Suburban Neighborhoods and Endocrine Disruption in Developing Green Frogs

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ABSTRACT

In recent decades there has been a growing focus on endocrine disrupting chemicals (EDCs), both naturally- as well as anthropogenically-derived, which alter hormonal regulation in animals. EDCs can have effects like increases in breast cancer proliferation in humans, infertility in female livestock, and the production of eggs in male frog and fish testes (testicular oocytes – TOs). Research has traditionally focused on agricultural landscapes and large wastewater treatment facilities as sources of these contaminants in the environment. However, recent work has found that male frogs in suburban neighborhoods have higher frequencies of endocrine disruption than frogs from other landscapes. As of yet we have limited knowledge as to which contaminants occur in suburban waterways or as to what their effects are on early development. Here, I assessed sex ratios and estradiol levels in metamorphosing green frogs. I used GIS software to locate suburban (treatment) and forested (control) ponds in southern Connecticut, measured sex ratios by assessing gross gonadal morphology, and am using enzyme-linked immunosorbent assays (ELISAs) to quantify estradiol and testosterone levels in whole-body frog extracts. Here I show that endocrine disruption is occurring early on in suburban frog development and that these abnormalities are associated with high frequencies of phytoestrogens, likely from heavy landscaping.

INTRODUCTION

Over the past two decades there has been an increasing awareness of the impact of environmental contaminants on hormonal regulation and function (Reeder et al. 1998, Hayes et al. 2002, Vajda et al. 2008, Skelly et al. 2010). These endocrine disrupting chemicals (EDCs) are known to come from a variety of sources such as pesticides, pharmaceuticals, detergents, plastics, or even naturally occurring plant chemicals. In human, the chemical triclosan, found in many types of toothpaste, can increase breast cancer proliferation (Gee et al. 2008). In a laboratory setting, EDCs have been shown to induce numerous effects including the growth of eggs (testicular oocytes – TOs) in male amphibian testes (Hayes et al. 2002), delayed ovarian maturation (Storrs and Semlitsch 2008), altered mating behavior (Hoffman and Kloas 2012), and deviant expression of sex hormones and regulatory enzymatic activity (Brande-Lavridsen et al. 2008). Furthermore, laboratory studies on EDCs have yielded demographic effects where a contaminant changes the proportion of females to males (sex ratio) of a population (Pettersson and Berg 2007, Hogan et al. 2008, Langlois et al. 2010, Tompsett et al. 2013). Field experiments have shown that the addition of 17α-ethynyl estradiol, the main ingredient in many birth control pills, to a lake could cause populations of minnows to become feminized and eventually collapse entirely (Kidd et al. 2007). Most field surveys have focused on systems with large, direct inputs to the environment. Indeed, these studies have often centered on amphibians in intense row crop agriculture (Hayes et al. 2002, Murphy et al. 2006, McDaniel et al. 2008) or fish from waste water treatment facility effluents (Vajda et al. 2008). Agricultural research tends to associate endocrine disruption with pesticides while studies on waste water effluent link endocrine disruption to EDCs from pharmaceuticals, plastics, or detergents (e.g., Vajda et al. 2008). However, recent work by Skelly et al. (2010) compared TO frequencies in adult green frogs (*Rana* clamitans) among land uses and found higher frequencies of TOs in suburban neighborhoods rather than the agricultural fields or urban areas implicated in previous studies. Because. The high rates of endocrine disruption reported by Skelly et al. (2010) imply a more insidious and nuanced source of endocrine disrupting chemicals in suburban neighborhoods than that purported in other human-altered environments. Whereas row crop agriculture is characterized by pesticides applied over vast monocultures and waste water treatment facilities disperse effluent from numerous households into the environment, the water in small suburban ponds may be potentially influenced by a suite of factors including road runoff, landscaping, or potentially via subterranean effluent from individual household waste removal. To my knowledge, these avenues of chemical contamination have been relatively unexplored in the endocrine disruption literature.

Here, I hypothesized that suburban pond chemistry would be influenced by septic tank or sewer line effluent and that such effluent would alter hormone regulation in developing green frogs. To test this, I used gas chromatography tandem mass spectrophotometry to measure known endocrine disrupting chemicals in ground water and surface water samples. I further sampled young, metamorphosing green frog cohorts to assess sex ratios, gonadal development, and whole-body sex steroid measurements. Early evidence from this study indicates that it is plant-derived chemicals, likely from landscaping, that is influencing pond water and that these chemicals are increasing the proportion of females in young frog cohorts.

METHODS

My approach was to understand the extent to which suburban households are associated with differences in the reproductive endocrinology of developing green frogs (*Rana clamitans*) relative to green frogs in natural, forested landscapes. I generally considered a pond to be potentially influenced by a suburban home if it was *peridomestic* in nature, meaning that it was within the general vicinity or “backyard” of a human household within a neighborhood. I constrained my pond selection to within a 30 km radius of the center of New Haven, Connecticut, USA. In order to determine suburban (treatment) and forested (control) ponds I estimated land cover around freshwater wetlands within my study area using a geographic information system (GIS – ArcMap 9.6). Specifically, I used US Fish and Wildlife Service National Wetland Inventory data to identify all wetlands within my study area as well as National Oceanic and Atmospheric Association (NOAA) 2006 land cover data to calculate the area of each land cover category within a 200 m buffer of each wetland. If a wetland’s buffer contained a combined land cover of 100% coniferous forest, deciduous forest, and mixed forest then I considered it a forested wetland and if it contained a combined land cover that was 70% or greater of medium intensity development, low intensity development, and open spaces developed land cover types then I considered it a suburban wetland. NOAA defines medium intensity development as land cover composed of 50-79% impervious surface and which contains small buildings such as single family housing or large sheds. Low intensity development is composed of 21-49% impervious surface as well as buildings similar to those in medium intensity developed cover but with roads and associated roadside vegetation. Developed open space is predominated by lawn grasses (e.g., lawns and golf courses) and is composed of less than 20% impervious surface. I further stratified the suburban ponds using University of Connecticut 1998 *sewer service area* GIS data. If a suburban pond was within 1km of a sewer line in the GIS I considered it to be potentially influenced by a sewer system. Similarly, if a suburban pond was further than 1km from a sewer line in the GIS, I considered it to be potentially influenced by a septic tank. At each suburban pond, I verified whether the household was on sewer service or a septic tank. I then randomized my lists of forested and suburban wetlands and visited them in sequential order. If *R. clamitans* larvae were present and I could obtain permission to a homeowner's property or to private forest land then I continuously surveyed wetlands until I collected metamorphic frogs.

Amphibians

Frog Sampling: Almost all studies on endocrine disruption (but see (Papoulias et al. 2013 for an exception) have focused on adult frogs. Because adult frogs readily move among ponds, there is limited inference into how particular contaminants at a site have affected these animals. Furthermore, because amphibians go through an extreme metamorphosis where they undergo a dramatic transformation from fish-like tadpoles to terrestrial adults in a very short period of time, studying young animals may be critical for understanding the impacts of EDCs. I collected prometamorphic frogs from each site at the appropriate stage of development. Specifically, I targeted frogs that were completing metamorphosis with the development of all four limbs and the presence of a reduced tail (Gosner Stages 43-46, Gosner 1960). At each pond, I measured the surface water conductivity, a measurement of the number of dissolved ions in water and a proxy for an anthropogenic influence on waterways where increasing conductivity is related to increasing levels of human disturbance. I also took three water samples for analysis of trace elements and endocrine disrupting chemicals. I brought all frogs back to the lab and either stored them in their pond water overnight or I immediately euthanized them. Upon euthanizing frogs with tricaine methanesulfonate, I measured the length as snout-vent length (SVL - mm), immediately dissected the frogs, and recorded the sex based upon the inspection of the gonad and the macrostructure of a testis or ovary. I then immediately removed the left gonad from each frog and fixed it gonad in Bouin's solution. The rest of the frog was then snap frozen on dry ice and stored in a freezer at -80F for future hormone analysis.

Histological Analysis (in progress): I have processed all gonads through graded concentrations of ethanol and infused the tissues with paraffin wax. After processing, I embedded each gonad in a block of paraffin wax, trimmed the block the edge of the tissue, and am currently serially sectioning each kidney-gonad complex at 7 μm slices. I am currently mounting all sectioned gonads onto glass slides and staining them with Harris' hematoxylin and eosin. I will examine ovary histology for the level of maturity.

Hormone Analyses (in progress): Because metamorphosing frogs are too small to draw an adequate blood sample from, I am measuring whole-body levels of estradiol and testosterone, two dominant steroid hormones that are key to gonadal differentiation, sexual maturation, and body development. I am homogenizing frogs using a Kinematica Polytron 2500E homogenizer with a PT-DA 20/2 dispersing aggregate. For each frog, I am homogenizing the whole body with 0.5M sodium hydroxide and allowing the tissue to digest overnight. I then vortex the homogenized tissue, add 5mL of methanol, vortex for an additional 30 seconds, and centrifuge at 3000 rpm/4C for 20 minutes. I then snap freeze the sample in dry ice, decant the organic supernatant into a new test tube, and evaporate the methanol on low heat over night. I repeat this process once more and store the dried hormone extract at -80F until later analysis. To measure steroid levels, I rehydrate the hormone in enzyme immunoassay buffer and measure steroid hormone levels using enzyme-linked immunosorbent assays (ELISAs).

Water Testing: All water sampling and chemical analyses were done in collaboration with Geoffrey Giller, a MESc student at the Yale School of Forestry and Environmental Studies, as well as Dr. Larry Barber, geologist and aquatic chemist with the United States Geologic Survey (USGS).

Ground Water Collection: Because I hypothesized that septic tanks or sewer lines might be influencing surface water chemistry through subterranean hydrological flow, I placed groundwater wells at five forest, eight septic ponds, and four sewer ponds. I placed wells within a half meter of each pond and in line with the septic tank or the nearest portion of a sewer line. I dug a bore hole until I was in the water table and the water depth measured 30-80 cm. I inserted PVC well casings into each bore hole, filled the space between the casing and the surrounding dirt with sand, and created a water-proof bentonite seal at the top of each well. Prior to sampling, I developed the well using a peristaltic pump to remove 10-20 well-volumes worth of water. For future analysis, I pumped water into two amber glass containers and one sterile plastic container and stored the samples in a refrigerator.

Surface Water Collection: I filled two amber glass containers and one sterile, plastic container with surface water. For consistent sampling, I waded to the deepest possible portion of each pond and held each container under the surface until full and capped the bottle underwater.

Long-Term Sampling Devices: At a subset of ponds, I inserted portable organic chemical integrated samplers (POCIS) into both the ground water well as well as in the surface water. I left these samplers in the field for four weeks. Although these ponds have no flow, and therefore do not permit calculations of chemical concentrations, these devices will at least provide a qualitative presence or absence description of chemical contaminants in each pond.

Chemical Analysis: We measured the herbicide atrazine as well as the pharmaceuticals sulfamethoxazole and carbamazepine via ELISA. All other analytes (pharmaceuticals, phytochemicals, surfactants, hormones, and cholesterol) were analyzed with gas chromatography tandem mass spectrophotometry (GC MSMS).

Statistical Analysis

I used a nonparametric Wilcoxin Mann Whitney test to assess differences in sex ratios between forested ponds and suburban ponds in general. I further assessed differences in sex ratios by using a Kruskal-Wallis rank sum test to compare sex ratios between forest, septic, and sewer as well as a nonparametric, post-hoc multiple comparisons test between each pond type using the command *kruskalmc* in the R package “pgirmess”. To my knowledge, there has yet to be a large-scale analysis of sex ratio for a juvenile amphibian, and so the statistical power needed the determine differences in the sex ratio of wild animal populations is largely unknown.

Because of this, I was conservative in my analysis and only used ponds with at least 16 individuals sampled so as to maximize sample size and the power of my sex ratio analyses.

For frog length, I used all ponds in the analysis and compared SVL among forest, septic, and sewer ponds for both males and females separately. Again, I used a Kruskal-Wallis test with post-hoc multiple comparisons of SVL in each land use for either sex.

When lab analyses on gonads and steroid concentrations are complete, I will similarly test for normality of the data and analyze for differences among land uses using either ANOVA or a nonparametric analysis. Most importantly, I will use principal components analysis (PCA) along with a multivariate analysis of covariance (MANCOVA) to analyze how different predictor variables (land cover categories, sewer or septic, contaminant concentrations, etc.) influence different biological endpoints (sex ratios, hormones levels, SVL, etc.).

RESULTS

In total, I collected frogs from five forest ponds, seven septic ponds, and three sewer ponds (Fig. 1 Top). I took water samples from five forest, eight septic ponds, and four sewer ponds (Fig. 1 Bottom). In the end, most ponds had both frog and water samples but some only had one or the other. I have reported all detected organic chemicals for surface waters in Table 1 and ground waters in Table 2.

Forest ponds had a sex ratio of 36.5% female (± 8% 1 SD) wile suburban ponds had a sex ratio of 50.26% female (±9.11% 1 SD). Within suburbia, septic ponds had a sex ratio of 49.07% female (± 9.6% 1 SD) and sewer ponds had a sex ratio of 52.63% female (± 9.33% 1 SD). Suburban frog cohorts had higher proportions of females than forest frog cohorts ($W=5$, $p=0.05$, Fig. 2). There was no detectable difference in sex ratio between forest, septic, and sewer frog cohorts (KW $\chi^2 = 4.2582$, $df=2$, $p=0.1189$, Fig. 3) although there was a trend towards higher sex ratios in sewer ponds. Males and females were shorter ($p<0.05$, Fig. 4) in sewer ponds relative to either septic or forest ponds. Frogs from septic ponds were no different in length than frogs from forest ponds.

DISCUSSION

These early results indicate demographic effects of suburban land use on frog cohorts. Most importantly, suburban ponds in general had a greater proportion of females in offspring cohorts with sewer ponds yielding the greatest proportion of females. Interestingly, natural pond sex ratios were only 35% female, even though theory suggests that natural sex ratios should be 50% of either sex (Trivers and Willard 1973). This suggests that natural sex ratios, for green frogs at least, are male biased and that amphibians may be able to modulate sex ratios based on environmental cues. There is currently limited knowledge as to whether sex ratio fluctuations negatively impact populations or whether endocrine disruption early in life has implications for future reproductive success. However, developing a deeper understanding as to how human land use alters demographics of populations and what this means for population viability is the logical progression from this finding. Furthermore, both males and females from sewer ponds were smaller than frog metamorphs from either septic or forested ponds, indicating that sewer ponds are not only producing fewer males, but that these males are also slightly smaller than their counterparts in forest ponds.

Most endocrine disruption literature has focused heavily on contaminants derived from pesticide application (e.g., Hayes et al. 2002) as well as pharmaceuticals, plastics, and detergents from waste water treatment facilities (e.g., Vajda et al. 2008). Following this theme, my study design explored the hypothesis that septic tanks and sewer lines influenced suburban surface water chemistry through inputs of contaminants like pharmaceuticals or surfactants. Although equilenin, an estrogen extracted from pregnant mare's urine and best known for its use in hormone replacement therapy, was present in many suburban pond waters, it was only found twice in ground water. Because of this, and the rest of the chemical data, I found little support for the hypothesis that sewer lines or septic tanks are contributing contaminants to suburban surface waters. Rather, suburban pond chemistry was often characterized by the presence of biochanin A, coumestrol, daidzein, estrone, and formononetin. Estrone is a natural estrogen produced by the ovaries of all vertebrates and may therefore be a signal of inputs from other wildlife like waterfowl and turtles inhabiting the ponds. Interestingly, biochanin A, coumestrol, daidzein, and formonenetin are all plant-derived chemicals. These chemicals have been increasingly studied for their presence in human foods where they cause an estrogen-like effect (e.g., Benlhabib et al. 2002), or in animal diets where they can alter hormone levels (Tucker et al. 2010, Opalka et al. 2012, Skipor et al. 2012) and sex ratios (El Sayed et al 2012) thus giving them the term *phytoestrogens*. Naturally, many plants from the families Asteraceae, Poaceae, and Fabaceae (specifically the leguminae) exude these chemicals from their roots. The function of these compounds is either allelopathic, a form of chemical warfare where plants exclude each other from a particular area (Inderjit and Dakshini 1992), or to encourage symbiotic mycorrhizal root colonization for increased nutrient uptake (Nair et al. 1991, Zhang and Smith 1995, Ozan et al. 1997, Lee et al. 2012, Wang et al. 2012). Because these chemicals are exuded by plant roots, and because there was often one or more of these phytoestrogens in the ground water in suburban ponds, it appears that landscaping, through biology and not additional chemicals, may act a major source of EDCs into surface waters. It is likely that forest ponds did not have any detectable levels of phytoestrogens because the forests studied here were composed of older trees. The plants best studied for heavy exudation of phytochemicals are grasses (e.g., maize; Ozan et al. 1997), asters (e.g., *Pluchea*; Inderjit and Dakshini 1992), and legumes (e.g., soy or clover; Zhang and Smith 1995, Tucker et al. 2010, Wang et al. 2012). In more natural systems without heavy landscaping or an agricultural influence, it may be expected that only early successional species and colonizing plants may produce high levels of phytoestrogens. This would likely have to do with the ecology of the plants where early successional species would exude phytoestrogens to discourage other plants from invading their rooting zones and also to increase the amount of usable nutrients available in the soil through symbiosis with mycorrhizal fungi. This means that older forests may actually not actually add many of these phytoestrogens to the soil and ponds. If it is true that anthropogenically altered plant communities can be exuding hormonally-active chemicals into surface waters, then it may be important to reevaluate whether phytoestrogens, in addition to pesticides, may be influencing agricultural ponds as well. Furthermore, this work opens up a relatively new domain in urban ecology. Urban development may come with a novel set of ecosystem processes whereby landscape plant communities alter soil, hydrological, and aquatic processes. A further exploration of plant-soil interactions and the effects on aquatic, or even subterranean, faunal life may be an important step in understanding the dynamics of urban ecosystems.

Interestingly, by segregating suburban sites into septic and sewer fed households, this study likely partitioned suburban neighborhoods by relative age of development. Simply put, it is likely that neighborhoods on sewer lines have been experiencing higher levels of urbanization, or suburbanization, relative to septic tank based neighborhoods. These areas may have been developed for longer periods of time while the context of septic-fed households may imply that these landscapes have more recently been developed. If this is true, then it may mean that continued landscaping the around a pond over time may actually increase the concentration of phytoestrogens leaching into waterways. Future work should be open to exploring plant community composition of suburban landscaping as well as the social decisions that go into current landscaping practices.

CONCLUSION

Here I provide evidence that frog populations from suburban neighborhoods are composed of higher proportions of females than in naturally occurring ponds. Furthermore, I show that suburban pond chemistry is most likely linked to plants from landscaping and is less likely to be influenced by septic tanks or sewer lines. Future work will need to assess whether endocrine disrupting chemicals in altered landscapes affects population viability through changed sex ratios and altered hormonal regulation.

NOTE TO THE HIXON CENTER: My laboratory analyses are almost complete. I expect to have these data analyzed by June 2013 and an updated report to the Hixon Center not long after. I thank the Hixon Center for their patience with this delay.

ACKNOWLEDGMENTS

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Figures

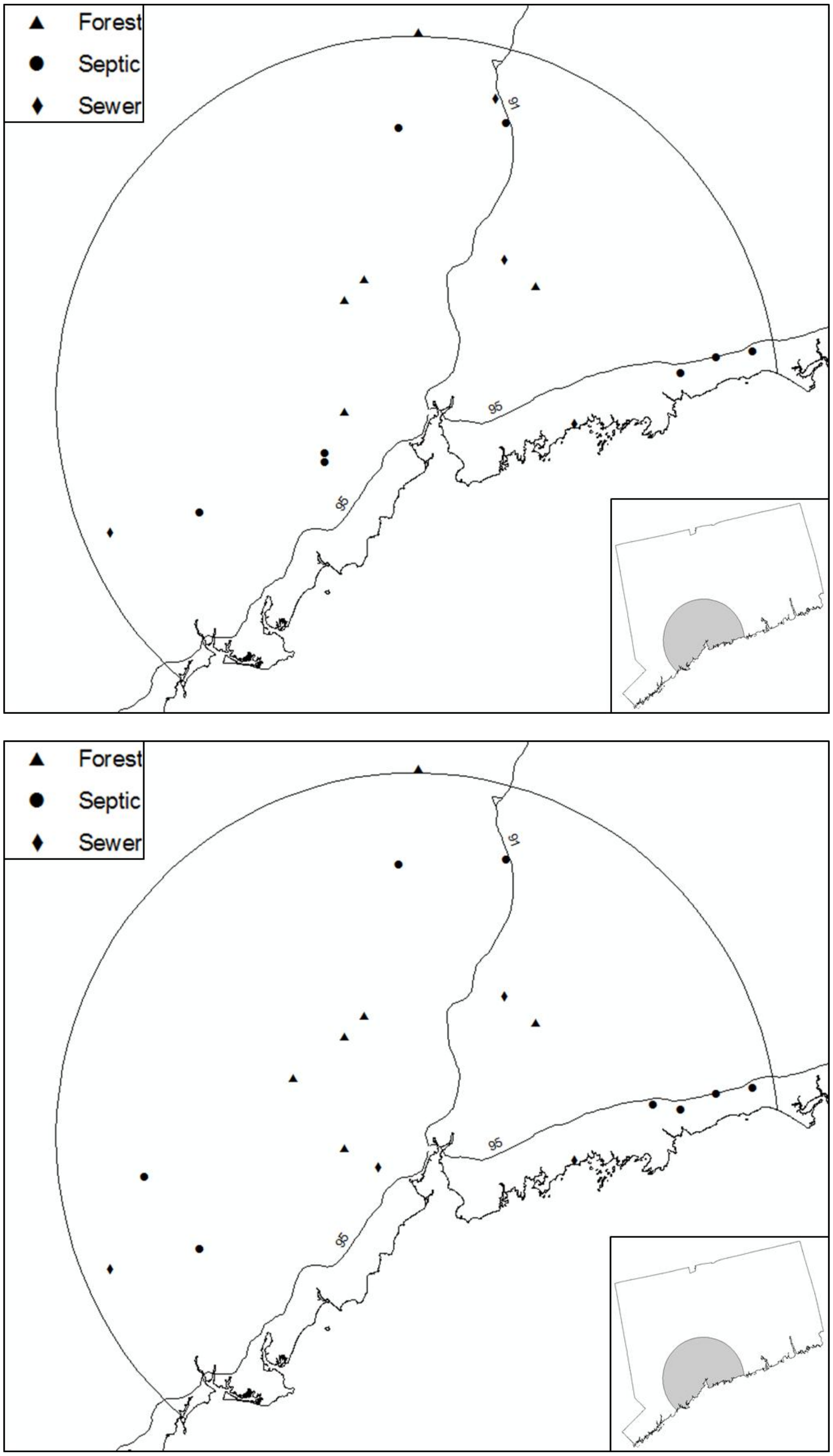


Fig. 1 – Map of ponds with frogs sampled (top) and map of ponds where surface and ground waters were samples (bottom). Note that some ponds where frogs were sampled do not have water samples and some ponds with water samples do not have frog samples.

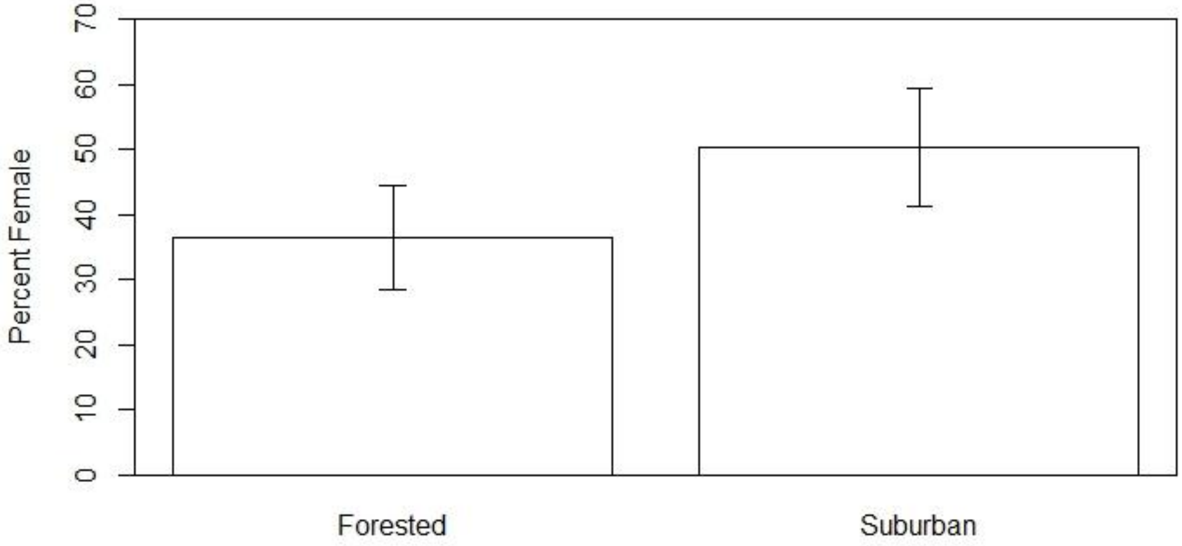


Fig. 2 - Difference (p=0.05035) in the sex ratio between forest and suburban frog cohorts.

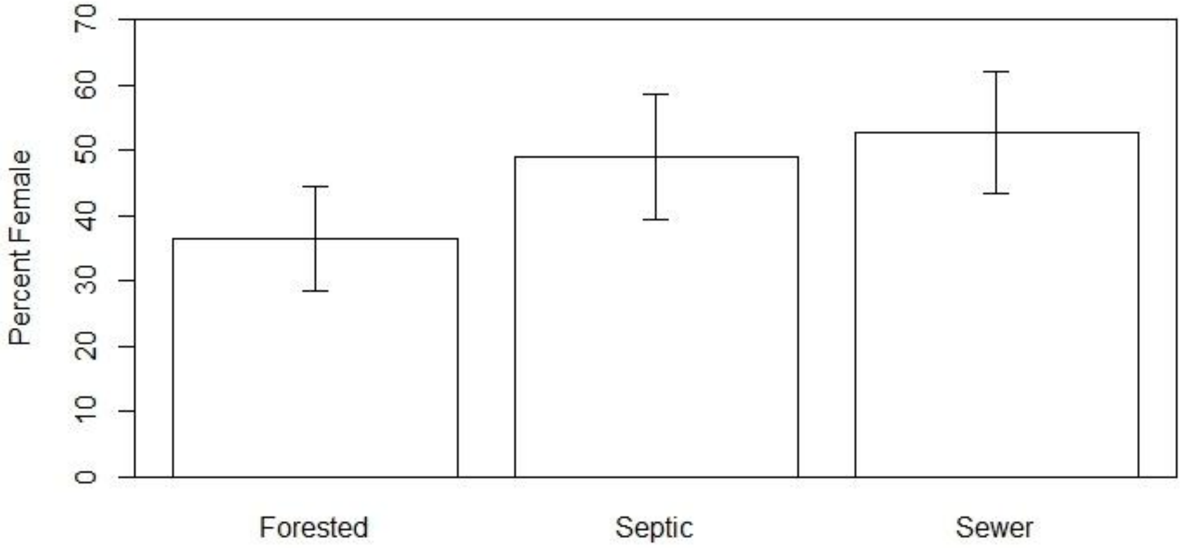


Fig. 3 - Proportion of females in forest, septic, and sewer frog cohorts. There is a trend towards higher proportions of females in sewer ponds.

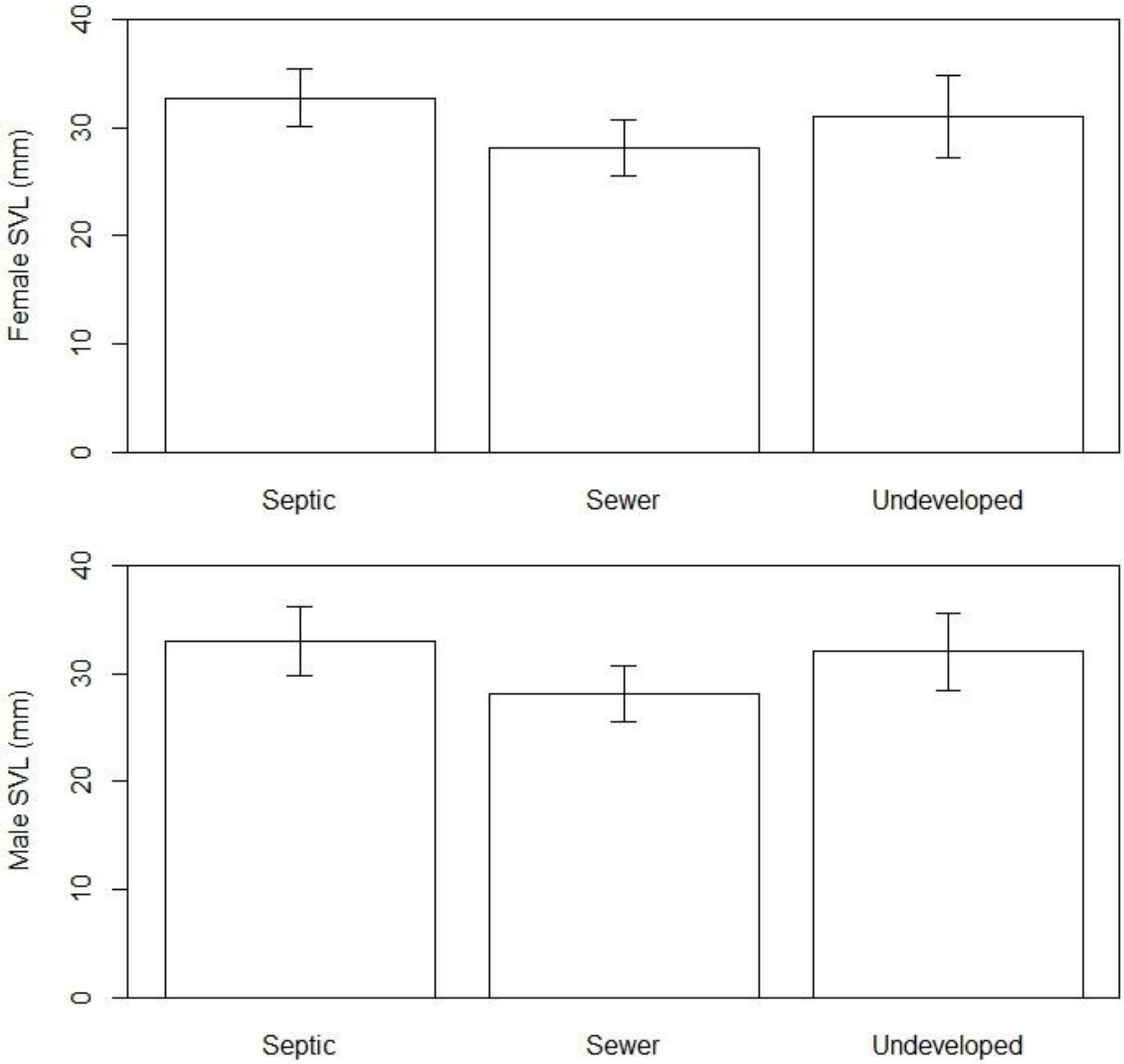
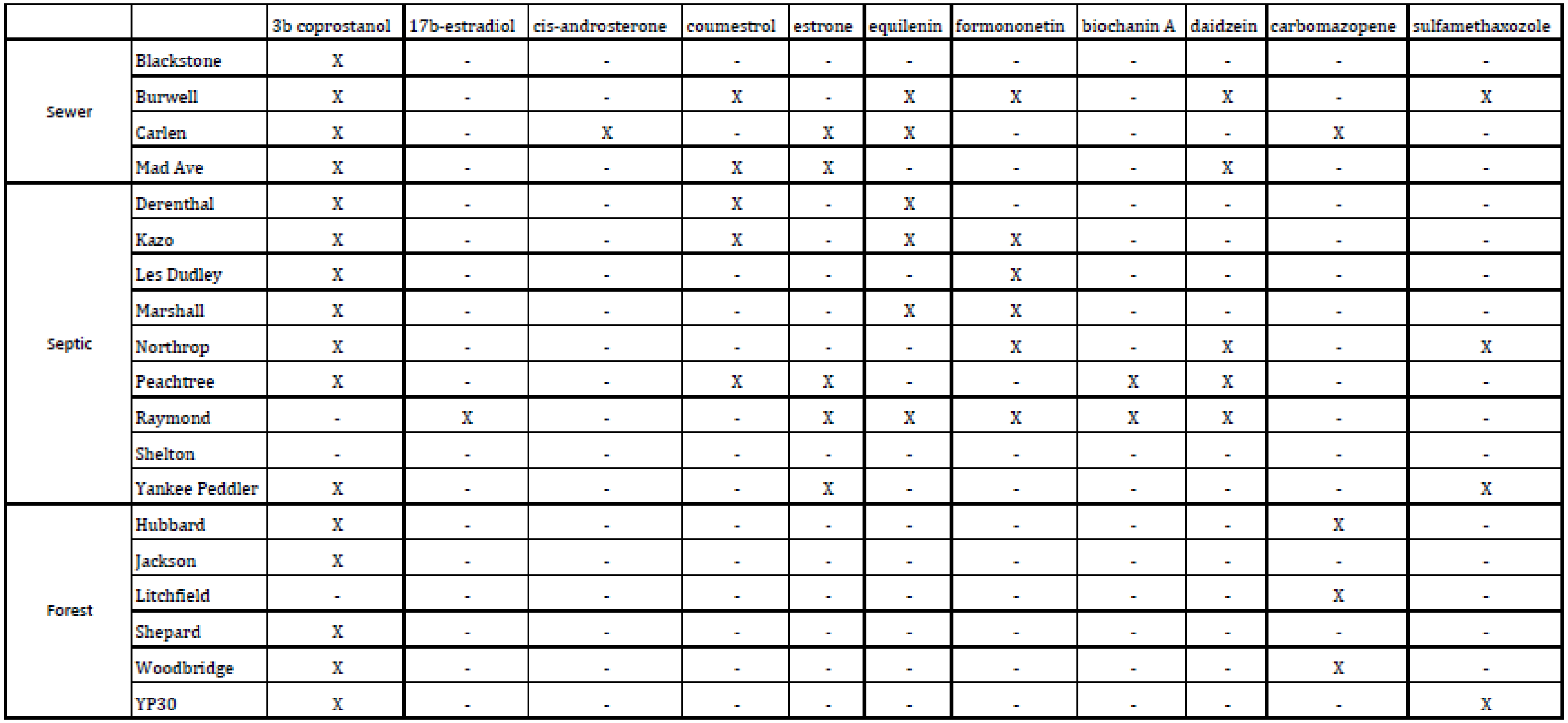
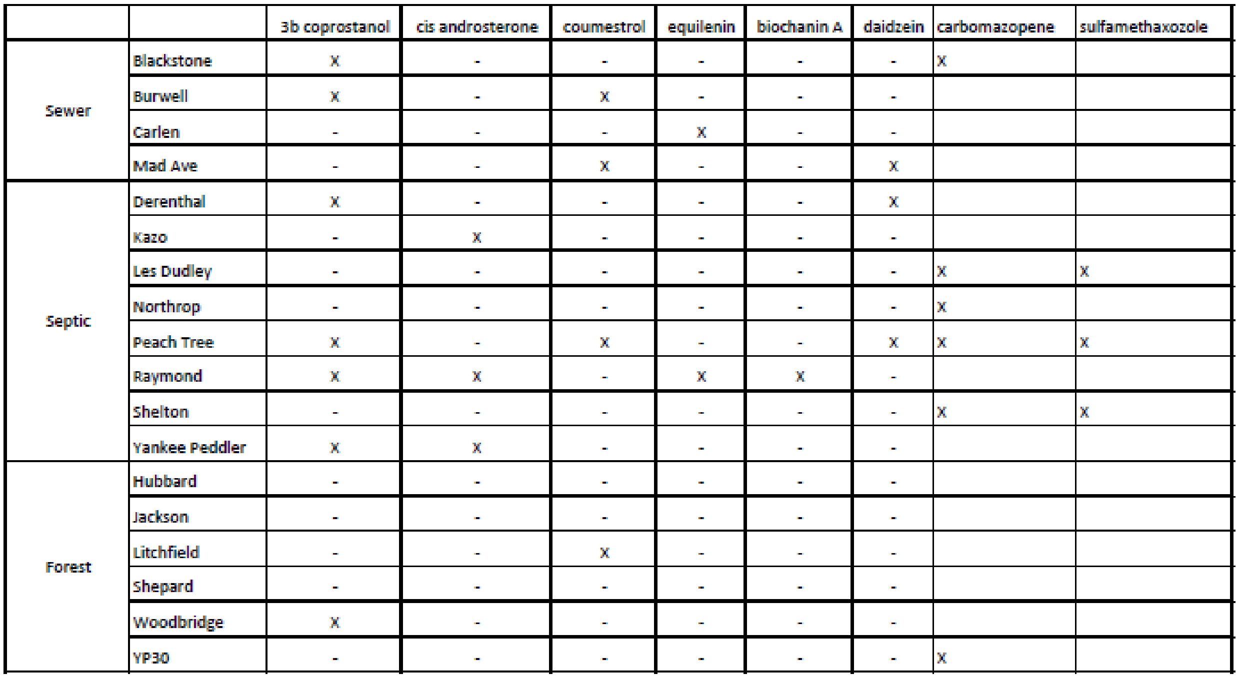


Fig. 4 - Snout-Vent Length of female (top) and male (bottom) frogs. Frogs from sewer ponds were smaller than frogs from either septic or undeveloped ponds.



		3b coprostanol	17b-estradiol	cis-androsterone	coumestrol	estrone	equilenin	formononetin	biochanin A	daidzein	carbomazopene	sulfamethaxozole
Sewer	Blackstone	X	-	-	-	-	-	-	-	-	-	-
	Burwell	X	-	-	X	-	X	X	-	X	-	X
	Carlen	X	-	X	-	X	X	-	-	-	X	-
	Mad Ave	X	-	-	X	X	-	-	-	X	-	-
Septic	Derenthal	X	-	-	X	-	X	-	-	-	-	-
	Kazo	X	-	-	X	-	X	X	-	-	-	-
	Les Dudley	X	-	-	-	-	-	X	-	-	-	-
	Marshall	X	-	-	-	-	X	X	-	-	-	-
	Northrop	X	-	-	-	-	-	X	-	X	-	X
	Peachtree	X	-	-	X	X	-	-	X	X	-	-
	Raymond	-	X	-	-	X	X	X	X	X	-	-
	Shelton	-	-	-	-	-	-	-	-	-	-	-
	Yankee Peddler	X	-	-	-	X	-	-	-	-	-	X
Forest	Hubbard	X	-	-	-	-	-	-	-	-	X	-
	Jackson	X	-	-	-	-	-	-	-	-	-	-
	Litchfield	-	-	-	-	-	-	-	-	-	X	-
	Shepard	X	-	-	-	-	-	-	-	-	-	-
	Woodbridge	X	-	-	-	-	-	-	-	-	X	-
	YP30	X	-	-	-	-	-	-	-	-	-	X

Table 1 - Surface Water Contaminants. An “X” denotes that the chemical was detected. Note that cholesterol and dihydrocholesterol were ubiquitous but are not indicated here



		3b coprostanol	cis androsterone	coumestrol	equilenin	biochanin A	daidzein	carbomazopene	sulfamethaxozole
Sewer	Blackstone	X	-	-	-	-	-	X	
	Burwell	X	-	X	-	-	-		
	Carlen	-	-	-	X	-	-		
	Mad Ave	-	-	X	-	-	X		
Septic	Derenthal	X	-	-	-	-	X		
	Kazo	-	X	-	-	-	-		
	Les Dudley	-	-	-	-	-	-	X	X
	Northrop	-	-	-	-	-	-	X	
	Peach Tree	X	-	X	-	-	X	X	X
	Raymond	X	X	-	X	X	-		
	Shelton	-	-	-	-	-	-	X	X
	Yankee Peddler	X	X	-	-	-	-		
Forest	Hubbard	-	-	-	-	-	-		
	Jackson	-	-	-	-	-	-		
	Litchfield	-	-	X	-	-	-		
	Shepard	-	-	-	-	-	-		
	Woodbridge	X	-	-	-	-	-		
	YP30	-	-	-	-	-	-	X	

Table 2 - Fig 4. Ground Water Contaminants. An “X” denotes that the chemical was detected. Note that cholesterol and dihydrocholesterol were ubiquitous but are not indicated here

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