and Hildebrand's collections. There was a 1.421 female to male ratio in Greenville wastewater effluent populations, whereas the ratio was 20.521 at broad run, 22.521 the coastal studies Institute Pond in Manteno, and 4.4 to 1 in Hildebrand's populations from 1927. It is normal to have a female bias in the sex ratio of this species because males die earlier in females get larger than males. This result is consistent with my hypothesis that exposure to EDC's can result in masculine features in females because I classified males bio looking for a gonopodium in females exhibited male characters due to EDC's, then the sex ratio would be expected to decrease. Further supporting evidence consisted of females that had a ratio of anal fin with an link similar to that of males in the sewage treatment plant effluent water.

Acknowledgments

I would sincerely like to thank the organizations that made this study possible: East Carolina University for allowing me to use their lab and equipment as well as Dan Tracey at Greenville Utilities for permitting me to take samples on their property and for providing me with primary resources for my research. I would like to thank Sid Mitra for helping me analyze water samples and Dr. Susan McRae for guiding me through completing this Biology Honors Thesis. I would also like to thank my Ichthyology classmates who helped me collect water samples and my friends and family who supported me along the way. I am especially grateful to my project mentor, Dr. Joseph Luczkovich, who shared his knowledge and guidance and helped me every step of the way in completing this project.

Table of Contents

List of Tables

List of Figures

Introduction

Mosquitofish *Gambusia affinis* (Poeciliidae) are ovoviviparous, or live-bearing, fish. This makes them model organisms for studying endocrine disrupting compounds (EDC's) due to their sexual dimorphism. There are many distinguishing features between male and female mosquitofish, females are larger than males; they have deeper bodies and grow throughout their life. Their max growth depends on fertility of water, including food supply and water temperature. Females can also be identified by a dark blotch above their vent and a small, rounded anal fin. The most noticeable difference is that male mosquitofish develop an elongated anal fin, or gonopodium, upon maturity for copulation.

The gonopodium is described by the elongation of anal fin rays 3,4, and 5 and a termination complex (Deaton and Cureton 2011). Fin ray 4 is the center of the gonopodium and grows the longest and ray 3 develops a new bone so it thickens (Angus et al., 2001). The appearance of this gonopodium should only be observed in males but studies have found that females may develop a structure resembling the gonopodium as well if they are exposed to androgen-mimicking EDCs (Deaton and Cureton 2009). In normal situations, naturally produced androgen hormones bind with cell membrane receptors, which in turn controls male sexual development. However, an assortment of manufactured sources such as pesticides, plastics, pharmaceuticals, various household and industrial chemicals contain endocrine disruptors, which may mimic this process (Apodaca and Mattessich 1997). Also, due to the fact that these compounds are chemically similar to testosterone and estrogen receptor binding sites, they can alter the pattern of synthesis as well as the metabolism of endocrine hormones and modify hormone receptor levels (Lagana, 2003). Since there are a

large variety of suspected EDC's it is possible that certain environments may contain a mixture of multiple EDC's.

When female mosquitofish are exposed to such compounds, secondary male characteristics may appear. These induced alterations of sexuality in mosquitofish have been found downstream of pulp mill and sewage treatment effluent due to the presence of phytosterols in the water (Bortone and Davis 1994). Phytosterols are steroid compounds that originate from trees and plants and become separated during the process of wood pulp preparation (Bortone and Davis 1994). These compounds are unaffected by the water treatment the pulp mill conducts to decontaminate the water bacteriologically, and mosquitofish can come in contact with these compounds in their natural environment (Bortone and Davis 1994). Phytosterols were shown to masculinize mosquitofish in the study by these authors who observed masculinized characteristic in females after only three weeks of exposure, resulting in females with intermediate secondary sexual characteristics. The environmental stress of androgen-mimicking compounds can be measured by the degree of masculinization of the secondary sexual characteristics in mosquitofish, noteably the development of gonopodia in females. Due to this phenomenon, mosquitofish serve as excellent bioindicators for EDCs.

Phytosterols are now increasingly taken as over the counter dietary supplements as natural remedies to lower cholesterol levels for the treatment of hypercholesterolemia and cardiovascular diseases (Nieminen et al. 2001). Due to the fact that humans only absorb approximately 5% of phytosterols ingested, roughly 95% will be excreted (Nieminen et al. 2001). Human excretion, along with flushing of expired drugs, agricultural runoff, pharmaceuticals and personal care products (PPCPs), industrial waste sources, and solid waste combine to form a complex mixture of chemicals in waste water treatment plant

effluents (Sadler et al. 2013). These compounds are now being classified as "emerging contaminants" and receiving an increase in scientific and public interest as they are being discovered in surface waters, groundwater, wastewater treatment plant effluents, and drinking water (Sadler et al. 2013). Although these compounds are usually found in small concentrations, and the direct impact on human health is low, the full effect is not yet completely known (Sadler et al. 2013). However, a substantial amount of evidence has supported the hypothesis that EDCs in wastewater effluent can lead to harmful reproductive disorders in wildlife and humans. In humans, EDC effects consist of decreasing sperm count in males, increasing rates of breast cancer in women, and the increase in certain reproductive system abnormalities (Lagana, 2003). According to Apocdaca and Mattessich (1997), the effects found in wildlife include: reduced fertility, altered sexual behavior, modified immune system, altered bone density and structure, and cancers of the reproductive tract among others. Also, endocrine disruptors have a long half -in the environment, allowing them to accumulate within organisms and potentially threaten their survival and fitness (Apocdaca and Mattessich 1997).

Study Objectives

This study seeks to find evidence indicating that this phenomenon is happening to local mosquitofish populations living in the effluent of Greenville Utilities' Wastewater Treatment Plant (GWWTP). Due to the increased use of phytosterols as well as other steroids and testosterones in medications as well as other suspected EDCs that could potentially end up in sewage plants, I predict that EDCs will be present in the water in the clear well, effluent stream and the river downstream of GWWTP. The objective of this project will be to test the hypothesis that mosquitofish are being affected by EDCs in the effluent of GWWTP. Alternatively, the null hypothesis is that water from the GWWTP will not have an effect on

the fish .The hypothesis and null hypothesis will be tested by analyzing sex ratios and anal fin ray length and width ratios in mosquitofish populations of the GWWTP and in comparison to control (reference) populations.

First, in phase 1, I established two kinds of controls for comparison with the fish collected. Hildebrand (1932) observed the sex ratios of mosquitofish from the Beaufort area of North Carolina in 1927, which I will use for the expected sex ratios, because his research was conducted before the widespread use of many of the suspected EDC's that would alter the ratios. In addition, I will compare sex ratios of mosquitofish collected at nearby sites, Broad Run (in Pitt County, NC) and Coastal Studies Institute (in Manteo, NC). The anal fin morphological results from mosquitofish collected will be compared to the wild control mosquitofish collected by Angus et al. (2001) in Alabama. In the study conducted by Angus et al., means and ranges of anal ray ratios of wild-collected mosquitofish were recorded to be compared to laboratory-bred mosquitofish fed 11-ketotestosterone. They began feeding the female fish 11-ketotestosterone in concentrations of 20, 40, 60, 80, and 100 µg/g in their trout food when they were 2 months old for 180 days. The anal fin ray 3:4 width and 4:6 length ratios of the females began to grow similar to that of males after being exposed to concentrations as little as 20 µg/g. These testosterone-fed females' fin ray ratios were significantly different than the females in the wild populations. The results of statistical comparisons will be used to conclude whether EDC's are present in the sewage effluent at GWWTP.

Phase 2, Dr. Sid Mitra, Department of Geological Sciences at ECU, assisted me in analyzing water samples taken at the time of fish collection in March 2014 from the water effluent of GWWTP to identify specific compounds present. In phase 2, we used gas

chromatograph/mass spectrometry(GC/MS) to identify organic compounds in the GWWTP wastewater and to determine if any EDCs were present.

Previous Research on this Topic at ECU

The occurrence of pharmaceuticals and personal care products at GWWTP has been tested in the past (Mitra, 2009) (Table 1). Small amounts of caffeine (4.7-12.5 µg/L) were detected, along with Ibuprophen (0.02-0.21 µg/L), and non-quantifiable concentrations of sucralose and 17B estradiol. 17B estradiol is an EDC. However, testosterone was not detected in that study. Most contaminant studies such as the Mitra (2009) study are preliminary, in that concentrations of organic compounds may vary over time, and only a small sample of the millions of gallons of wastewater passing through treatment plants can be analyzed.

Date	Type of sample	Amt. Extracted	Acetaminophen	Sucralose	17B-Estradiol	Ibuprophen	Caffeine
1/26/10	GWTP-finished	.45 L	ND	NQ	NQ	ND	ND
2/17/10	GWWTP-influent	.7 L	3.18 ug/L	ND	ND	ND	ND
2/17/10	GWWTP-influent	.7 L	ND	ND	ND	.21 ug/L	12.5 ug/L
2/17/10	GWWTP-aeration	.5 L	ND	ND	ND	ND	7.04 ug/L
2/17/10	GWWTP-clear well	.5 L	ND	ND	ND	ND	4.71 ug/L
2/17/10	GWWTP-effluent	.5 L	ND	ND	ND	ND	6.78 ug/L
3/15/09	SW Tar River	.485 L	ND	ND	ND	.02 ug/L	ND

Table 1: SW (surface water), ND (non-detectable), NQ (non-quantifiable). Caffeine was found in a majority of the sites, with the largest concentrations.

Methods

Phase 1:

A small seine (3.2 mm mesh) and dip net was used for collecting mosquitofish samples from the effluent of the GWWTP (N 35.59969, W 77.30127), Broad Run (N 35.62281, W 77.267856), and the Coastal Studies Institute (CSI) pond in Wanchese, NC (N 35.8738, W 75.6606) was used for a reference sample. The water temperature, salinity, dissolved oxygen, and pH of the environments where the samples were taken were measured using a YSI meter (Model 85 or 556) and exact location pf collection was determined using a Garmin GPS. After collection, samples were euthanized using MS-222 (43 mg to every 100 ml of water) and brought to the lab for analysis. The standard lengths, from snout to the last vertebrae, of all the fish were then measured in millimeters. The fish were viewed under a microscope for sex determination, secondary sexual characteristic observations, and to take pictures of the anal fin of all fish collected. Each individual was classified as male or female, based on the presence of a gonopodia among other secondary sexual characteristics. Those that measured less than 20 mm were classified as juveniles and not included in the sex ratio to avoid classifying an immature male as a female, this was the same procedure used by Hildebrand (1932). The University of Texas Health Science Center at San Antonio (UTHSCSA) ImageTool was used to measure the anal fin ray 4 and 6 lengths, and anal fin ray 3 and 4 widths (at the point where ray 4 bifurcates) of all fish from photographs taken at 64X magnification and recorded. The UTHSCSA Imagetool was calibrated using a stage micrometer, measurements were made in mm. Observations were recorded on any and all unusual secondary sex characteristics. Statistical analyses were then conducted on morphological characteristics (t-test) and sex ratios (chi squared) compared to what was

expected from Hildebrand (1927) sex ratios from North Carolina and Angus et al. (2001) anal fin morphological ratios from wild mosquitofish collected in Alabama. My null hypothesis was that there was not a significant difference between the sex ratios or morphological ratios of the fish collected in this study compared to Hildebrand's and Angus at al.'s ratios.

Phase 2:

To further determine if EDC's were present at the time of fish collection, grab samples of water were collected in March 2014 from the influent, clear well tank, effluent, and down-stream of the effluent at GWWTP as well as from Broad Run (a reference site). The samples were collected using clean, disposable, plastic beaker and stored in pre-ashed (450 degrees Celsius for 4 hours in a muffle furnace) amber, glass bottles, for in-field environmental readings and subsequent laboratory analysis of PPCPs. A YSI 556-multimeter was used in the field to measure temperature, DO, pH, and conductivity of the water upon collection. Care was taken during sampling to avoid the use of PPCP products, and polyethylene gloves were worn, in an attempt to prevent sample contamination. At least 500 mL of water was collected at each site for PPCP, total dissolved nitrogen (TDN), and total organic carbon (TOC) analysis. Samples were stored on ice within a cooler and immediately filtered upon returning to the lab using pre-ashed filters. After filtration, a 20 ML aliquot of the filtrate was acidified with 2N hydrochloric acid (HCl) to remove the inorganic carbon, and immediately analyzed for TDN/DOC. The remaining filtrate was acidified with 4N HCl for preservation, and ethyl acetate was added to the filtrate for liquid-liquid extraction. Samples were stored at 4 degrees Celsius until further processing, within 7 days.

The filter for each sample was retained, however due to time constraints, those have yet to be analyzed. Liquid-liquid extraction for PPCPs was performed using a separatory

funnel and ethyl acetate as the solvent. Each sample was extracted three times. The extract was evaporated to dryness using a Rotavap as well as an N-evap and reconstituted gravimetrically in pyridine. Each sample was divided into two so that a derivitizing agent N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) could be added. The BSTFA derivativing agent was heated for one hour prior to mass spectrometer analysis. Derivitization was necessary to detect polar compounds on GC_MS as derivitizing rends these compounds less polar and more amendable to GC analysis. Samples were analyzed using a Shimadzu QP2010 gas chromatograph-mass spectrometer (GC/MS) in scan mode. The GC oven was heated from 50 to 320° C at a rate of 10 degrees per minute, and held for 25 minutes.

Quality Assurance and Statistical Analysis

Multiple quality assurance measures were taken as part of this study. Each compound was identified based on both its specific retention time and mass-to-charge ratios of previously recorded compounds configured into the software system of the QP2010 GC/MS. Compounds were not quantified unless their signal-to-noise ratio exceeded 5:1. The area and height were also recorded for each compound to estimate the quantity detected. However, due to time constraints and absence of standards normally used to get quantities, we were unable to quantify the amounts present at this time. Thus, the results here represent a 'scan' of compounds present in GWWTP, but is not intended to be an estimate of compounds present.

Results

Sex Ratios

There was a significant difference in the sex ratios of the mosquitofish population at GWWTP compared with the ratio expected from Hildebrand's research. The female to male ratio was 1.43:1 at GWWTP with slightly more females, however pre-EDC NC population had a greater female bias of 4.4:1; this gave a chi squared of 27.17 and a p-value of 1.863*10e-7 (Table 2). Hildebrand's data from October and March were also compared to the fish collected from GWWTP collected in October 2013 and March 2014 (Table 2). Although a female bias was observed at GWWTP, it was less than half of the bias Hildebrand found in his average, indicating that a normal population could have been masculinized. Hildebrand's October 1927 ratios were used for comparison with the fish collected at Broad Run because the fish were only collected in October 2013. There was a significant female bias in sex ratio at Broad Run, because out of the 43 fish collected, only 2 were males, giving a female to male ratio of 20.5 to 1, compared with Hildebrand's 2.75 to 1 ratio (Table 3). The female bias at Broad Run was very large; almost seven times what was expected, indicating possible feminization. Hildebrand's October 1927 ratios were also used in comparison to the fish collected at CSI since the fish were only collected in October 2014. There was no significant difference in sex ratio at the CSI Pond when compared with Hildebrand (1927) . The female bias (2.5:1) was similar to what was expected; indicating EDC's may not be having an effect at CSI.

Anal Fin Morphology

The 3 to 4 anal fin ray width ratios at CSI the females had a median below 1, similar to the juveniles, but the juveniles had a larger range, and the males had the largest range and

the median was around 1.45 (Figure 1). At GWWTP the 3:4 ratio of the females overlapped the ratio of the males, although the median ratio of the males was 1.5 and the females was 1. The mean 3:4 ratio was significantly different from fish collected by Angus et al. (2001) in males and females of both the CSI pond and GWWTP (Table 5). The mean male 3:4 ratio was significantly smaller at CSI (1.5) and even more significantly at GWWTP (1.43) than what was expected (2.73) according to the study by Angus et al. (2001). The females widths were slightly larger than expected compared to Angus at al. mean of .84. At CSI, the mean was .88 and the mean was more significantly larger at GWWTP (1.01).

At CSI the mean length ratio of anal fin ray 4 to ray 6 of the males at CSI (2.35) wasn't far off the expected (2.27) from Angus et al. (2001) (Table 6). The mean male 4:6 ratio at GWWTP was 1.99, more significantly less compared to what was expected. The mean female 4:6 ratio was expected to be 1.01 from Angus et al. (2001). However, the mean ratio was significantly larger at CSI (1.3) and GWWTP (1.2). The females 4:6 anal fin ray length ratio had a median around 1.3 with a small range and few outliers, the juveniles had a higher median around 1.45 as well as a larger range, and the males' median was around 2.7 (Figure 2). At GWWTP the 4:6 ratio of the females range overlapped the males' large range of around 1.25 to above 2.5.

Water Samples

Clear-well and Effluent samples had a low TOC to TDN ratio (1.13 and 1.05) and Near Tar River and Broad Run had high TOC to TDN ratio (12.7 and 4.38) (Table 7). The compounds of interest have been highlighted in table 8, the include: Carbamazepine, Phthalic acid, Pyridine, Hexadecanoic acid, Caffeine, and Heptadecanoic acid. The areas shown correlate with the amount present in the water sample, revealing that the largest amount of

potential EDC found was Heptadecanoic acid with an area of 15,516,806 at Broad Run and the second largest was Caffeine with an area of 4,611,676 found near Tar River.

Table 2: Counts of the number of females and males collected from GWWTP in October 2013 and March 2014 compared with the expected numbers from Hildebrand's (1927) overall collection of males and females in NC. Probability-value of a chi-square test of the null hypothesis of no difference in sex ratio is given.

	GWWTP	Hild.'s Avg. (expected)
Females	50	83554
Males	35	19596
F to M ratio	1.43 to 1	4.4 to 1
chi squared	27.1708	
p value	1.863*10e-7	

Table 3: Counts of the number of females and males collected from Broad Run in October 2013 compared to Hildebrand's October 1927 sample in NC. Probability-value of a chi-square test of the null hypothesis of no difference in sex ratio is given.

	Broad Run	Hild.'s Oct. (expected)
Females	41	31674
Males	2	11614
F to M ratio	20.5 to 1	2.75 to 1
chi squared	10.7741	
P value	0.001029	

Table 4: Counts of the number of females and males collected from the CSI pond in October 2013 compared to Hildebrand's October 1927 sample in NC. Probability-value of a chi-square test of the null hypothesis of no difference in sex ratio is given.

	CSI Pond	Hild's Oct. (expected)
Females	20	31674
Males	8	11614
F to M ratio	2.5 to 1	2.75 to 1
chi squared	0.0433	
p value	0.8352	

Morphological Ratios

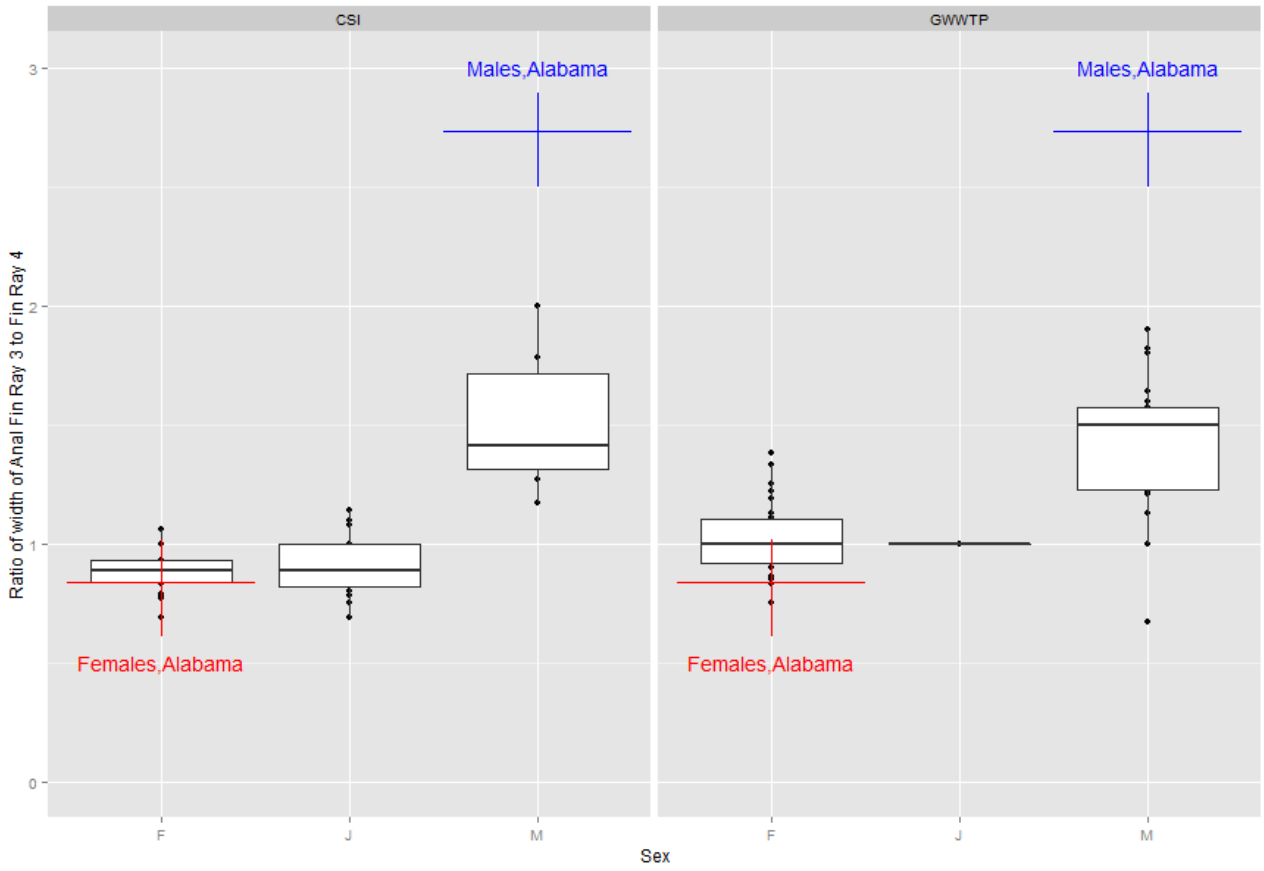

Figure 1: The medians and ranges of the width of anal fin ray 3 to ray 4 ratios of the female, male and juvenile mosquitofish from CSI and GWWTP. For comparison, the mean ratio and range of male and female mosquitofish collected in Alabama from Angus et al 2011 study means are indicated by the red (female) and blue (male) horizontal lines and ranges are shown as vertical bars.

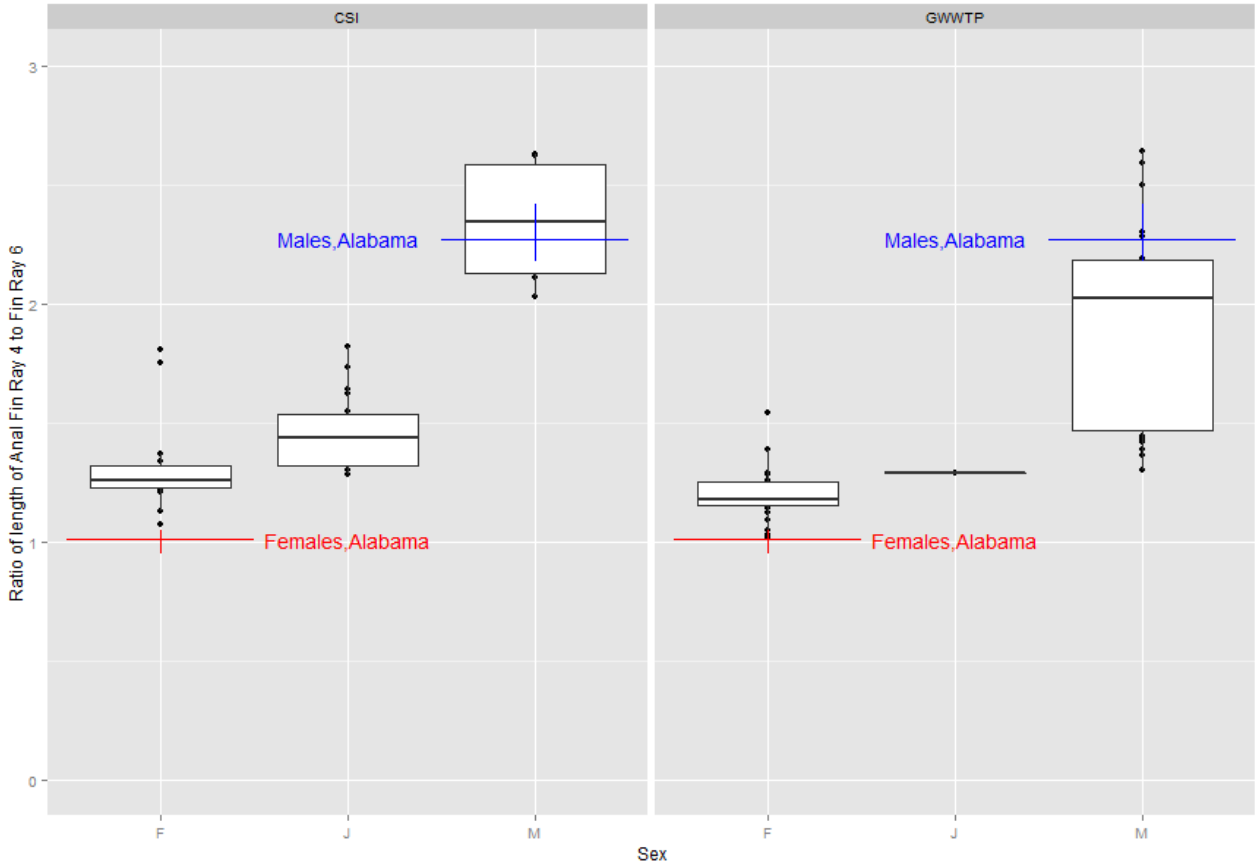

Figure 2: The medians and ranges of the length of anal fin ray 4 to ray 6 ratio of the female, male and juvenile mosquitofish from CSI and GWWTP. For comparison, the mean ratio and range of male and female mosquitofish collected in Alabama from Angus et al 2011 study means are indicated by the red (female) and blue (male) horizontal lines and ranges are shown as vertical bars.

Table 5: The observed mean of 3:4 anal fin ray width ratio of male and female mosquitofish from CSI and GWWTP. Mu (μ) is the mean 3:4 ratio from Angus et al. (2001) wild mosquitofish from Alabama. T-values were computed using a one-sample t-test; the probability of the difference in means being due to chance alone is given

Analysis of width ratios Observed Compared to Angus et al. Study (2001)

Location	Gender	Observed Avg.	mu	t-value	p-value
CSI	Male	1.5075	2.73	-12.0545	6.17e-06
CSI	Female	0.8865	0.84	2.2931	0.03342
GWWTP	Male	1.427	2.73	-20.6709	1.94e-15
GWWTP	Female	1.0145	0.84	8.2595	1.54e-09

Table 6: The observed mean of 4:6 anal fin ray length ratio of male and female mosquitofish from CSI and GWWTP. Mu (μ) is the mean 3:4 ratio from Angus et al. (2001) wild mosquitofish from Alabama. T-values were computed using a one-sample t-test; the probability of the difference in means being due to chance alone is given

Analysis of length ratios Observed Compared to Angus et al. Study (2001)

Location	Gender	Observed Avg.	mu	t-value	p-value
CSI	Male	2.3475	2.27	0.8401	0.429
CSI	Female	1.306	1.01	7.5103	4.23e-07
GWWTP	Male	1.9915	2.27	-3.9148	7.96e-4
GWWTP	Female	1.195	1.01	10.4755	4.99e-12

Water Analysis Results

Table 7: Total organic carbon, total dissolved nitrogen, and their ratio for each water sample.

Sample ID	TOC (mg/L)	TDN (mg/L)	TOC/TDN
Clear Well	5.593	4.95	1.13
Near Tar River	6.529	0.5143	12.695
Effluent	4.921	4.672	1.053
Broad Run	8.905	2.033	4.38

Table 8: The compounds found via a (GC-MS scan) in the water samples as well as the area of the peaks detected.

Location	Compound Name	Area
Clearwell	Dibutyl phthalate (plasticizer)	22757634
Clearwell	Hexadecanoic acid (Palmitic acid, fatty acid found in animals)	1600130
Clearwell	Oleic acid (fatty acid)	1650277
Clearwell	Octadecanoic acid (Stearic acid, saturated fatty acid)	1994904
Clearwell	Carbamazepine (anti-seizure/neuralgia drug)	838539
Clearwell	1,2-Benzenedicarboxylic acid (Phthalic acid- used in production of dyes, perfumes etc.)	1623048
Clearwell	Dotriacontane	1341675
Clearwell	Pyridine (organic compound, can be made from coal or tar)	728052
Clearwell	Dibutyl phthalate (plasticizer)	21266005
Clearwell	Dotriacontane	716119
Clearwell	Hexadecanoic acid, octadecyl ester (chemical component in tobacco)	36043469
River	Caffeine	4611676
River	Dibutyl phthalate	26163111
River	Hexadecanoic acid	2577516
River	butyl boronate	30676451
Effluent	Dibutyl phthalate (plasticizer)	11974988
Effluent	Dibutyl phthalate (plasticizer)	11129174
Broad Run	2,4-Pentadienenitrile	633491
Broad Run	Dibutyl phthalate (plasticizer)	1966712
Broad Run	Mono(2-ethylhexyl) ester (plasticizer)	1139239
Broad Run	Dibutyl phthalate (plasticizer)	1482040
Broad Run	Ethyl iso-allocholate	642414
Broad Run	Heptadecanoic acid, heptadecyl ester (chemical component in tobacco)	15516806

Discussion

After conducting statistical analyses of the data collected in phase one, the null hypothesis that there is no significant difference from the sex ratios of Hildebrand's research was rejected at both the GWWTP and the Broad Run sites. The fish from GWWTP seemed to be slightly masculinized, indicating that most of the EDCs likely to end up in sewage effluent are androgen-mimicking compounds. Conversely, at Broad Run, just downstream from GWWTP, the fish appeared to be highly feminized. Since this stream is surrounded by farmland, my proposed explanation for this is that agricultural runoff may be the cause. There could be a number of explanations for the variation in sex ratios. More extensive predation on the smaller males could cause an increase in the female bias. As well as Hildebrand's (1927) "seasonal variation" hypothesis where males are more numerous in the spring for mating and then thin out mid-summer, "The thinning out of males, therefore, appears to be nature's process of eliminating "surplus" animals". I would like to take more samples to better verify the ratios I obtained. However, the control site chosen (CSI) showed no significant difference from that to be expected which gives me reason to believe that it is probable that EDC's are present at GWWTP as well as Broad Run. The results on sex ratio require further investigation.

The morphological analyses revealed that the width and length ratios for male and female fish in both CSI and GWWTP were significantly different from Angus et al.'s research, except for the male length ratio from CSI. Since the masculine characteristics of the males were significantly lower at these sites, I have reason to believe that I may have incorrectly classified some females as males based on morphological features. In order to

confirm their true gender I would need to dissect suspected fish to identify whether they have ovaries or testes. If previously classified males are found to be females, this would further support that EDC's are present. However, since I was comparing mosquitofish from North Carolina to mosquitofish from Alabama, it is possible for there to be a natural gradient in anal fin lengths and widths.

Before going into the water analysis results it is necessary to go through the stages of water treatment at Greenville Utilities. The waste water received goes through three treatment processes. First, water passes through screens to remove heavy solids. Second, any remaining solid material is removed by supplying oxygen in an Aeration Tank which "stimulates the growth of helpful microorganisms, which consume organic matter in the wastewater" (Greenville Utilities Commission, 2014). Due to the fact that human waste is high in nutrients such as nitrogen and phosphorus, this step is necessary to avoid any harmful effects to aquatic life when this water is returned to Tar River. "The water then moves to a Secondary Clarification Tank that allows the microorganisms and solid wastes to form clumps and settle to the bottom" (Greenville Utilities Commission, 2014). In the third treatment, the water passes through a deep-bed sand filter prior to being disinfected by an Ultraviolet Disinfection System in order to prevent bacteria reproduction. At this point the water is in a "clear well" where one sample was obtained, then moves into an effluent drainage ditch where the "effluent" sample was obtained, then joins a stream that flows into Tar River where the "near-tar-river" sample was obtained. Another sample was taken from Broad Run for a reference site.

As shown in table 7, the total organic carbon (TOC) to total dissolved nitrogen (TDN) ratios was the highest in the water sample collected near the Tar River (12.695) and was also high in the Broad Run sample (4.38). This was expected since there should be less nitrogen

in these samples as well as more sources of carbon since the majority of the water from these locations has not received treatment. Due to the fact that the sample collected near Tar River had the highest TOC to TDC ratio, this may be an indication that the water is more polluted since many EDCs are high in carbon content. The Clear Well and Effluent water samples TOC to TDC ratios were almost 1 to 1 since human excretion and solid waste are high in nitrogen.

Potential Effects of Compounds Present at GWWTP

Before discussing water analysis results, it is important to note that the plasticizers found may have resulted from contamination during the extraction process so these compounds cannot be assured to be naturally occurring in the site sampled. Also some compounds detected are commonly detected lipids from biological organisms, often bacteria, thus most likely did not derive from any sort of anthropogenic pollution such as agriculture or sewage. Thus, compounds suspected to result from contamination or to be naturally occurring rather than pollution will not be discussed.

The water sample analysis from the GC-MS scan revealed that carbamazepine, a synthetic drug used to combat seizures/ neuralgia/depression, was present in the clear well sample. A study by Oetken et al. (2005) found that exposure to this drug caused a suppression of pupation in frogs (Cophixalus *riparius)* larvae. Oetken et al. believed that this was due to an "interference with a physiologic pathway first activated in this life stage or to some modulation of endocrine functions". Also, phthalic acid was detected in the clear well sample, which is used in the production of manufactured products such as dyes and perfumes. Another compound, hexadecanoic acid, which is primarily used to create soaps and cosmetics, was detected in the clear well sample. The last significant product identified in the clear well sample was pyridine, which is an organic compound that can be used as a

solvent in the production of paint, rubber, pharmaceuticals, herbicides, and many other potential EDCs (DCASR, 1983).

No notable compounds were detected in the effluent ditch sample; however caffeine was detected in the near-Tar-River sample. This was not a surprise due to the fact that caffeine was also found in the previous study by Mitra and the high intake of caffeine in the diet of the majority of the population of Greenville. The most notable compound identified in the Broad Run sample was heptadecanoic acid, a chemical component in tobacco. The source of this compound could be from local tobacco fields. Also, according to a study by Hecht (2002), tobacco compounds have been quantified in the urine of smoker and non-smokers exposed to tobacco smoke. The measurements of these compounds are being used for on-going tobacco and cancer research. To the best of my knowledge, the effects of these compounds on aquatic life have not been investigated.

Future Research

The preliminary research conducted in this study should be continued to further investigate this topic. For future research, the method for analyzing whether or not the water from GWWTP is affecting mosquitofish could be improved. There are various options that could be implemented, such as bringing mosquitofish into the lab and exposing them to compounds found present in the water through water analysis, either by feeding the compounds to the fish (as was done in the study by Angus et al.) or by putting the compounds directly into the water. A population of juvenile mosquitifish could also be caged in the effluent water so that their morphological features could be monitored for any changes. Furthermore, it would be interesting to research whether any of the mosquitofish from Hildebrand's study in 1927 were saved in a museum. By measuring the morphological

features of those fish, a more accurate "true morphology" of mosquitofish could be used for statistical analysis due to the fact that it is nearly impossible to find a natural population of mosquitofish that have not been exposed to EDCs.

Additionally, the water samples obtained in this study are classified as "grab samples" which simply means that only one sample was taken at one time from each site. For future research it would be beneficial to take "composite samples" where multiple samples are taken throughout a day at specified time-intervals. This would be a more accurate sample since the compounds going through GWWTP are sure to fluctuate throughout the day. However, due to the fact that compounds were still detected from 500-700 mL grab samples that were collected in this study, proves that further research is necessary. Concentrations were not able to be calculated at this time. However, even if the concentrations of the compounds found may be low in the water, persistent chemicals are picked up by microorganisms from sediments, which are consumed by zooplankton, which are consumed by small fish (including mosquitofish). These persistent compounds accumulate exponentially in the body fat of these animals as they move up the food chain and can be up to 25 million times greater in top predators (Colborn, 1997). Although these compounds do not seem to be affecting adults, their offspring are becoming victimized. Most cases found can be linked to "disruption of the endocrine system" which plays a major role in the development of offspring (Colborn, 1997). Therefore, research on EDCs present in water that organisms are exposed to as well as the sources of these EDCs is crucial to attempt to prevent these compounds from affecting future generations.

Literature Cited

Angus, Robert A., Heather B. Mcnatt, W.mike Howell, and Shearlyn D. Peoples. "Gonopodium Development in Normal Male and 11-Ketotestosterone-Treated Female Mosquitofish (Gambusia Affinis): A Quantitative Study Using Computer Image Analysis." General and Comparative Endocrinology 123.2 (2001): 222-34.

Apodaca, Jennifer, and John Mattessich. "The Effects of Endocrine-disrupting Chemicals on Survivorship and Fecundity." (1997): n. pag.

Author Unknown. "Wastewater Treatment Process." - Greenville Utilities Commission. Greenville Utilities, n.d. Web. 29 Apr. 2014.

Bortone, Stephen A., and William P. Davis. "Fish Intersexuality as Indicator of Environmental Stress." BioScience 44.3 (1994): 165-72

Chinathamby, Kavitha, Mayumi Allinson, Fujio Shiraishi, Andreas L. Lopata, Dayanthi Nugegoda, Vincent Pettigrove, and Graeme Allinson. "Screening for Potential Effects of Endocrine-disrupting Chemicals in Peri-urban Creeks and Rivers in Melbourne, Australia Using Mosquitofish and Recombinant Receptor–reporter Gene Assays." Environmental Science and Pollution Research 20.3 (2013): 1831-841.

Colborn, Theo, Dianne Dumanoski, and John Peterson. Myers. Our Stolen Future: Are We Threatening Our Fertility, Intelligence, and Survival?: A Scientific Detective Story. New York: Dutton, 1996.

DCASR. "IARC Monographs on the Evaluation of Pyridine." International Agency for Research on Cancer: World Health Organization, Lyon, France (1983)

Deaton, Raelynn, and James C. Cureton. "Female Masculinization and Reproductive Life History in the Western Mosquitofish (Gambusia Affinis)." Environmental Biology of Fishes 92.4 (2011): 551-58.

Hecht, S. S. "Human Urinary Carcinogen Metabolites: Biomarkers for Investigating Tobacco and Cancer." Carcinogenesis 23.6 (2002): 907-22.

Hildebrand, Samuel F. "Sex Ration in Gambusia." Biological Bulletin 53.5 (1927): 390-404
Nieminen, Petteri, Anne-Mari Mustonen, Pirji Lindstron-Seppa, Juha Asilainen, Helena Mussalo-Rauhamaa, and Jussi V. K. Kukkonen. "Phytosterols Act as Endocrine and Metabolic Disruptors in the European Polecat (Mustela Putorius)." Toxicology and Applied Pharmacology 178.1 (2002): 22-28.

Mitra, Siddartha, and Spruill, Richard. "Baseline Assessment of the Occurence and Composition of Pharmaceuticals and Personal Care Products in the Tar River and Greenville Water Treatment and Wastewater Treatment Plants, Greenville, North Carolina." A Report Submitted to the Greenville Utilities Commission.

Oetken, M., Nentwig, G., Löffler, D., Ternes, T., & Oehlmann, J. (2005). Effects of pharmaceuticals on aquatic invertebrates. part I. the antiepileptic drug carbamazepine. Archives of Environmental Contamination and Toxicology, 49(3), 353-61. doi:http://dx.doi.org/10.1007/s00244-004-0211-0

Sadler, Mary, Lamya Badr, and Ben Stanford. "Facing the Future: A Perspective on Emerging Contaminants and Water Quality." NC Currents Fall 2013: 60-64.