AN ASSESSMENT OF A CONSTRUCTED WASTEWATER TREATMENT WETLAND COMPLEX AS URBAN AMPHIBIAN HABITAT

by

ALINA MARCELA RUIZ

(Under the Direction of John C. Maerz)

ABSTRACT

The rapid growth of urban areas is largely responsible for simultaneous increases in demand for potable water and habitat loss for wildlife. Constructed wetlands are an efficient way for growing communities to reclaim wastewater for reuse while providing potential wildlife habitat. However, discharging treated wastewater into wetlands may expose animals to pollutants, as well as unnatural temperature and nutrient gradients. My thesis examines diversity and performance of anuran tadpoles at Panhandle Road Constructed Wetlands in Clayton County, Georgia. I compared diversity, size at metamorphosis, and prevalence of visible abnormalities of tadpoles from PRCW and reference ponds that do not receive treated wastewater. In comparison to reference ponds, diversity and size at metamorphosis was similar or larger at PRCW. However, tadpoles in ponds initially receiving treated wastewater showed delayed development and visible symptoms of a novel hypercalcification disorder, suggesting that recognition of treatment wetlands as suitable wildlife habitat may be premature.

INDEX WORDS: Constructed wetlands, Amphibians, Wastewater treatment, Contaminants, *Rana catesbeiana*, Nutrient gradient, Species diversity, Tadpole development, Soft-tissue mineralization, Hypercalcification

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DEDICATION

I would like to dedicate this thesis to my father, Juan, who taught me the value of hard work and persistence. My mother, Alba, who always made me laugh when I felt like I was going to fall apart. And my big brother, "Juany," for always taking care of his little sister, no matter how old I get. Lastly, I would like to extend a special dedication to my grandmother, Blanca Lilian, who is currently battling breast cancer in Colombia, South America….I wish I could be there with you, besos y abrazos.

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CHAPTER 1

AMPHIBIAN SPECIES DIVERSITY IN WASTEWATER TREATMENT WETLANDS: RELATIONSHIP TO NUTRIENT AND TEMPERATURE GRADIENTS

Introduction

In recent decades, scientists, land managers, and the general public have increasingly recognized the value of wetlands for treatment of wastewater or runoff before it reaches rivers and lakes and is ultimately reused for public consumption (Brix, 1994; Mitsch and Gosselink, 2000). This recognition has led to the use of constructed wetlands for wastewater treatment. Constructed wetlands may be built on sites where a wetland did not previously occur, or as part of a network of natural wetlands (Brix, 1994; Moshiri, 1993). Constructed wetlands are generally low maintenance systems composed of one or more treatment ponds, or cells, built in a partially controlled environment. They offer an effective means of integrating wastewater treatment and resource enhancement, often at a cost that is competitive with conventional wastewater treatment alternatives (U.S. Environmental Protection Agency, 1993). Constructed wetlands typically have lower construction, operation, and maintenance costs than conventional mechanical treatment systems, and they are especially suited for smaller communities, due to their ease of operation (Environmental Protection Division, 2002).

In addition to their water quality treatment benefits, constructed wetlands may also provide valuable habitat for wildlife, as well as areas for public education and recreation (U.S. Environmental Protection Agency, 1993). The structure and function of constructed wetlands closely resembles that of natural wetlands, and in urban and suburban environments where natural wetlands are generally rare, constructed wetlands are attractive to many animal species. A recent review of the North American Treatment Wetland Database reported 824 species of invertebrates, 78 species of fish, 21 species of amphibians, 31 species of reptiles, 361 species of birds, and 22 species of mammals among 257 wastewater treatment wetland sites (Knight et al., 2001). Constructed wetlands are also used in various educational programs that range from general environmental awareness to research and graduate courses in wetland management (Mitsch and Gosselink, 2000; Moshiri, 1993).

Another potential benefit of constructed wetlands is their use as quasi-natural laboratories for ecological research. It would be financially and logistically prohibitive for an ecologist to construct replicated wetlands at a scale comparable to that of wastewater treatment wetlands. Constructed wetlands are potentially ideal for environmental research because replicated units are often constructed with similar soils, hydrologic properties, and plant communities and systems with sequential wetland units create environmental gradients. For example, the progressive removal of nutrients can create nutrient/productivity gradients along a system of constructed wetlands. Therefore, wastewater treatment wetlands provide an experimental opportunity to study patterns of animal community structure or population performance across environmental gradients.

Amphibians are popular focal organisms for studies of wetlands. Larval amphibian communities have been studied extensively by ecologists in natural and experimental systems (Werner, 1998; Wilbur, 1997). It is notable that the overwhelming majority of studies focus on the roles of predators and competition in larval amphibian community structure (Werner, 1998; Wilbur, 1997). There have been studies on adult amphibian species richness in relation to annual rainfall and litter volume (Allmon, 1991; Woinarski et al., 1999), but studies examining the role of nutrient availability or productivity on larval amphibians are noticeably lacking despite extensive attention to the relationship between nutrient availability and community structure in other systems.

The majority of studies on nutrient availability and species diversity have focused on plant communities and major biogeochemical processes, such as carbon and nitrogen cycling (Bardgett et al., 1999; Barrett et al., 2006). With regards to wetlands, nearly all research has focused on relationships between plant species richness and nutrient availability. For example, Xu et al. (2007) grew freshwater wetland plant communities at different nutrient levels. After two growing seasons, plant species richness showed a unimodal relationship with N: P. That is, wetland plant species richness was greatest at intermediate nutrient levels (Xu et al., 2007). Other studies showing similar trends have been documented in plant communities in tropical and arid environments (John et al., 2007; Perroni-Ventura et al., 2006; Theodose and Bowman, 1997).

My research utilized a replicated nutrient gradient within a wastewater treatment wetland complex to examine relationships between nutrient diversity and larval amphibian species diversity. Specifically, I examined whether species richness and diversity varied within treatment wetlands as a function of proximity to discharged treated wastewater (nutrient levels are highest closest to discharge points), and whether diversity measures were correlated with actual measures of nutrient availability. Further, I documented whether larval amphibian species within treatment wetlands were comparable to those observed in non-wastewater application reference ponds. Given that species richness of other organisms (plants) show a unimodal relationship with nutrient concentrations, I hypothesized that larval amphibian diversity would show a unimodal relationship with nutrient concentrations. Because differences in diversity between reference ponds and wastewater treatment wetlands, and among wetlands within the treatment wetland complex, will depend on where along the unimodal curve different ponds occur, I could not make a priori predictions about diversity differences between reference ponds and wastewater treatment wetlands, or among wetlands within the treatment wetland complex.

Materials and Methods

Study site

My study took place within the Panhandle Road Constructed Wetlands (PRCW) in the southern portion of Clayton County, a large residential suburb immediately south of Atlanta, Georgia. PRCW is one of several constructed wetland complexes built and managed by the Clayton County Water Authority (CCWA). The PRCW was ideal for this research because it provides three replicate systems that function independently once treated wastewater is discharged into them. Secondarily treated wastewater from the Shoal Creek Water Reclamation Facility is discharged into PRCW through a distribution box that divides the flow into three parallel systems: North, Central, and South (Figure 1.1). Once discharged into each system water is not exchanged among systems. The systems flow from east to west along an elevation drop of approximately 30 m before discharging into the Flint River floodplain. Each system has 6-8 individual wetland "cells" set up in sequence so that treated wastewater flows through the input pipe into Cell 1, then Cell 2, and on until the last cell of each system. Each cell in PRCW has alternating shallow/deep zones planted with species of emergent aquatic vegetation including pickerelweed (*Pontederia cordata*), arrowhead (*Sagittaria lancifolia*), cattail (*Typha latifolia*), four species of bulrush (*Scirpus* spp.), and cutgrass (*Zizaniopsis miliacea*). The deep zones in each cell contain submerged aquatic vegetation such as coontail (*Ceratophyllum demersum*), bushy pondweed (*Najas guadalupensis*), water-thread pondweed (*Potamogeton diversifolius*), and fragrant white water lily (*Nymphaea odorata*). For each cell, planting pattern of emergent vegetation was based on the direction of flow between each deep zone. Water from all systems empty into a 400-acre reservoir, which then releases water south, either into the Flint River or into the reservoir of a water production plant.

For comparison purposes, I sampled four reference ponds in Athens-Clarke County, Georgia, about 70 miles east of Clayton County; three in the Whitehall Experimental Forest, a fourth near the University of Georgia Golf Course. All reference ponds were also artificial impoundments that receive inputs of water from primary streams.

Larval Sampling

Between January and July 2006, I conducted repeated samples of tadpoles from cells 1, 3, and 5 in each of the three PRCW systems. Additional samples from reference ponds were collected in June and July 2006. On each occasion, I performed dipnet sweeps for 21 minutes in each cell. Tadpoles were killed by emersion in 1% tricaine methanesulfonate, MS-222, and preserved in 10% neutral buffered formalin. All specimens were later examined in the laboratory to determine species.

Species Diversity

I used Simpson's index of diversity and Simpson's Measure of Evenness (Krebs, 1999) to compare species diversity among ponds and evaluate relationships between species diversity and nutrient concentrations. It has been shown that small samples (< 30) could give unreliable estimates of diversity, especially when less than 10 samples were counted (Krebs, 1999), so estimates of diversity for ponds with few tadpoles may be unreliable. Further, because we had a limited number of possible species in these communities, and some species may have low detection probabilities, inferences drawn from analyses of diversity indices should be tempered.

To determine whether diversity measures were correlated with measures of nutrient availability at the PRCW, I calculated three different measures of diversity based on proximity to discharge of treated wastewater. Simpson's index of diversity was calculated for the first, third, and fifth cells of the PRCW. For each cell location, I combined all individuals collected from either the first, third, or fifth cells in each of the three PRCW systems. I used these totals to calculate proportions of each species found at each cell location of the three PRCW systems.

Temperature and Nutrient Analysis

During each tadpole sampling period, I measured water temperature and collected replicate water samples. Water temperatures were measured approximately 0.5 meters deep with a bulb thermometer. Three 30-ml water samples were collected monthly from each cell, and then stored in opaque bottles in a darkened refrigerator at ~3°C for later analyses (approximately six weeks). These samples were analyzed at the University of Georgia's Analytical Chemistry Laboratory in the Institute of Ecology. A persulfate digest was performed on each water sample to determine total nitrogen and total phosphorus (University of Georgia, Institute of Ecology, 2007). After water samples were brought to room temperature, 1 ml of oxidizing reagent was mixed into 5 ml of each water sample. Digest tubes were shaken, then placed in autoclave for 30 minutes on liquid cycle. After tubes cooled, an Alpkem ™ continuous flow colorimetric analyzer was used to determine nitrate and orthophosphate concentrations. The values from duplicate water samples taken from cells on the same visit to the PRCW were averaged.

<u>Statistical Analysis</u>

To determine whether nutrient levels differed among cells during my sampling period, I used a repeated measures analysis of variance (ANOVA). The effect of cell location (first, third, or fifth), date, and the cell location by date interaction for total nitrogen, total phosphorus, and temperature was measured (Sokal and Rohlf, 1987). To determine if the distribution of larval amphibian species differed among cells, I used a Chi-squared test (Sokal and Rohlf, 1987). I then used visual examinations of scatter plots with fitted polynomial curves to evaluate the hypothesis that the relationship between larval amphibian diversity and nutrient availability was unimodal. If results did not appear unimodal, and instead appeared linear, I used standard linear regression approaches to determine if linear relationships were statistically significant. All statistical analyses, except for the Chi-squared test, were calculated using STATISTICA, Version 6.0 (StatSoft, Inc., 2003).

Results

Between January and July 2006, 1661 tadpoles and metamorphs were collected from the PRCW and four reference ponds: 1023 *Rana catesbeiana*, 366 *Rana sphenocephala*, 184 *Hyla cinerea*, 84 *Rana clamitans*, and 4 *Acris* species (presumably *A. crepitans*). Four species, 1409 total specimens, were collected from the PRCW: *Rana catesbeiana, Rana sphenocephala, Rana clamitans* and *Hyla cinerea*. Five species, 252 total specimens, were collected at the reference ponds: *Rana catesbeiana, Rana clamitans, Rana sphenocephala, Acris* sp., and *Hyla cinerea*.

Capture rates (tadpoles per minute) were highest in cell N1 of the PRCW and Pond 3 of the reference ponds (Table 1.1). *Rana catesbeiana* had the highest relative abundance in most ponds, while *Hyla cinerea* and *Acris* sp. had the lowest relative abundance (Table 1.2; Figure 1.2). The distribution of larval amphibian species significantly differed among the first, third, and fifth cells of the PRCW ($x^2 = 216.03$; $P < 0.001$). Mean Simpson's index of diversity for the PRCW and reference ponds were 0.475 (±1 SE = 0.037) and 0.297 (± 0.136), respectively. Simpson's measure of evenness yielded 0.579 for the PRCW and 0.331 for the reference ponds.

Total nitrogen and total phosphorus concentrations were significantly positively correlated among all ponds (r = 0.929, $P < 0.001$). Within the PRCW, nutrient levels varied among wetland cells during some but not all sampling dates (Table 1.3; Figure 1.3). Among all pond types, mean total nitrogen and mean total phosphorus concentrations were lower among reference ponds compared to the PRCW (Figure 1.3). Mean total nitrogen concentration and mean total phosphorus concentration for reference ponds were 0.364 (±1 SE = 0.073) mg/L and 0.009 (± 0.003) mg/L, respectively. Mean nutrient concentrations in the first, third, and fifth cells of the PRCW were 3.267 (±1 SE = 0.235), 1.705 (± 0.474), and 1.418 (± 0.280) mg/L for total nitrogen and 1.267 (± 0.110), 0.922 (± 0.243), and 0.547 (± 0.099) mg/L for total phosphorus, respectively. Within the PRCW, total nitrogen and total phosphorus concentrations were measurably higher in the first cells compared to the third or fifth cells, which was consistent with expectations; however, third cells had similar nutrient concentrations to fifth cells (Figure 1.3). Though differences in nutrient concentrations appeared to vary temporally (Figure 1.3), there were no significant effects of date, or interactions between cell and date, for total nitrogen or total phosphorus (Table 1.3).

Temperatures also differed among first, third, and fifth cells for part of the year (Figure 1.3). Cell and date had significant effects on temperature, and I found a significant cell by date interaction (Table 1.3). Between January and April, temperatures were warmest in the first cells, intermediate in the third, and lowest in the fifth cells. During this period, water temperatures in the first cells and third cells were significantly warmer than air temperatures indicating the water was artificially warm. During the months of May through July when air temperatures were hotter, water temperatures converged (Figure 1.3).

Overall, larval amphibian diversity appeared to show a unimodal relationship among pond types (Figure 1.4). However, within the PRCW, species diversity tended to decline with increasing nutrient concentrations. The relationship between larval amphibian diversity and total nitrogen ($r = 0.830$, $P = 0.377$) was not statistically significant, nor was the relationship between species diversity and total phosphorus ($r = 0.983$, $P = 0.119$); however, the relationship between total phosphorus and species diversity was clearly strong, as indicated by the correlation coefficient (Figure 1.4).

Discussion

Constructed wetlands are increasingly being used to treat wastewaters and promoted as habitat for wildlife. My research suggests that larval amphibian diversity is negatively correlated with nitrogen and phosphorus levels in constructed wetlands. I qualify that because Simpson's index of diversity is intended for communities with large numbers of species, my estimates of diversity may have limited informative value. Further, I have not accounted for some species having potentially low detection. However, assuming these estimates are correct there are two ways you can view these results. The negative view suggests that the discharge of wastewaters rich in nutrients into wetlands could have a negative effect on larval amphibian diversity. This could be problematic at sites where wastewater is discharged directly into all cells within a constructed wetland complex or in cases where wastewaters are discharged into natural wetlands. On the other hand, the positive view suggests that the removal of nutrients from treated wastewater in primary wetlands has a positive effect on amphibian diversity in wetlands farther from the discharge point. Therefore, more distant wetland cells create habitats that appear to support more diverse larval amphibian assemblages and collectively, the gradient of wetlands at PRCW generates a variety of larval amphibian communities.

Currently, the mechanisms driving observed relationships between nutrient concentrations and larval amphibian diversity in the PRCW are not known. Temperature gradients in the winter months at the PRCW may also have a confounding effect on larval amphibian diversity. It is possible that these temperature gradients, in combination with nutrient gradients, are a driving force behind these relationships. In natural wetlands, nutrient concentrations range from 1 to 2 mg/L for nitrogen and 0.1 to 0.7 mg/L for phosphorus, approximately (Kadlec and Knight, 1996). Since nutrient concentrations are above natural ranges in the first cells of the PRCW, one might expect a more diverse amphibian community because high nutrients would support high levels of algal growth; however, some studies have suggested that high levels of phosphorus and nitrogen increase developmental risk to amphibians (Taylor et al., 2005). If amphibian species are differentially sensitive to the negative effects of high nitrogen or phosphorus levels, then this might reduce larval diversity in these environments. The dominance of bullfrog tadpoles in high nutrient cells may also be indicative of a change in predator gradients linked to nutrient levels. In studies of interactions among bullfrogs and other large *Rana* sp. such as green frogs (*R. clamitans*), bullfrog tadpoles (*R. catesbeiana*) were favored in the absence of macroinvertebrate predators such as dragonfly naiads (Werner and McPeek, 1994). The bullfrog appears to be more vulnerable to *Anax* sp. because it is more active than the green frog and consequently has higher encounter rates with predators (Eklov and Werner, 2000; Werner and McPeek, 1994). Additionally, larval amphibian predators can be more sensitive to some contaminants than amphibians. Because invertebrates are highly sensitive to insecticides, elimination of this predator may bolster the ability of bullfrogs to invade new habitats and reduce community diversity (Boone and Semlitsch, 2003). Variations in the occurrence of green treefrogs (*Hyla cinerea*) at the PRCW may also be linked to the absence of predators, since tadpole mortality rates tend to increase linearly with predator density (Gunzburger and Travis, 2005). I did not quantify differences in predator communities among cells, but if macroinvertebrate predators are sensitive to high nitrogen or phosphorus levels, then the differences in larval communities along a nutrient gradient may be linked to predator-mediated effects.

Finally, I must consider the possibility that other factors correlated with nutrient levels are driving patterns. The water discharged into these cells not only contains high amounts of nutrients, but large amounts of contaminants such as pharmaceutical and other wastes that may be affecting the performance and community structure of amphibians at the PRWC (Gros et al., 2007). Amphibians are known to be differentially susceptible to pesticides, so it is reasonable that other contaminants might be responsible for observed patterns. Although pesticides have been shown to negatively affect amphibian populations, for most pesticides, tests on amphibians are rare and conducted only for short durations (1 to 4 days) and without natural stressors (Relyea, 2005). My study at the PRCW exposes amphibians to year-round exposure of potential contaminants. The presence of chemicals could alter community function, and thereby affect biodiversity (Boone and Semlitsch, 2001; Boone and Semlitsch, 2003). Differences in susceptibility to stresses may explain why some amphibian populations decline while others appear unaffected, such as American bullfrog populations. Susceptibility to a contaminant may be mediated by length of the larval period and time of breeding (Boone and Semlitsch, 2001).

The absence of common amphibian species at the PRCW, such as gray treefrogs (*Hyla chrysoscelis/versicolor*) and American toads (*Bufo americanus*) could be attributed to either the presence of contaminants or predators. Takahashi (2007) provided evidence that concentrations of herbicide expected to be found in the field can alter oviposition site choice by amphibians. In his study, gray treefrogs avoided oviposition in contaminated pools and placed the majority of their eggs in control pools, which suggests that breeding adults may be able to prevent lethal exposure of herbicide to their offspring through oviposition site selection (Takahashi, 2007).

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Table 1.1. Number of tadpoles collected per minute from the Clayton County Water Authority (CCWA), Panhandle Road Constructed Wetlands and four reference ponds in 2006.

Location	Jan	Feb	Mar	Apr	May	Early June	Late June	July
CCWA N1	0.24	0.38	0.95	1.19	0.55	3.62	1.62	1.97
CCWA C1	0.00	0.05	0.05	1.33	0.31	0.02	0.19	1.16
CCWA S1	0.00	0.14	0.38	0.29	0.12	0.05	0.05	0.25
CCWA N3	0.33	0.10	1.19	0.52	0.60	0.33	0.79	2.71
CCWA C3	0.00	0.00	0.33	0.14	1.26	0.26	0.40	1.19
CCWA S3	0.00	0.19	0.00	0.19	0.17	0.19	0.64	0.21
CCWA N5	0.00	0.00	0.29	0.62	0.83	0.43	0.36	0.46
CCWA C5	0.00	0.05	0.24	1.29	0.88	0.17	0.55	0.63
CCWA S5	0.00	0.00	0.29	0.43	0.21	0.21	0.10	0.06
Ref R1						0.07	0.02	0.11
Ref R2						0.14	0.36	0.06
Ref R3						1.31	2.21	0.44
Ref R4						0.12	0.83	

Note: Sampling began at reference ponds on June 14, 2006. Data not collected from Reference pond R4 in July.

Table 1.2. Relative abundance of amphibian species collected from the Clayton County Water Authority (CCWA), Panhandle Road Constructed Wetlands and four reference ponds in 2006.

		Relative abundance				
Location	N	*Rana catesbeiana*	*Rana clamitans*	*Rana sphenocephala*	*Hyla cinerea*	*Acris* sp.
CCWA N1	425	0.62	0.02	0.35	0.01	0.00
CCWA C1	125	0.63	0.01	0.36	0.00	0.00
CCWA S1	42	0.79	0.14	0.07	0.00	0.00
CCWA N3	288	0.51	0.02	0.09	0.38	0.00
CCWA C3	166	0.67	0.01	0.15	0.17	0.00
CCWA S3	63	0.81	0.02	0.17	0.00	0.00
CCWA N5	117	0.54	0.10	0.12	0.24	0.00
CCWA C5	141	0.37	0.01	0.53	0.09	0.00
CCWA S5	42	0.76	0.00	0.24	0.00	0.00
Ref R1	11	0.55	0.00	0.00	0.27	0.18
Ref R2	25	0.92	0.00	0.00	0.00	0.08
Ref R3	176	0.69	0.27	0.05	0.00	0.00
Ref R4	40	1.00	0.00	0.00	0.00	0.00

Table 1.3. Summary of repeated measures analysis of variance of nutrients and temperatures from the Clayton County Water Authority, Panhandle Road Constructed Wetlands in 2006.

Variable	*df*	*MS*	*F*	*P*
Nitrogen				
Cell location	2	17.816	8.297	0.019
Date	5	4.925	1.573	0.198
Cell location x Date	10	1.471	0.470	0.896
Phosphorus				
Cell location	2	2.335	4.813	0.057
Date	5	5.102	8.967	<0.001
Cell location x Date	10	0.982	1.725	0.121
Temperature				
Cell location	2	13.238	7.619	0.023
Date	5	286.306	474.541	<0.001
Cell location x Date	10	4.635	7.682	<0.001

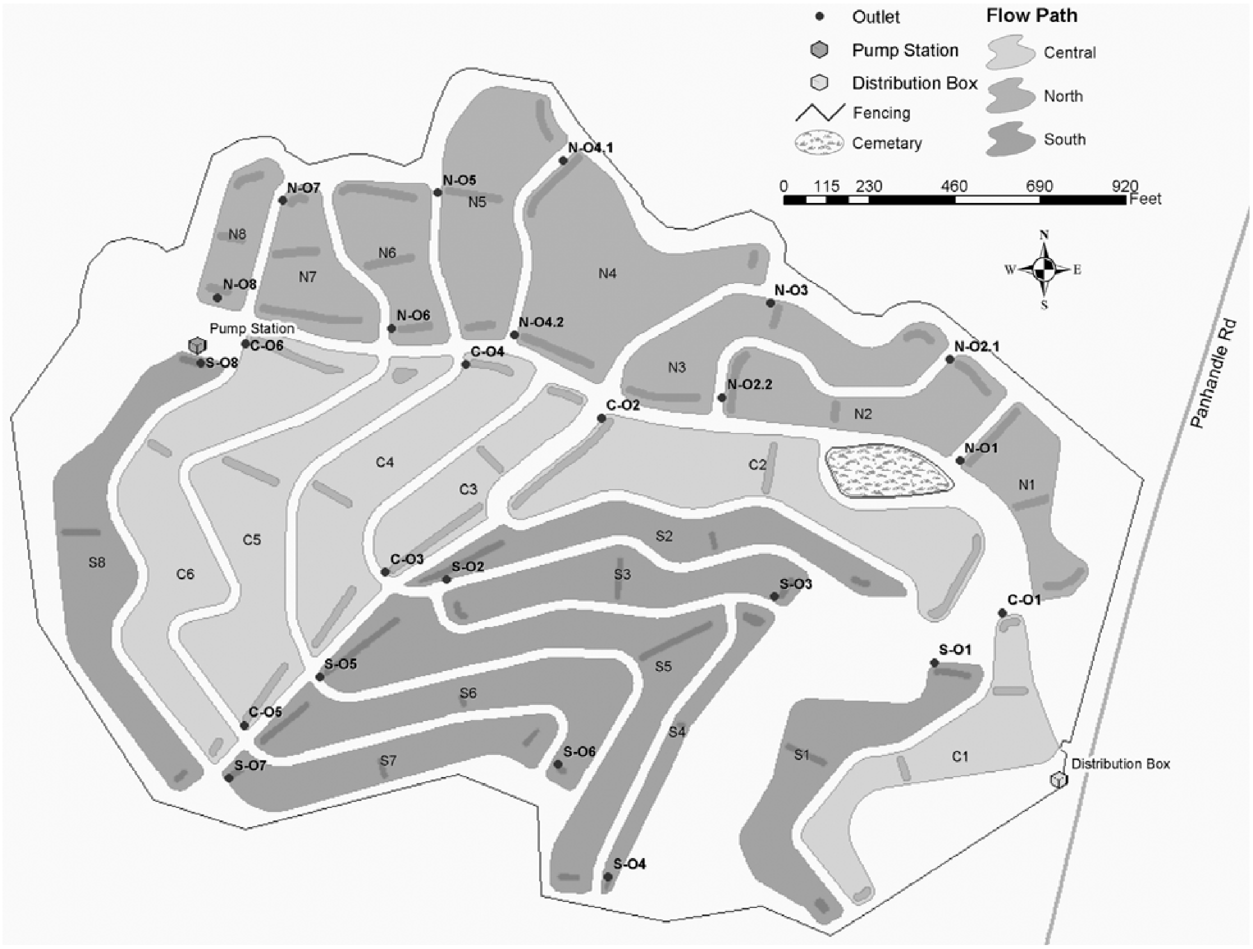

Figure 1.1. Map of the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Includes three separate systems (N-north, C-central and S-south) and their associated cells (number of cell refers to its sequence of water; water enters N1, flows to N2, flows to N3, etc.).

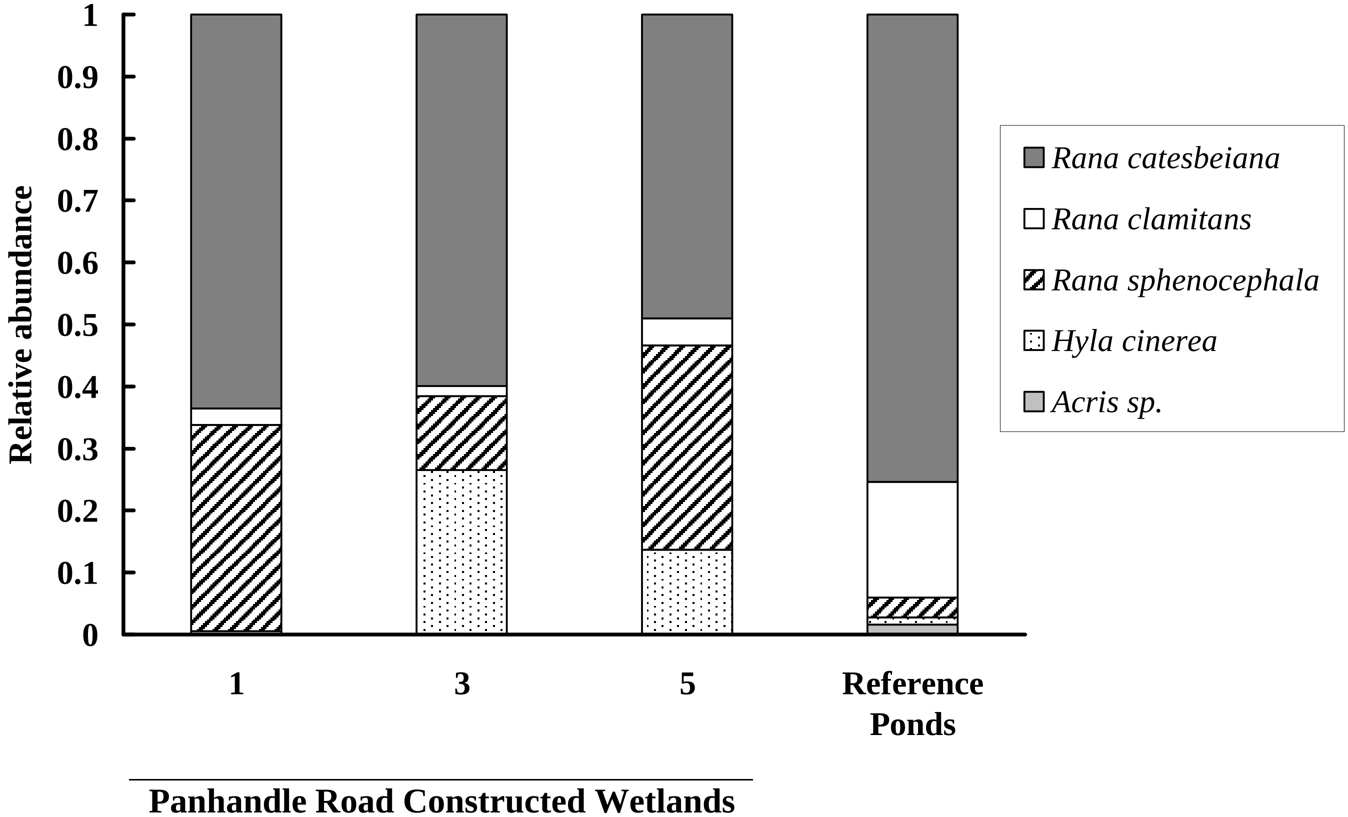

Figure 1.2. Mean relative abundance of amphibian species in the first, third, and fifth cells of the Clayton County Water Authority, Panhandle Road Constructed Wetlands and four reference ponds in 2006.

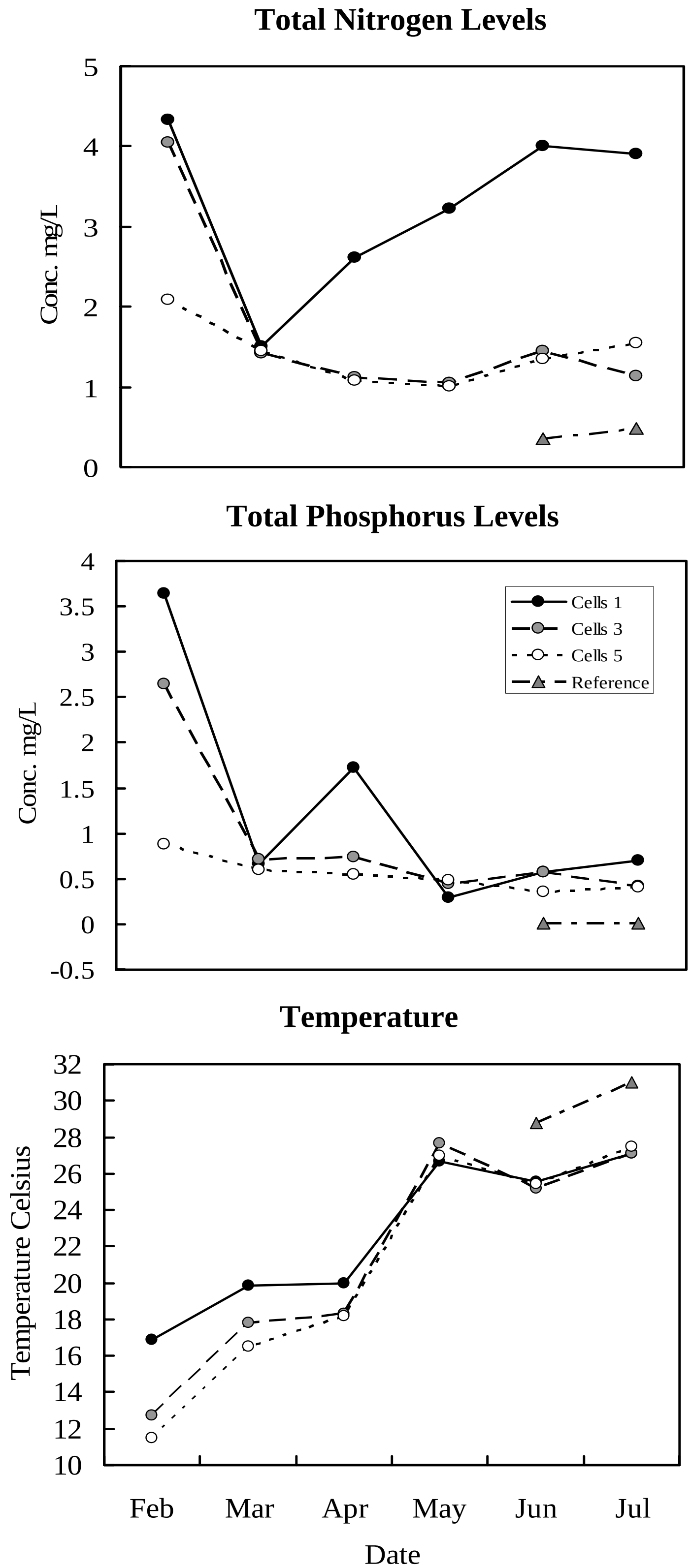

Figure 1.3. Mean total nitrogen levels, mean total phosphorus levels, and mean temperatures at the Clayton County Water Authority, Panhandle Road Constructed Wetlands and four reference ponds in 2006.

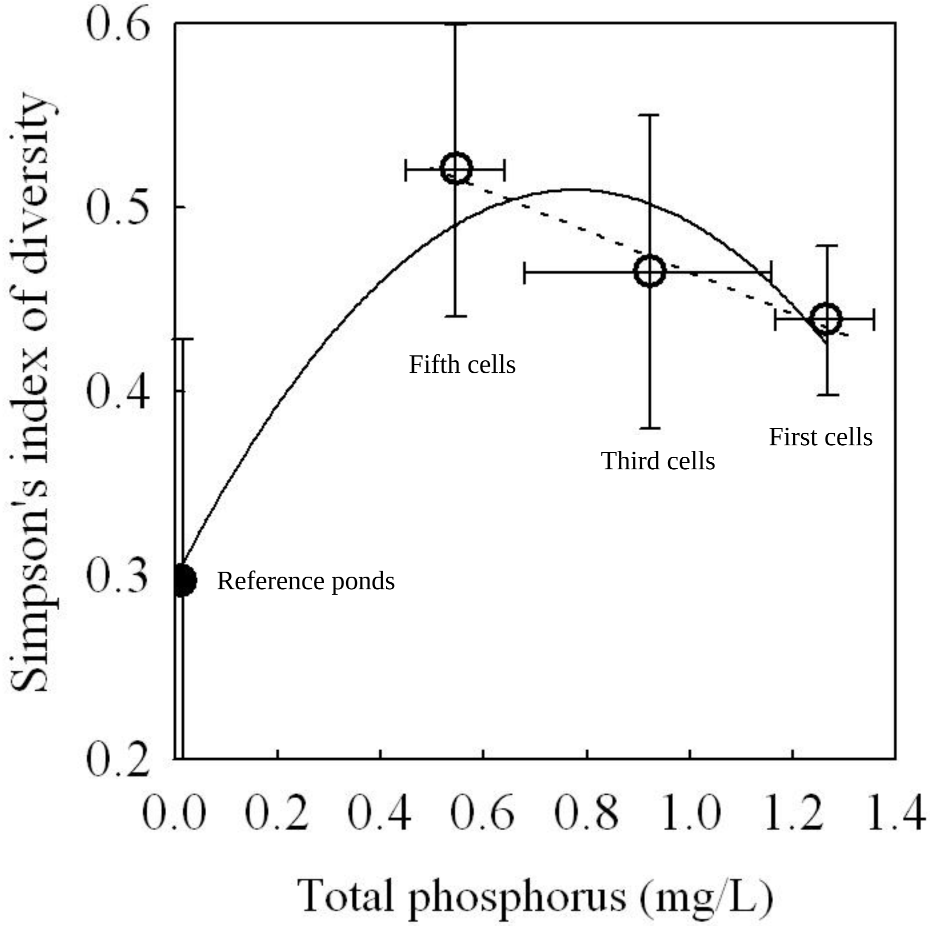

Figure 1.4. The relationship between mean total phosphorus (mg/L) and mean Simpson's index of diversity in the first, third, and fifth cells of the Clayton County Water Authority, Panhandle Road Constructed Wetlands (PRCW) and four reference ponds in 2006. Solid curve is a unimodal curve fitted to all points. Dashed line is a linear function of the three points from PRCW.

CHAPTER 2

PERFORMANCE OF LARVAL *RANA CATESBEIANA* IN WASTEWATER TREATMENT WETLANDS

Introduction

The growth of human populations in urban areas places simultaneous pressure on limited natural resources and habitat available to support wildlife. For example, demand for water has resulted in significant diversions of this resource from rivers and the mining of groundwaters. In many cities, population growth may not be matched by growth in infrastructure, especially in wastewater management (Kaseva, 2004), resulting in the return of inadequately treated wastewater to rivers and lakes. In addition to the acquisition of natural resources often resulting in degraded environmental conditions (Foley et al., 2005), expanding urban environments also result in the loss of habitat available for wildlife. Urbanization creates progressively smaller and more isolated habitat patches within a matrix of residential, commercial, and industrial development resulting in population fragmentation (Wilcox and Murphy, 1985). Increases in road traffic and recreational use of natural areas can result in higher mortality rates for wildlife, and predation by domestic animals may be high in urban environments (Gagne and Fahrig, 2007; Woods et al., 2003). In the United States, urbanization has been cited as a major cause of more than half of threatened or endangered species declines (Czech et al., 2000; Millennium Ecosystem Assessment, 2005). Even local species extirpations of common or cosmopolitan species have been reported in urban environments as a result of localized habitat degradation

(Urban et al., 2006). Solutions to the growing pressures of urbanization on natural resources and wildlife present a significant challenge.

Wastewater treatment wetlands provide potential solutions to increasing demand for water use in urban areas, and for mitigation of natural habitats lost through urban development. Wetland habitats, which support high species diversities and are essential for many organisms, have been and continue to be one of the most highly impacted habitats in North America (Gibbons et al., 2006). The loss of wetlands in the United States began over two centuries ago. Wetlands were regarded as habitats that bred disease, restricted overland travel, and impeded the growth of agriculture, so settlers, commercial interests, and governments agreed that wetlands should be drained and used for other purposes (U.S. Geological Survey, 2005). Wetland loss accelerated in the 1950s when the United States passed laws that permitted the destruction of wetlands to allow for mass expansion of agriculture (Erickson et al., 1979). These policies led to wetland area losses averaging 550,000 acres each year from the mid-1950s to the mid-1970s (Office of Technology Assessment, 1984). This dramatic loss in wetland area was eventually addressed in 1972 by the Clean Water Act. The Clean Water Act imposed regulations requiring replacement of wetlands that were destroyed through agriculture, construction, or urbanization (National Research Council, 2001). Also wetland functions, such as water quality, wildlife habitat, and hydraulic functions must be replaced (U.S. Geological Survey, 2005). Despite these regulations, a recent review of mitigation efforts in the United States shows a net loss of wetland area and function, even though 'no net loss' is the national policy and compensatory measures are mandatory (Zedler, 2004).

Generally, the primary function of wastewater treatment wetlands is to remove nutrients from treated wastewater before waters are discharged into rivers, lakes, or reservoirs (Greenway, 2005). The oldest documented use of a constructed wetland for wastewater purification was in 1904 (Brix, 1994), and wetland treatment systems have grown in use in different parts of the world since the 1950s (Verhoeven and Meuleman, 1999). The use of wetlands for wastewater treatment is beneficial because wetland treatment is a lower-cost alternative to conventional secondary treatment processes, mainly because forced aeration is not needed when using wetland treatment systems (Gersberg et al., 1986), and they also do not require advanced technology or highly-skilled personnel to operate (Al-Omari and Fayyad, 2003; Hiley, 1995; Juwarkar et al., 1995). More recently, governments and the public recognized that constructed wetlands not only provide a lower-cost solution to an increasing need to clean and recycle water, but they also provide potentially valuable wetland habitat in areas where this habitat is otherwise declining (Knight et al., 2001).

Though considered a secondary function, the use of constructed wetlands as wildlife habitat is often widely publicized by local agencies. Even the United States Environmental Protection Agency has promoted the value of constructed wastewater wetlands as wildlife habitat (U.S. Environmental Protection Agency, 1993). The portrayed “value” of constructed wetlands for wildlife may, however, be premature. Superficially, these wetlands are attractive to wildlife because they offer abundant water and growing plants, components which provide the basis for an ecological food web (Knight, 1997); however, information on performance of wildlife in wastewater treatment wetlands is lacking. During the summer of 1992, the U. S. EPA conducted a pilot study of wildlife usage and habitat functions of constructed wetlands. This study represents the only known attempt to critically compare habitat and wildlife usage between treatment wetland sites. Wildlife studies in treatment wetlands such as this measured abundance of species, but lack information concerning wildlife health and performance (Knight et al., 2001). Furthermore, the limited information concerning wildlife in wastewater treatment wetlands deals predominately with avian species.

Despite their well known linkage to wetlands, amphibian performance in wastewater treatment wetlands has received scant attention. The global decline of amphibian populations is relatively well known, even among the general public. The causes of amphibian declines are multi-faceted and vary from location to location, but habitat loss and degradation is a common and significant contributor (Boyer and Grue, 1995; Brinson and Malvarez, 2002; Fisher and Shaffer, 1996; Lehtinen et al., 1999; Pechmann et al., 2001; Sparling et al., 2000; Urban et al., 2006). Many also view amphibians as "sentinels" of environmental degradation, and are used increasingly as bioindicators of environmental quality (DeGarady and Halbrook, 2006; Wasonga et al., 2007). I am aware of a single study examining the performance of amphibians in wetlands that receive treated wastewater effluent. Laposata and Dunson (1998) reported reduced numbers of amphibian egg masses and larval amphibian survival in wetlands irrigated with treated wastewater; further research on amphibians conducted by Laposata and Dunson (2000) at the same wetlands revealed similar results. Though this is only a single treatment wetland location, it suggests that wastewater irrigation may have detrimental effects on amphibian populations. This study also indicates that we should be cautious in promoting the value of constructed wetlands for amphibians or other wildlife species.

The objectives of my research were to evaluate the "performance" of tadpoles in a constructed wastewater treatment wetland. Specifically, I compared the development rates and size at metamorphosis of larval bullfrogs (*Rana catesbeiana*) in a complex of wastewater treatment wetlands to larval bullfrogs in several constructed reference ponds that do not receive treated wastewater. I also examined bullfrog tadpole performance within the wastewater treatment complex to evaluate whether performance varied as a function of proximity to the treated wastewater discharge point. I chose larval bullfrogs because my research at the treatment wetlands revealed that bullfrogs were the dominant larval amphibian at the site and the only species found in all wetlands throughout the complex (see Chapter 1). I focused on development rates and size at metamorphosis because both are considered key metrics for predicting the effects of larval environments on amphibian populations. Extended development periods can indicate poor larval environments and is often associated with reduced larval survival and small size at metamorphosis (Alford, 1999). Size at metamorphosis is widely accepted as a positive predictor of survival to first reproduction (Berven, 1990; Diana et al., 2000). Because wetlands receiving treated wastewater will be high in nutrients, which should have a positive effect on algal productivity, and are warmer than other wetlands for most of the year (see Chapter 1), I hypothesized that bullfrog tadpoles in the wastewater treatment wetlands would be larger at metamorphosis compared to reference ponds. Similarly, because nutrient concentrations and water temperatures would be higher in wetlands closer to the wastewater discharge point, I hypothesized that bullfrog tadpoles in wetlands closer to the discharge point would develop more rapidly and be larger at metamorphosis than tadpoles from treatment wetlands farther from the discharge point.

Materials and Methods

<u>Study site</u>

My study took place within the Panhandle Road Constructed Wetlands (PRCW) in the southern portion of Clayton County, a large residential suburb immediately south of Atlanta, Georgia. PRCW is one of several constructed wetland complexes built and managed by the

Clayton County Water Authority (CCWA). The PRCW was ideal for this research because it provides three replicate systems that function independently once treated wastewater is discharged into them. Secondarily treated wastewater from the Shoal Creek Water Reclamation Facility is discharged into PRCW through a distribution box that divides the flow into three parallel systems: North, Central, and South (Figure 2.1). Once discharged into each system water is not exchanged among systems. The systems flow from east to west along an elevation drop of approximately 30 m before discharging into the Flint River floodplain. Each system has 6-8 individual wetland "cells" set up in sequence so that treated wastewater flows through the input pipe into Cell 1, then Cell 2, and on until the last cell of each system. Each cell in PRCW has alternating shallow/deep zones planted with species of emergent aquatic vegetation including pickerelweed (*Pontederia cordata*), arrowhead (*Sagittaria lancifolia*), cattail (*Typha latifolia*), four species of bulrush (*Scirpus* spp.), and cutgrass (*Zizaniopsis miliacea*). The deep zones in each cell contain submerged aquatic vegetation such as coontail (*Ceratophyllum demersum*), bushy pondweed (*Najas guadalupensis*), water-thread pondweed (*Potamogeton diversifolius*), and fragrant white water lily (*Nymphaea odorata*). For each cell, planting pattern of emergent vegetation was based on the direction of flow between each deep zone. Water from all systems empty into a 400-acre reservoir, which then releases water south, either into the Flint River or into the reservoir of a water production plant.

For comparison purposes, I sampled four reference ponds in Athens-Clarke County, Georgia, about 70 miles east of Clayton County; three in the Whitehall Experimental Forest, a fourth near the University of Georgia Golf Course. All reference ponds were also artificial impoundments that receive inputs of water from primary streams and were known to contain high densities of larval bullfrogs.

Larval Sampling

Between January and July 2006, I conducted repeated samples of tadpoles from cells 1, 3, and 5 in each of the three PRCW systems. Additional samples from reference ponds were collected in June and July 2006. On each occasion, I performed dipnet sweeps for 21 minutes in each cell. Tadpoles were killed by emersion in 1% tricaine methanesulfonate, MS-222, and preserved in 10% neutral buffered formalin. In the lab, I weighed each animal, determined the Gosner (1960) stage, and took dorsal and lateral photos of each tadpole using a Canon Power Shot G6 digital camera mounted onto a stage. I used Program Image J to measure body length of each tadpole from the tip of the snout to the mid-insertion point on the tail, which was typically in vertical alignment with the vent (Pechmann et al., 2001; Van Buskirk and McCollum, 2000).

Because the relationship between Gosner stage and mass is unimodal for tadpoles and declines significantly between metamorphic Gosner stages 40 and 46, I could not use mass as my measure of projected metamorph size. However, mass at metamorphosis is positively correlated with body length at metamorphosis, and unlike mass, tadpole body length remains relatively unchanged beyond Gosner stages 38-39 (Maerz, unpublished data; Ruiz, unpublished data in Figure 2.2; D. Skelly, personal communication). Therefore, I used body length for all tadpoles of Gosner stage 39 or greater as my measure of size at metamorphosis.

Statistical Analysis

I used a nested ANOVA to test the hypotheses that bullfrog size at metamorphosis was larger at PRCW compared to reference ponds and that size at metamorphosis would be greater within PRCW cells closer to the wastewater discharge point. To test the hypotheses that tadpole growth would be greater in within PRCW cells closer to the wastewater discharge point, I calculated the mean Gosner stage of tadpoles for each cell on each date and then used a repeated measures ANOVA with cell (1, 3 or 5) as a between groups factor. All statistical analyses were calculated using STATISTICA, Version 6.0 (StatSoft, Inc., 2003).

Results

Of the 1661 tadpoles I collected from PRCW and reference ponds, 1023 were bullfrog tadpoles. Cell 3 in the Central system was drained for maintenance shortly after sampling started, so this system was excluded from our analysis of development rates. Also, because no bullfrog metamorphs were collected from Cell 1 of the Central system, data on metamorphic body size was only available for cells 3 and 5.

Mean metamorphic body length of bullfrogs was larger at PRCW compared to reference ponds (MS= 296.200, $F_{1, 147}$=13.402, P<0.001; Figure 2.3), and varied significantly among cells within the PRCW complex (MS= 141.400, $F_{10, 147}$=6.397, P<0.001; Figure 2.3). There were no significant differences in metamorphic body length between any reference ponds (Figure 2.3). Contrary to my hypothesis, the variation in metamorph body size with PRCW was not related to proximity to the discharge point. That is, metamorph size was not greater among 1st cells compared to 3rd or 5th cells (Figure 2.3). Instead, most of the variation in body size was attributable to the different systems of cells within PRCW. Metamorph size tended to be smaller in cells from the North systems, intermediate in the Central system, and largest in the South system (Figure 2.3). It was the South system that accounted for the larger size of PRCW metamorphs compared to reference ponds. Metamorph sizes from the North and Central systems were similar to those observed in the reference ponds (Figure 2.3).

Within North and South PRCW systems, the proximity of cells to the treated wastewater discharge point had a significant effect on tadpole development rates, though the pattern was opposite than what we had hypothesized (*MS*= 23.850, $F_{2,20}$=4.107, *P*= 0.032; Table 2.1, Figure 2.4). Mean Gosner stage increased with sampling date in all cells (*MS*=322.093, $F_{1,10}$=33.681, *P*<0.001), but increased more rapidly in the 5th cells, which were most distant from the discharge point, than in the 1st cells that directly received the treated wastewater (Cell X Date Interaction: *MS*=23.921, $F_{2, 20}$=4.119, *P*=0.032). By mid June, most tadpoles in the 5th cells were metamorphic (Gosner stage > 39), and most tadpoles were metamorphic in the 3rd cells by early July. By contrast, few tadpoles from first cells were metamorphic by late July (Figure 2.4).

Discussion

Size at metamorphosis and larval development rate are important determinants of amphibian fitness and population viability, and therefore can be used to compare the quality of larval environments (Semlitsch et al., 1988). This is the first study to measure such performance metrics for amphibians inhabiting wastewater treatment wetlands. My study shows that bullfrog size at metamorphosis is variable among wastewater treatment wetlands, but appears to be as large, or larger, than bullfrogs from reference ponds. The potential for larger-sized metamorphs in treatment wetlands may be a product of higher nutrient inputs to those systems, although we did not see evidence of a relationship between nutrient levels and metamorph size among cells within the treatment wetland complex. That is, metamorphs in the 1st cells, which receive direct inputs of treated wastewater, and have the highest nutrient availability, were not larger than individuals from cells farther from the discharge point with lower nutrient levels.

Though metamorph size did not vary as a function of distance from the wastewater discharge, tadpole development did. More specifically, tadpole development was slowest in 1st cells despite higher nutrient levels. The Wilbur-Collins model of amphibian metamorphosis predicts that high food environments should favor slower development in favor of a longer growth period and ultimately a larger size at metamorphosis (Alford and Harris, 1988). Despite widespread acceptance of this model, empirical support for facultative shifts in growth and development in response to food availability remain limited (Alford and Harris, 1988); in fact, development rate and size at metamorphosis are often positively correlated among environments. Because metamorph body size was not larger in first cells where development was slower, I do not think the delayed development was an adaptive response to increased resources. Instead, I hypothesize that more proximate exposure to discharged wastewater retarded the development of bullfrog tadpoles. In addition to nutrients, treated wastewater can contain high concentrations of contaminants including pharmaceutical and industrial by-products that affect aquatic organisms (Gros et al., 2007). If true, then this research suggests that proximate exposure to discharged wastewater may have deleterious effects on larval amphibians.

On the whole, the PRCW appears to provide habitat capable of supporting tadpole development and size at metamorphosis consistent with other types of wetlands. In this regard, the PRCW could be considered beneficial wildlife habitat. However, within the PRCW, tadpole development in cells that receive treated wastewater directly suggests that proximate exposure to treated wastewater has a negative effect on tadpoles. Intermediate development rates in the 3rd cells suggest that this negative effect persists, but is diminishing as wastewater moves through the wetland complex. These results are potentially important for a number of reasons. First, several constructed wetland complexes often discharge treated wastewater directly into all cells rather than into a single cell, allowing the water to move linearly through a system of wetlands before it leaves the site. My results suggest that this practice would reduce the value of these constructed wetlands for amphibians. Second, a number of water authorities discharge treated wastewater into natural rather than constructed wetlands. This might degrade natural wetlands as wildlife habitat. Use of natural wetlands should be discouraged because of their current conservation value (Verhoeven and Meuleman, 1999). Finally, some constructed wetlands, including PRCW, currently do not operate at capacity (PRWC currently operates at ~ 45%). Increasing treated wastewater loads to these facilities might extend any negative effects of exposure to treated wastewater farther along the wetland complex, ultimately reducing its potential value as amphibian habitat.

The potential for constructed wetlands to solve some of the water resource problems facing urban communities is great, but the portrayal of these constructed wetlands as dually beneficial to wildlife may be premature and is certainly in need of more critical investigation (Knight et al., 2001). Although, if these wastewater treatment wetlands had not been constructed by the Clayton County Water Authority, this area of land may not serve as available wildlife habitat in an urban community. To be fair, the primary function of these wetlands is to clean wastewater to prevent eutrophication or for human reuse, so the decision to construct wetlands may rest with this function alone. However, we should be cautious in statements regarding the value of these wetlands for wildlife and the potential for them to do inadvertent harm. My study is only the second I am aware of to investigate the potential effects of treated wastewater on amphibians, and both studies have found some evidence of potential harm. Amphibians and other wildlife that feed within these wetlands including birds, reptiles, and mammals may also be exposed to various contaminants. Such possibilities need greater attention as the use of constructed wetlands increases.

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Table 2.1. Summary of repeated measures analysis of variance of tadpole development at the Clayton County Water Authority, Panhandle Road Constructed Wetlands in 2006.

Variable	*df*	*MS*	*F*	*P*
Cell location	2	23.850	4.107	0.032
Date	1	322.093	33.681	<0.001
Cell location x Date	2	23.921	4.119	0.032

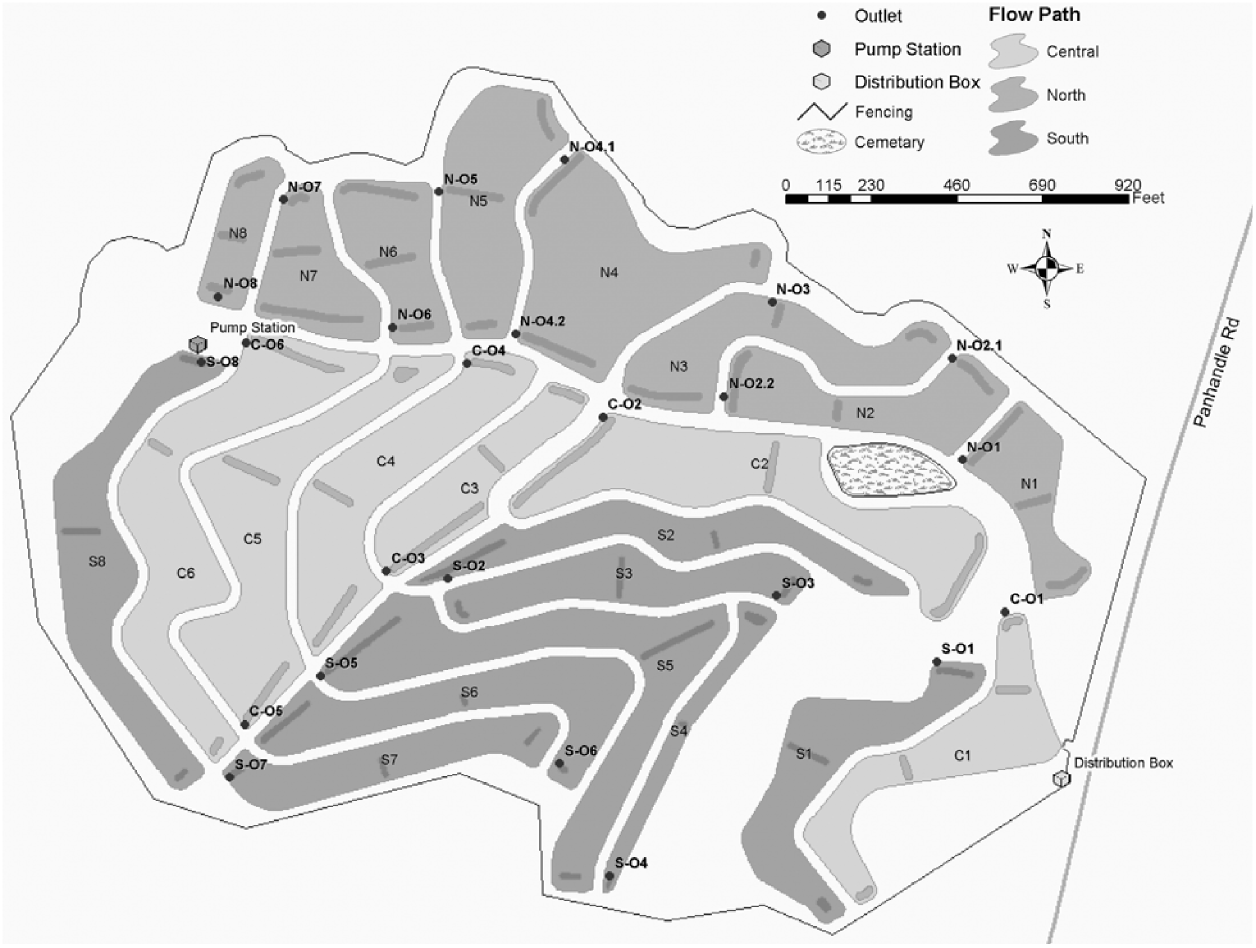

Figure 2.1. Map of the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Includes three separate systems (N-north, C-central and S-south) and their associated cells (number of cell refers to its sequence of water; water enters N1, flows to N2, flows to N3, etc.).

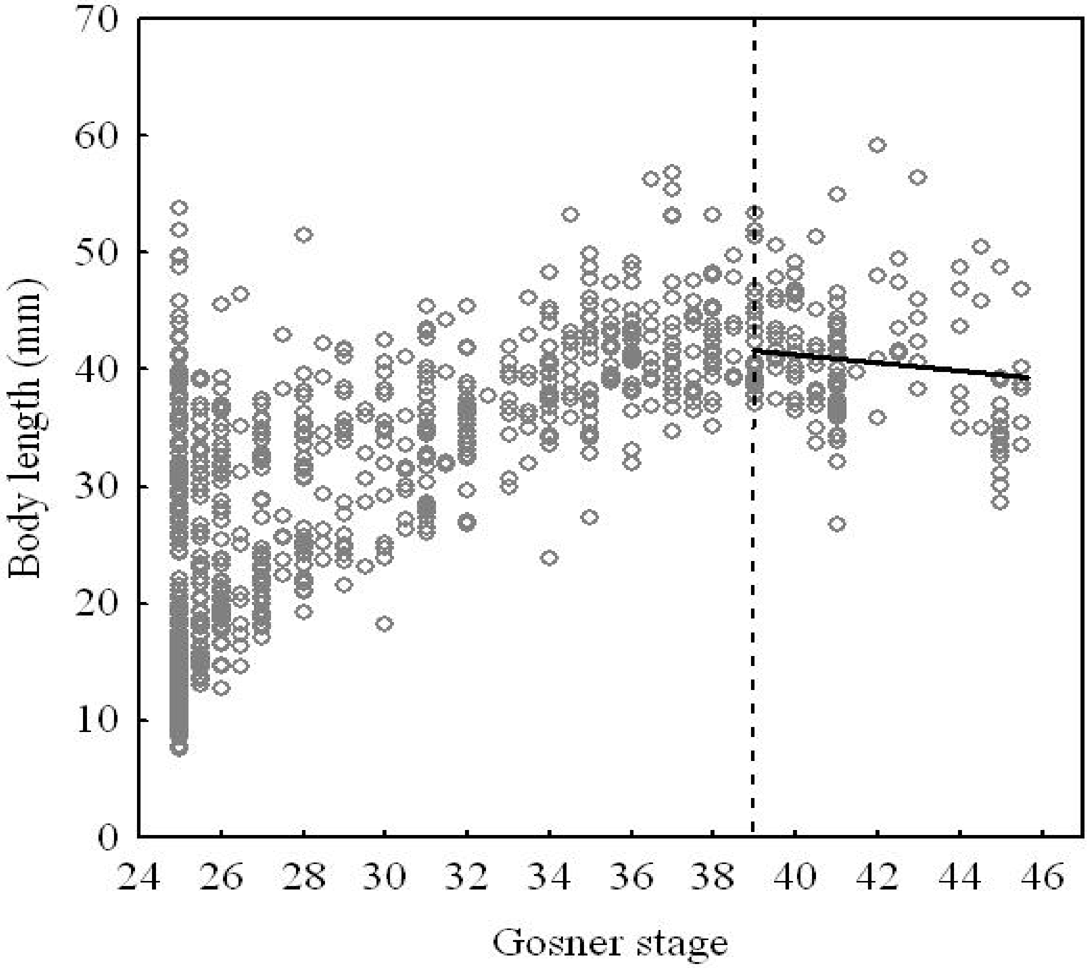

Figure 2.2. Body lengths at various Gosner stages of *Rana catesbeiana* from the Clayton County Water Authority, Panhandle Road Constructed Wetlands and four reference ponds in 2006. Solid line is the linear regression of body length on Gosner stage for tadpoles ≥ stage 39. Vertical dashed line indicates stage 39. There is no significant change in body length between Gosner stages 39 and 46 (<1 mm).

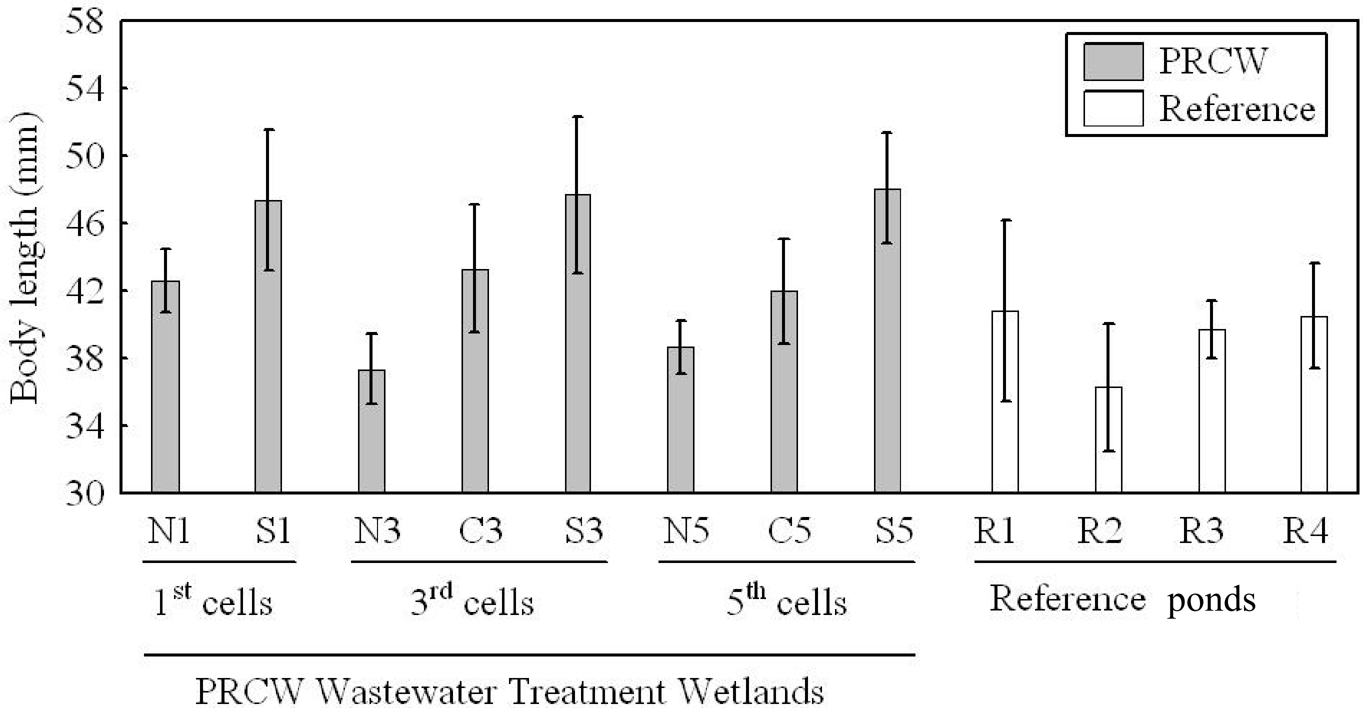

Figure 2.3. Mean body lengths of *Rana catesbeiana*, Gosner stages ≥ 39, from the Clayton County Water Authority, Panhandle Road Constructed Wetlands and four reference ponds in 2006. No bullfrog metamorphs were captured in Cell C1.

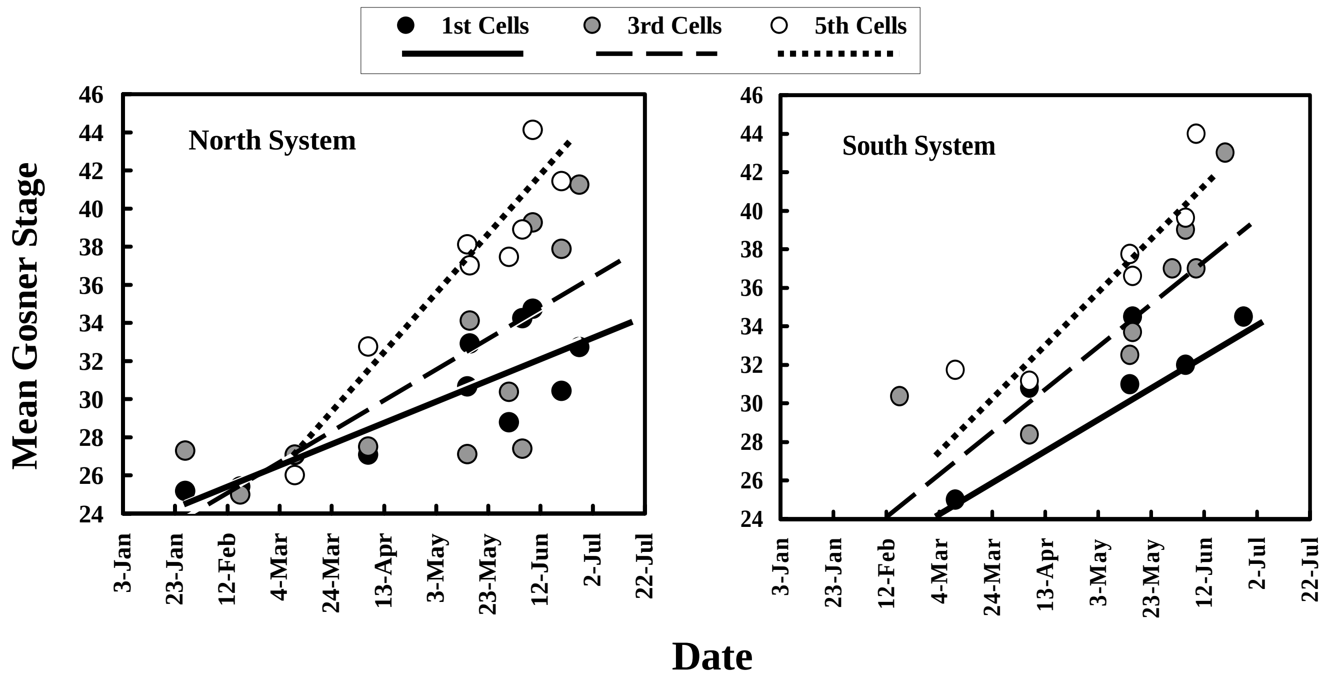

Figure 2.4. Mean Gosner stage through time of the North and South systems of the Clayton County Water Authority, Panhandle Road Constructed Wetlands in 2006. Central system excluded due to incomplete data.

CHAPTER 3

OCCURRENCE OF A NOVEL CALCIUM DISORDER IN LARVAL AMPHIBIANS FOUND IN WASTEWATER TREATMENT WETLANDS

Introduction

Concern over worldwide declines in amphibian populations has been growing since the late 1980s. Reasons for these declines are attributed to habitat loss, introduced predators and competitors, fungal disease, parasites, ultra-violet radiation, and contaminants (Ankley et al., 2002; Johnson et al., 2002; Sparling et al., 2000; Tietge et al., 2001). Because amphibians have highly permeable skin, acute and subacute effects of water-borne pollutants have received particular attention from researchers as a factor contributing to amphibian population declines. For example, sublethal concentrations of toxins may indirectly cause death by increasing susceptibility of eggs and larvae to pathogenic organisms and disease, or by inhibiting the ability of larvae to avoid predators (Blaustein et al., 1994; Carey and Bryant, 1995; Hopkins et al., 2000). Toxins may also have thyroid-disrupting, androgenic, or estrogenic properties that can impair or inhibit future reproduction by disrupting developmental processes (Carey and Bryant, 1995; Coady et al., 2005; Goulet and Hontela, 2003; Hayes et al., 2003; Hayes et al., 2002; Howe et al., 2004).

Eggs and newly hatched larvae are the amphibian life stages most sensitive to environmental contaminants (Bishop et al., 1999), and the aquatic habitats where amphibian eggs and larvae develop are impacted by a wide variety of pollutants. Table 3.1 provides an overview of research into abnormal growth and development patterns in amphibians and their links to aquatic pollution sources. The majority of studies focus on agricultural pollutants. These studies demonstrate that high nitrate concentrations associated with fertilizer runoff can inhibit hatching success and tadpole survivorship (Hecnar, 1995), and pesticide residues can cause higher rates of larval mortality, axial deformities, limb deformities, and endocrine disruption (Alvarez et al., 1995; Cooke, 1981; Hayes et al., 2002; Kiesecker, 2002; MacKenzie et al., 2003; Ouellet et al., 1997; Taylor et al., 2005). There are far fewer studies of aquatic pollutants outside of agricultural settings (Table 3.1); however, runoff from coal-burning power plants, road-salting practices, and coal-tar based pavement sealers used in parking lots have all been linked to reduced performance or higher rates of abnormalities in larval amphibians (Bryer et al., 2006; Hopkins et al., 2000; Rowe et al., 1996; Sanzo and Hecnar, 2006). Missing from the literature are any reports of amphibian abnormalities linked to "residential" pollutants in urban and suburban habitats.

In urban areas, constructed wetlands can be important amphibian habitat. Constructed wetlands are used to control storm water runoff in residential areas, and are used increasingly as a tertiary treatment for treated wastewater. Larvae in urban constructed wetlands may be exposed to pollutants from road and lawn runoff, and, in the case of wastewater treatment wetlands, may be exposed to a variety of contaminants, including pharmaceuticals and household cleaning products (Gros et al., 2007). Data on the types and rates of larval amphibian abnormalities in urban constructed wetlands is very limited. Recent studies show that trace levels of pharmaceuticals from wastewaters cause endocrine disruptions of fishes (Metcalfe et al., 2001; Mimeault et al., 2005; Nash et al., 2004), and recent laboratory studies show that compounds in common household goods can affect tadpole development (Veldhoen et al., 2006).

Finally, in the previous chapter, I presented data indicating that proximate exposure to treated wastewater discharged into constructed wetlands is retarding the development of bullfrog tadpoles. Data on other types of abnormal larval development in constructed wetlands is lacking.

The purpose of this paper is to report on the frequencies of visible external abnormalities in tadpoles colleted from a network of constructed wetlands in Clayton County, Georgia. In particular, a novel hypercalcemic disorder that accounted for the overwhelming majority of observed abnormalities. Symptoms of hypercalcification included extensive soft tissue mineralization and the formation of large calcium nodules in tail musculature and around mouthparts. This "hypercalcemic" disorder has never been reported in the literature, although qualitatively similar epithelial nodules, some characterized as papillomas, were observed in the Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*) (Hiler et al., 2005). Within the constructed wetland complex, the frequency of visibly hypercalcemic tadpoles declined with increasing distance from the treated wastewater discharge point. Bullfrog tadpoles, which have the longest larval periods of any species observed at the site, also accounted for nearly all tadpoles with visible signs of hypercalcification.

Materials and Methods

Study site

My study took place within the Panhandle Road Constructed Wetlands (PRCW) in the southern portion of Clayton County, a large residential suburb immediately south of Atlanta, Georgia. PRCW is one of several constructed wetland complexes built and managed by the Clayton County Water Authority (CCWA). The PRCW was ideal for this research because it provides three replicate systems that function independently once treated wastewater is discharged into them. Secondarily treated wastewater from the Shoal Creek Water Reclamation Facility is discharged into PRCW through a distribution box that divides the flow into three parallel systems: North, Central, and South (Figure 3.1). Once discharged into each system water is not exchanged among systems. The systems flow from east to west along an elevation drop of approximately 30 m before discharging into the Flint River floodplain. Each system has 6-8 individual wetland "cells" set up in sequence so that treated wastewater flows through the input pipe into Cell 1, then Cell 2, and on until the last cell of each system. Each cell in PRCW has alternating shallow/deep zones planted with species of emergent aquatic vegetation including pickerelweed (*Pontederia cordata*), arrowhead (*Sagittaria lancifolia*), cattail (*Typha latifolia*), four species of bulrush (*Scirpus* spp.), and cutgrass (*Zizaniopsis miliacea*). The deep zones in each cell contain submerged aquatic vegetation such as coontail (*Ceratophyllum demersum*), bushy pondweed (*Najas guadalupensis*), water-thread pondweed (*Potamogeton diversifolius*), and fragrant white water lily (*Nymphaea odorata*). For each cell, planting pattern of emergent vegetation was based on the direction of flow between each deep zone. Water from all systems empty into a 400-acre reservoir, which then releases water south, either into the Flint River or into the reservoir of a water production plant.

For comparison purposes, I sampled four reference ponds in Athens-Clarke County, Georgia, about 70 miles east of Clayton County; three in the Whitehall Experimental Forest, a fourth near the University of Georgia Golf Course. All reference ponds were also artificial impoundments that receive inputs of water from primary streams.

Larval Sampling

Between January and July 2006, I conducted repeated samples of tadpoles from cells 1, 3, and 5 in each of the three PRCW systems. Additional samples from reference ponds were collected in June and July 2006. On each occasion, I performed dipnet sweeps for 21 minutes in each cell. Tadpoles were killed by emersion in 1% tricaine methanesulfonate, MS-222, and preserved in 10% neutral buffered formalin. In the lab, I weighed each animal, determined the Gosner (1960) stage and species, and took dorsal and lateral photos of each tadpole using a Canon Power Shot G6 digital camera mounted onto a stage. Detailed descriptions of visible external abnormalities were also documented.

Diagnosis of Abnormality

A set of tadpoles with and without tail and gular region mineralized nodules were sent to the University of Georgia's Southeastern Cooperative Wildlife Disease Study for evaluation. Histopathology reports investigated the potential cause(s) of the mineralization and the presence of a parasite or infectious disease. A microscopic examination of tadpole tissues was performed to determine the extent of tissue mineralization and to look for signs of distrophic calcification (calcification caused by disruptive damage to tissues from injury). Crystallography of mineralized nodules was performed by the Gerald V. Ling Urinary Stone Analysis Laboratory at the University of California.

To further evaluate potential sources of the hypercalcemic disorder, the vitamin D levels of blood serum in tadpoles and water samples from PRCW were analyzed by the toxicology lab at Michigan State University. Serum and water were analyzed for $25(OH)D_3$ and $1,25(OH)_2D_3$ by radioimmunoassay. Specificity data, as reported by the manufacturer, indicated 0.8% cross-reactivity with vitamin D_3 and D_2 and 2.5% cross-reactivity with the 1,25-diOH metabolites of those forms of vitamin D. The assay has 100% cross-reactivity with 25-OH, 24,25-diOH, and 25,26-diOH metabolites of vitamin D_3 and D_2. The sensitivity of the assay was 5 nmol/liter, defined as the average calculated concentration at 90% of total binding for 10 assay runs (Rumbeiha et al., 2000). Lastly, calcium concentrations of blood serum and water samples were determined using a* *Roche BMC Hitachi 912 automated chemistry analyzer in the Clinical Pathology Lab at the University of Georgia College of Veterinary Medicine.

Results

Of the 1661 tadpoles collected from PRCW and reference ponds, 199 showed some form of visible abnormality. Of the abnormal specimens, 192 (97%) were captured at PRCW; 7 specimens with visible abnormalities were captured at the reference ponds. Among all the specimens with visible abnormalities, 185 (93%) were American bullfrog, *Rana catesbeiana*. External abnormalities observed at the reference ponds included tail and limb malformations and ventral discoloration. External abnormalities observed at PRCW included lateral curvatures of the spine, malformed and extra limbs, missing eyes, edema (fluid retention), ventral discoloration, and nodules of calcium salts on the tail musculature and gular region (Table 3.2; Figure 3.3 abc). Of the 192 visibly abnormal tadpoles from PRCW, 82 (43%) were afflicted with multiple abnormalities, and 111 (58%) had externally visible hypercalcemic nodules. Of tadpoles with hypercalcemic symptoms, 98% were American bullfrog; the remaining 2% were Southern leopard frog, *Rana sphenocephala*, tadpoles. All (100%) hypercalcemic tadpoles were collected from the PRCW. I did not observe any tadpole from a reference wetland with visible signs of hypercalcification. Within PRCW, the frequency of bullfrog tadpoles with visible hypercalcemic abnormalities was highest (range 12%-52%) among the three 1st cells that received direct discharge of treated wastewater, and then declined dramatically (range 1%-2%) by the 3rd cells (Figure 3.2). I did not observe any tadpoles in the 5th cells with visible external symptoms of hypercalcification. Species collected at PRCW which did not develop externally visible hypercalcemic nodules include green frog, *Rana clamitans,* and green treefrog, *Hyla cinerea*.

Postmortem examinations of 15 bullfrog tadpoles with and without grossly visible nodules were performed at the Southeastern Cooperative Wildlife Disease Study. Tadpoles exhibiting external lesions were similarly affected by variably chalky, mineralized nodules in the soft tissue of the tail or gular region, or, in some instances deforming the caudal vertebrae. Microscopic examinations of tadpoles lacking large exophytic nodules revealed they were suffering internally from the same hypercalcemic disorder, but with less severe lesions. Results indicated that there was no evidence of a parasite or an infectious agent associated with the calcified tissues, though serial sections of the tissues were examined microscopically. Internal and external nodules found in all tadpoles consisted of fragments of variably mineralized material with macrophages, giant cells fibroblasts, and variable numbers of lymphocytes. Inflammatory cells infiltrate smaller aggregates, but form a thin margin of tissue encapsulating the largest fragments. Encased in a similar inflammatory response, were small mineralized aggregates scattered throughout the renal interstitium, liver, pancreas, and stomach. Very small mineralized foci scattered throughout the heart of some tadpoles were associated with minimal or no inflammation. In some areas, individual connective tissue fibers or muscle fibers were affected suggesting some element of metastatic mineralization. Some individual myofibers in the gular region were lightly granular and basophilic (consistent with partial mineralization).

Crystallography showed nodules were 100% apatite [$Ca_5(PO_4)_3(OH, F, Cl)$], this crystalline species is formed from calcium salts. In addition, tadpoles from PRCW tested positive for chytrid fungus.

Further analysis of tissues by the toxicology lab at Michigan State University indicated higher levels of vitamin D in tadpoles with nodules, 6 nmol/L of 25- OH vitamin D_3. Tadpoles from reference ponds did not have detectable levels of vitamin D. The toxicology lab at Michigan State University is currently in the process of evaluating levels of vitamin D in the water samples collected from PRCW and reference ponds. Water concentrations of calcium at PRCW were within ranges observed in reference ponds, and serum calcium levels were similar among all tadpoles submitted from PRCW and reference ponds (K. Keel, SCWDS, UGA, unpublished data).

Discussion

This study shows an alarmingly high frequency of hypercalcification among bullfrog tadpoles proximately exposed to discharged treated wastewater at PRCW. A search of the literature and consultation with a large number of wildlife veterinarians and world authorities on tadpoles indicated that this abnormality has never been reported in natural systems, even ones with high levels of anthropogenic disturbance. Further, we failed to find even a single tadpole with the same disorder during concurrent sampling of reference ponds. Finally, visibly hypercalcemic tadpoles were nearly restricted to the 1st cells within PRCW, which receive direct inputs of treated wastewater, and nearly all afflicted tadpoles were American bullfrog, which have the longest larval periods of all species observed at the site including the other *Rana* species. These results suggest strongly that the cause of the hypercalcification is prolonged exposure to contaminants in treated wastewater.

The 1st cells at PRCW contain the highest amount of nitrogen, phosphorus, BOD, and TSS levels. In addition, these ponds have the warmest temperatures in the winter months (see Chapter 1). Although high nitrate levels are sub-acutely toxic to tadpoles (Rouse et al., 1999), the many studies on nitrogen pollution in agricultural systems have not reported hypercalcemic disorders in tadpoles. Additionally, other studies conducted in agricultural systems report limb deformities in amphibians (Ouellet et al., 1997), but no abnormalities similar to the hypercalcemic disorder found in tadpoles at PRCW. This could indicate that other pollutants not present in agricultural wastewaters may be causing the hypercalcemic disorder found at PRCW. In the previous chapter, tadpole development varied as a function of distance from the wastewater discharge point in a manner consistent with hypercalcemic frequencies; development was slowest in 1st cells despite higher nutrient levels. In addition, we now know from companion research that bullfrog tadpoles in the first cells at PRCW show high levels of immune-related stress and more severe and higher levels of oral deformities than tadpoles from third or fifth cells (Davis et al., *unpublished data*). Collectively, these results demonstrate significant developmental stress on tadpoles in wetland cells receiving direct inputs of treated wastewater, and we believe that contaminants in treated wastewater affect aquatic organisms present in these systems.

Lab results indicate that the afflicted tadpoles have elevated vitamin D levels and possess nodules that are 100% apatite, leading us to believe that the tadpoles have somehow shifted physiologically to allow increased absorption of calcium. Because calcium levels in the water were reported within ranges of natural ponds, we suspect that a pollutant in the water has caused a physiological disorder, likely endocrine related, leading to the abnormal calcium accumulation and retention. Calcium regulation in a tadpole is mediated by the parathyroid hormone and calcitonin, a hormone produced by the thyroid gland. These hormones could be influenced by high levels of human hormones (e.g., estrogen) or hormone mimics in wastewater (Snyder et al., 2003). As amphibians pass through critical hormone-regulated developmental stages in their aquatic environment, habitats contaminated with endocrine-disrupting chemicals may have significant effects on individuals and populations (Hayes et al., 2006).

Whether hypercalcification in these tadpoles is caused by endocrine disruptors, contaminants, excess nutrients and temperatures, or a combination of these factors, the usual outcome of tadpoles afflicted with hypercalcification is fatal. Large calcified nodules in tails resulted in mortality while small calcified nodules in tails were lost at metamorphosis, but other calcified tissues such as in the mouth were retained among newly metamorphosed frogs (Figure 3.3a). As of now, we are uncertain as to whether retained nodules have possible sublethal clinical effects, or if metamorphs suffer from reduced fitness due to these nodules. All specimens kept for observation that retained calcified nodules died shortly after metamorphosis. Nodules in gular regions may limit effective feeding or breathing (because anurans rely on a bucco-pharyngeal pump). Further, tadpoles that exhibited gross hypercalcification also showed more subtle damage including mineralization of the heart musculature that we assume these animals will carry beyond metamorphosis. Calcification of heart tissues would most certainly negatively affect heart performance and ultimately frog fitness.

Because descriptions of these abnormalities are likely to raise alarm among the public, it is responsible to reinforce some of the results and the larger context of this study. Although wastewater wetlands may also provide potentially valuable wetland habitat for wildlife (Knight et al., 2001), the purpose of such wetlands is to clean water for public reuse. In addition to declines in nutrient loads as water passes from the initial to tertiary wetland cells (see Chapter 1), it is clear that whatever abnormalities tadpoles exhibited within the PRCW decline as water moves through the wetlands complex. That is, the wetlands are removing whatever substances are responsible for tadpole abnormalities before the water is discharged into the river or reservoirs for uptake and reuse. So our results, however horrifying, suggest the wetlands are functioning as intended.

The presumed wildlife value of constructed wetlands or the discharge of waste into natural wetlands is well-publicized. Superficially, these wetlands are attractive to wildlife because they offer abundant water and growing plants, components which provide the basis for an ecological food web (Knight, 1997). It is known that wastewater treatment plants serve as a pathway for aquatic contamination by pharmaceuticals (Gros et al., 2007), but the side effects on wildlife inhabiting these systems is only recently being investigated (Knight et al., 2001). For example, Fraker and Smith (2004) conducted a study examining the effects of common organic wastewater contaminants, such as caffeine, acetaminophen, and triclosan, on *Rana pipiens* tadpoles. Results show that tadpoles exposed to triclosan were less active than controls, likely affecting foraging efficiency and predator avoidance ability (Fraker and Smith, 2004). These studies indicate that we should be cautious in promoting the value of constructed wetlands for amphibians or other wildlife species.

This study documents a range of abnormalities found in the Clayton County Water Authority, Panhandle Road Constructed Wetlands. The majority of abnormalities found at PRCW have been previously documented in scientific literature. However, the bulk of the tadpoles that exhibited abnormalities were afflicted with a novel hypercalcemic disorder.

Therefore, this study indicates that constructed wetlands (and potentially natural wetlands) that receive direct discharge of treated wastewater may be harmful to some wildlife. Although, if these wastewater treatment wetlands had not been constructed by the Clayton County Water Authority, this area of land may not serve as available wildlife habitat in an urban community.

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Table 3.1. Summary of amphibian abnormalities published in scientific literature.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Altered behavior	Laboratory	*Xenopus laevis* *Bufo marinus* 4 species of Australian frogs	Tadpoles exposed to nonylphenol ethoxylate and alcohol alkoxylate in static-renewal acute toxicity tests showed reduced activity. These chemicals are nonionic surfactants that are frequently incorporated into pesticides formulations.	Mann and Bidwell 2001
Altered behavior	Laboratory	*Rana sylvatica*	Road salt (NaCl) had a toxic effect on tadpoles in both acute and chronic tests at environmentally realistic concentrations. Water used in experiments was taken from roadside ditches and semi-permanent farm and forest ponds. Results show that tadpoles had decreased survivorship, weight, and activity; they metamorphosed earlier and had increased developmental abnormalities as salt concentration increased.	Sanzo and Hecnar 2006
Altered growth and development	Laboratory	*Xenopus laevis*	Individual tadpoles were placed in containers of low, medium, and high concentrations of polycyclic aromatic hydrocarbons. PAHs are found in coal-tar based pavement sealers and are used on parking lots. Individuals in high concentrations died by day 6 and individuals in medium concentrations showed patterns of stunted growth and slower development compared to those in low concentrations.	Bryer et al. 2006
Altered growth and development	Laboratory	*Rana cascadae*	Wild caught tadpoles (Gosner stage 38-40) were exposed to sublethal concentrations of nitrite in the laboratory. Results show they developed slower and emerged at earlier developmental stages than control tadpoles.	Marco and Blaustein 1999
Altered growth and development	Laboratory	*Rana sylvatica*	Road salt (NaCl) had a toxic effect on tadpoles in both acute and chronic tests at environmentally realistic concentrations. Water used in experiments was taken from roadside ditches and semi-permanent farm and forest ponds. Results show that tadpoles had decreased survivorship, weight, and activity; they metamorphosed earlier and had increased developmental abnormalities as salt concentration increased.	Sanzo and Hecnar 2006
Axial deformities and lordosis	Agricultural sites	*Bufo americanus* *Rana pipiens* *Rana clamitans*	Elevated trace concentrations of organophosphorus pesticides were found downstream from a vegetable growing area. Tadpoles were also exposed to high levels of ammonia, phosphorus, particulates, BOD and TKN.	Bishop et al. 1999
Axial deformities and lordosis	Agricultural sites	*Rana temporaria*	Tadpoles in cages beside potato fields were exposed application of oxamyl, a carbamate nematicide and insecticide. Tadpoles showed vertical curvature and tail tip defects.	Cooke 1981

Table 3.1. Continued.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Axial deformities and lordosis	Coal-burning power plant	*Rana catesbeiana*	Sites contaminated with coal combustion wastes exhibited lateral curvatures of the spine in tadpoles. High tissue concentrations of potentially toxic trace elements including As, Cd, Se, Cu, and V were also measured.	Hopkins et al. 2000
Axial deformities and lordosis	Laboratory	*Rana perezi*	Tadpoles were kept for 14 weeks in water containing two sublethal levels of the carbamate insecticide ZZ-Aphox (R) or the organophosphate Folidol (R). The pesticides caused the animals to have malformations of the spinal column (scoliosis) and/or limbs (short and thick long bones with the epiphyses grossly twisted).	Alvarez et al. 1995
Axial deformities and lordosis	Laboratory	*Xenopus laevis*	The toxicity of bio-available Zn, Cu, Pb, and Cd on the life stages of embryos and tadpoles was investigated. Malformations were observed, along with a strong negative relationship between the increase in Cu concentrations and the hatching of the embryos.	Haywood et al. 2004
Axial deformities and lordosis	Laboratory	*Rana sylvatica*	Road salt (NaCl) had a toxic effect on tadpoles in both acute and chronic tests at environmentally realistic concentrations. Water used in experiments was taken from roadside ditches and semi-permanent farm and forest ponds. Results show that tadpoles had decreased survivorship, weight, and activity; they metamorphosed earlier and had increased developmental abnormalities as salt concentration increased.	Sanzo and Hecnar 2006
Axial deformities and lordosis	Outdoor mesocosms	*Hyla chrysoscelis*	Agrichemicals (atrazine, chlorpyrifos, and MSMA) and methyl mercury were added to mesocosms. Results show tadpoles and metamorphs with leg malformations, lordosis (bent tail), and missing eyes.	Britson and Threlkeld 1998
Edema	Agricultural sites	*Bufo americanus* *Rana pipiens* *Rana clamitans*	Elevated trace concentrations of organophosphorus pesticides were found downstream from a vegetable growing area. Tadpoles were also exposed to high levels of ammonia, phosphorus, particulates, BOD and TKN.	Bishop et al. 1999
Edema	Laboratory	*Xenopus laevis*	The toxicity of bio-available Zn, Cu, Pb, and Cd on the life stages of embryos and tadpoles was investigated. Malformations were observed, along with a strong negative relationship between the increase in Cu concentrations and the hatching of the embryos.	Haywood et al. 2004

Table 3.1. Continued.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Endocrine disruption	Laboratory	*Xenopus laevis* *Rana temporaria*	Larvae were exposed to the effluent of a regional sewage treatment plant. Exposure was carried out in triplicate per treatment (river water as a reference, mixtures of 1:2 and 1:12 sewage/river water, respectively) using 50-L glass aquaria. Results show exposure to sewage treatment work effluents disrupts the endocrine system under semifield conditions.	Bogi et al. 2003
Internal organ abnormalities	Agricultural sites	*Bufo americanus* *Rana pipiens* *Rana clamitans*	Elevated trace concentrations of organophosphorus pesticides were found downstream from a vegetable growing area. Tadpoles were also exposed to high levels of ammonia, phosphorus, particulates, BOD and TKN.	Bishop et al. 1999
Internal organ abnormalities	Laboratory	*Xenopus laevis*	Water samples were collected from wetlands that contained malformed frogs. Laboratory results indicated that water from the sites induced gut malformations and reduced embryo lengths in exposed larvae. The developmental delay observed was alleviated by supplementation with sodium and potassium.	Garber et al. 2004
Internal organ abnormalities	Laboratory	*Xenopus laevis*	Tadpoles were exposed to atrazine throughout larval development. Results showed that atrazine induced hermaphroditism and demasculinized the larynges of exposed males.	Hayes et al. 2002
Internal organ abnormalities	Laboratory	*Xenopus laevis*	Gonadal differentiation was observed in tadpoles exposed to estrogenic and antiestrogenic compounds. Results indicate that amphibians could be susceptible to altered gonadal differentiation when exposed to these compounds in aquatic environments impacted by agricultural, industrial, and municipal runoff.	MacKenzie et al. 2003
Internal organ abnormalities	Laboratory	*Xenopus laevis*	Tadpoles were exposed to polychlorinated biphenyls (PCB) from stage 46/47 to the completion of metamorphosis. Rate of forelimb malformations caused by PCBs was > 70%. Also, testes from more than a third of male frogs exposed to PCBs exhibited feminization to different degrees.	Qin et al. 2005
Lateral compression	Agricultural sites	*Bufo americanus* *Rana pipiens* *Rana clamitans*	Elevated trace concentrations of organophosphorus pesticides were found downstream from a vegetable growing area. Tadpoles were also exposed to high levels of ammonia, phosphorus, particulates, BOD and TKN.	Bishop et al. 1999

Table 3.1. Continued.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Missing and deformed limbs	Agricultural sites	*Rana clamitans* *Rana catesbeiana* *Rana pipiens* *Bufo americanus*	Of the 853 wild-caught metamorphs exposed to pesticide runoff, 106 exhibited varying degrees of ectromelia and ectrodactyly.	Ouellet et al. 1997
Missing and deformed limbs	Agricultural sites	*Hyla versicolor* *Pseudacris crucifer* *Rana pipiens* *Rana catesbeiana* *Rana clamitans* *Rana sylvatica*	Ponds along an urbanization gradient were sampled. Proximity to agricultural land was associated with more than doubling the risk of missing or malformed limbs in tadpoles. Agricultural runoff contained animal waste, pesticides, and fertilizers.	Taylor et al. 2005
Missing and deformed limbs	Agricultural Ponds and Laboratory	*Rana sylvatica*	Results of field and laboratory experiments link increased trematode infection and increased limb deformities to pesticide exposure. Deformities were more common at sites adjacent to agricultural runoff.	Kiesecker 2002
Missing and deformed limbs	Natural ponds and wetlands	Many ranids	Trematode parasite (*Ribeiroia ondatrae*) was found concentrated around the tissue of hind limbs in infected anurans. Malformations associated with infection included skin webbings, supernumerary limbs and digits, and missing or malformed hind limbs. The aquatic snail hosts (*Planorbella* spp.) were significant predictors of the presence and abundance of *Ribeiroia* infection.	Johnson et al. 2002
Missing and deformed limbs	Laboratory	*Rana perezi*	Tadpoles were kept for 14 weeks in water containing two sublethal levels of the carbamate insecticide ZZ-Aphox (R) or the organophosphate Folidol (R). The pesticides caused the animals to have malformations of the spinal column (scoliosis) and/or limbs (short and thick long bones with the epiphyses grossly twisted).	Alvarez et al. 1995
Missing and deformed limbs	Laboratory	*Rana pipiens*	Larvae were exposed from early embryonic stages through complete metamorphosis to varying natural sunlight regimes. Full sunlight caused approximately 50% mortality during early larval development. Exposure to sunlight also resulted in increased eye and limb malformations (missing or truncated digits). Filtration of sunlight with either glass or acrylamide both significantly reduced the incidence of malformed limbs.	Ankley et al. 2002

Table 3.1. Continued.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Missing and deformed limbs	Laboratory	*Xenopus laevis*	Tadpoles were exposed to polychlorinated biphenyls (PCB) from stage 46/47 to the completion of metamorphosis. Rate of forelimb malformations caused by PCBs was > 70%. Also, testes from more than a third of male frogs exposed to PCBs exhibited feminization to different degrees.	Qin et al. 2005
Missing and deformed limbs	Natural ponds and wetlands	*Rana septentrionalis*	After collecting frogs in the field, the authors concluded that the spectrum of abnormalities documented was remarkably similar to the range of abnormalities that has been reported as a result of exposure of developing vertebrates to exogenous retinoids. The authors also described a newly recognized malformation of the proximal-distal limb axis, a bony triangle.	Gardiner and Hoppe 1999
Missing and deformed limbs	Natural ponds and wetlands	Many ranids	Data shows that the recent increase in amphibian malformations results from a complex set of interactions among a parasite that causes malformations (*Ribeiroia*), the parasite's intermediate host (planorbid snails) and anthropogenic changes to the food web within which the snails are embedded (cultural eutrophication).	Johnson and Chase 2004
Missing and deformed limbs	Outdoor mesocosms	*Hyla chrysoscelis*	Agrichemicals (atrazine, chlorpyrifos, and MSMA) and methyl mercury were added to mesocosms. Results show tadpoles and metamorphs with leg malformations, lordosis (bent tail), and missing eyes.	Britson and Threlkeld 1998
Mortality	Laboratory	*Rana pipiens*	Larvae were exposed from early embryonic stages through complete metamorphosis to varying natural sunlight regimes. Full sunlight caused approximately 50% mortality during early larval development. Exposure to sunlight also resulted in increased eye and limb malformations (missing or truncated digits). Filtration of sunlight with either glass or acrylamide both significantly reduced the incidence of malformed limbs.	Ankley et al. 2002
Mortality	Natural ponds and wetlands	*Rana sylvatica* *Ambystoma maculatum* *Ambystoma jeffersonianum*	Wastewater-irrigated ponds were compared to natural temporary ponds to investigate how secondarily treated wastewater effluent affects amphibians.	Laposata and Dunson 2000

Table 3.1. Continued.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Mortality	Laboratory	*Xenopus laevis*	Individual tadpoles were placed in containers of low, medium, and high concentrations of polycyclic aromatic hydrocarbons. PAHs are found in coal-tar based pavement sealers and are used on parking lots. Individuals in high concentrations died by day 6 and individuals in medium concentrations showed patterns of stunted growth and slower development compared to those in low concentrations.	Bryer et al. 2006
Mortality	Laboratory	*Rana sylvatica*	Road salt (NaCl) had a toxic effect on tadpoles in both acute and chronic tests at environmentally realistic concentrations. Water used in experiments was taken from roadside ditches and semi-permanent farm and forest ponds. Results show that tadpoles had decreased survivorship, weight, and activity; they metamorphosed earlier and had increased developmental abnormalities as salt concentration increased.	Sanzo and Hecnar 2006
Mortality	Laboratory	*Rana pipiens* *Rana clamitans* *Rana septentrionalis*	Embryos and larvae were exposed to UV-B radiation to determine the effects of each wavelength range on embryo and larval survival. Ambient levels of solar radiation were found to be lethal to all three species under exposure conditions that eliminated shade and refuge.	Tietge et al. 2001
Mortality	Natural ponds and wetlands	*Rana pretiosa*	Mortality following forest spraying of DDT.	Kirk 1988
Mortality	Outdoor mesocosms	*Rana clamitans* *Rana catesbeiana*	The author examined how pH, predatory stress, and a single application of an insecticide (carbaryl) affected the survival and growth of tadpoles. Results show that stress from pH and predators can make carbaryl (and other pesticides) more lethal under laboratory conditions using repeated applications of carbaryl, but these stressors did not interact under mesocosm conditions using a single application of carbaryl.	Relyea 2006
Other external deformities	Natural ponds and wetlands	*Rana catesbeiana*	Tadpoles collected from a coal ash deposition basin (contaminated with As, Cd, Cr, Cu, Se and other elements) had a reduced number of labial teeth and deformations of labial papillae when compared with tadpoles from reference areas.	Rowe et al. 1996

Table 3.1. Continued.

Abnormality	Venue	Species Affected	Problem Summary	Citation
Other external deformities	Laboratory	*Rana pipiens*	Larvae were exposed from early embryonic stages through complete metamorphosis to varying natural sunlight regimes. Full sunlight caused approximately 50% mortality during early larval development. Exposure to sunlight also resulted in increased eye and limb malformations (missing or truncated digits). Filtration of sunlight with either glass or acrylamide both significantly reduced the incidence of malformed limbs.	Ankley et al. 2002
Other external deformities	Outdoor mesocosms	*Hyla chrysoscelis*	Agrichemicals (atrazine, chlorpyrifos, and MSMA) and methyl mercury were added to mesocosms. Results show tadpoles and metamorphs with leg malformations, lordosis (bent tail), and missing eyes.	Britson and Threlkeld 1998

Table 3.2. Abnormalities documented at the Clayton County Water Authority, Panhandle Road Constructed Wetlands in 2006.

Category	Symptom	% of abnormal tadpoles (n = 192)
Hypercalcification	tail nodules	41.1
	mouth nodules	39.1
	edema	19.8
Axial deformation	curvature of spine	21.9
Tail deformities	including angles/twists	13.5
Malformed limbs	multiple	2.1
	malformed	1.6
Belly discoloration	green or red spots	6.3
Missing eye	missing one eye	1.6
Exposed lungs	visible at arm holes	0.5

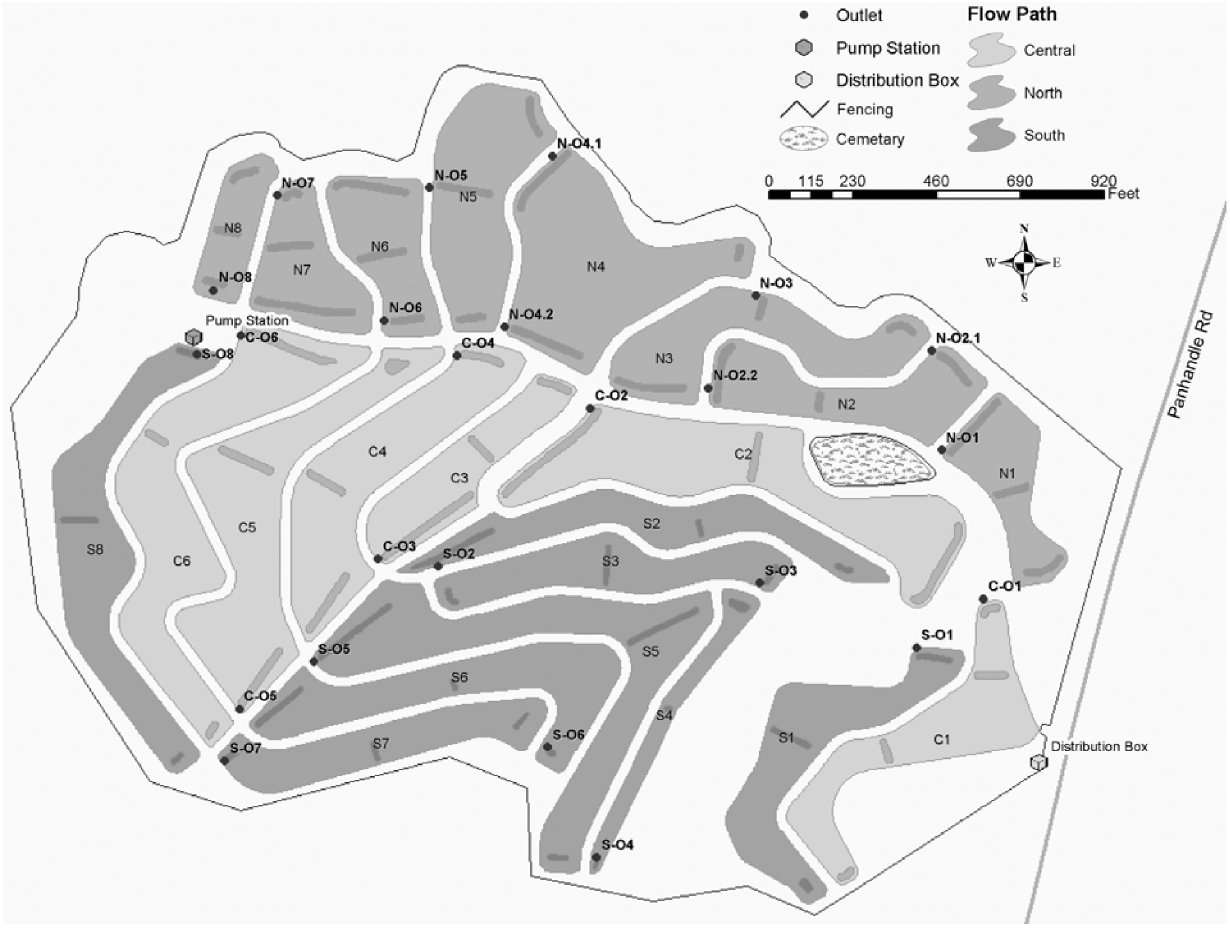

Figure 3.1. Map of the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Includes three separate systems (N-north, C-central and S-south) and their associated cells (number of cell refers to its sequence of water; water enters N1, flows to N2, flows to N3, etc.).

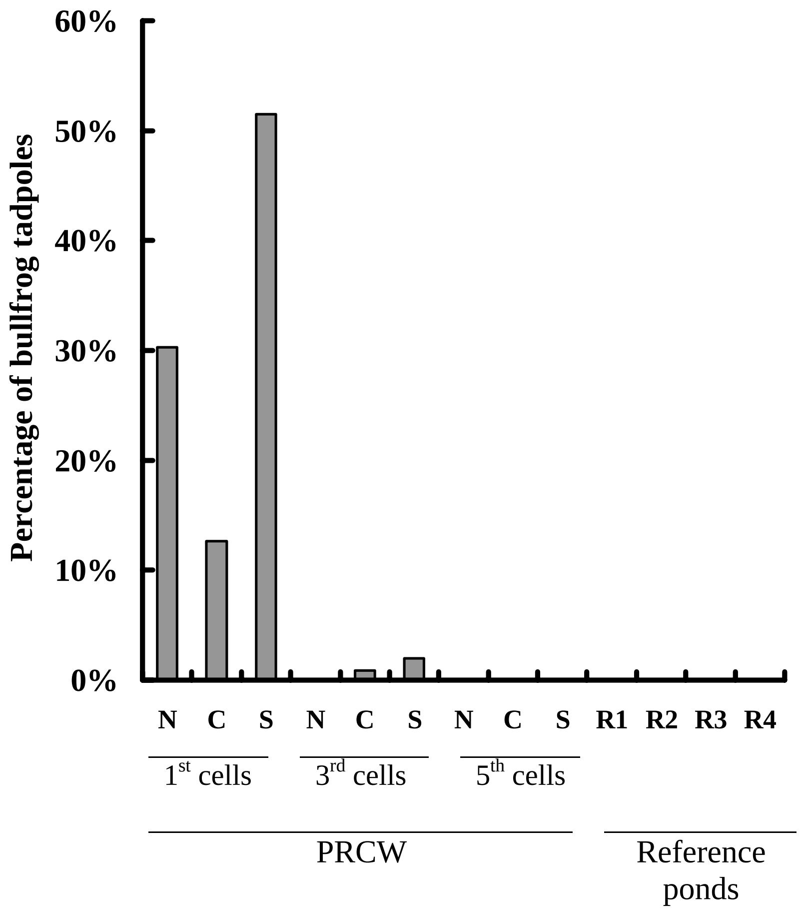

Figure 3.2. Prevalence of visible hypercalcemic nodules in *Rana catesbeiana* at the Clayton County Water Authority, Panhandle Road Constructed Wetlands and four reference ponds in 2006.

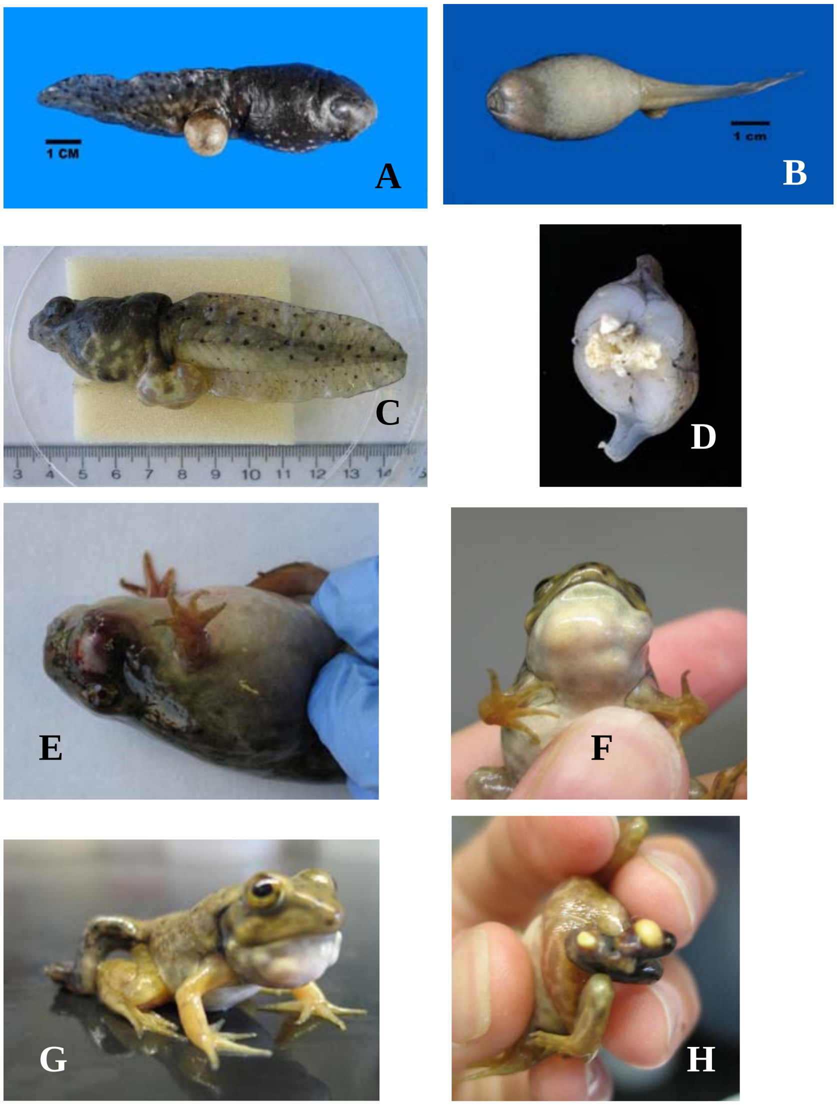

Figure 3.3a. Hypercalcemic disorders commonly observed among tadpoles at the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Pictured are (A) visible tail nodule, (B) visible mouth nodules, (C) fluid-filled abdominal sac, (D) internal deposits of calcium in tail muscle, (E) progressed mouth nodule, (F & G) calcified nodules in the gular region of a recently metamorphosed frog, and (H) calcified tail nodules of the same recently metamorphosed frog. All animals are American bullfrogs, *Rana catesbeiana*.

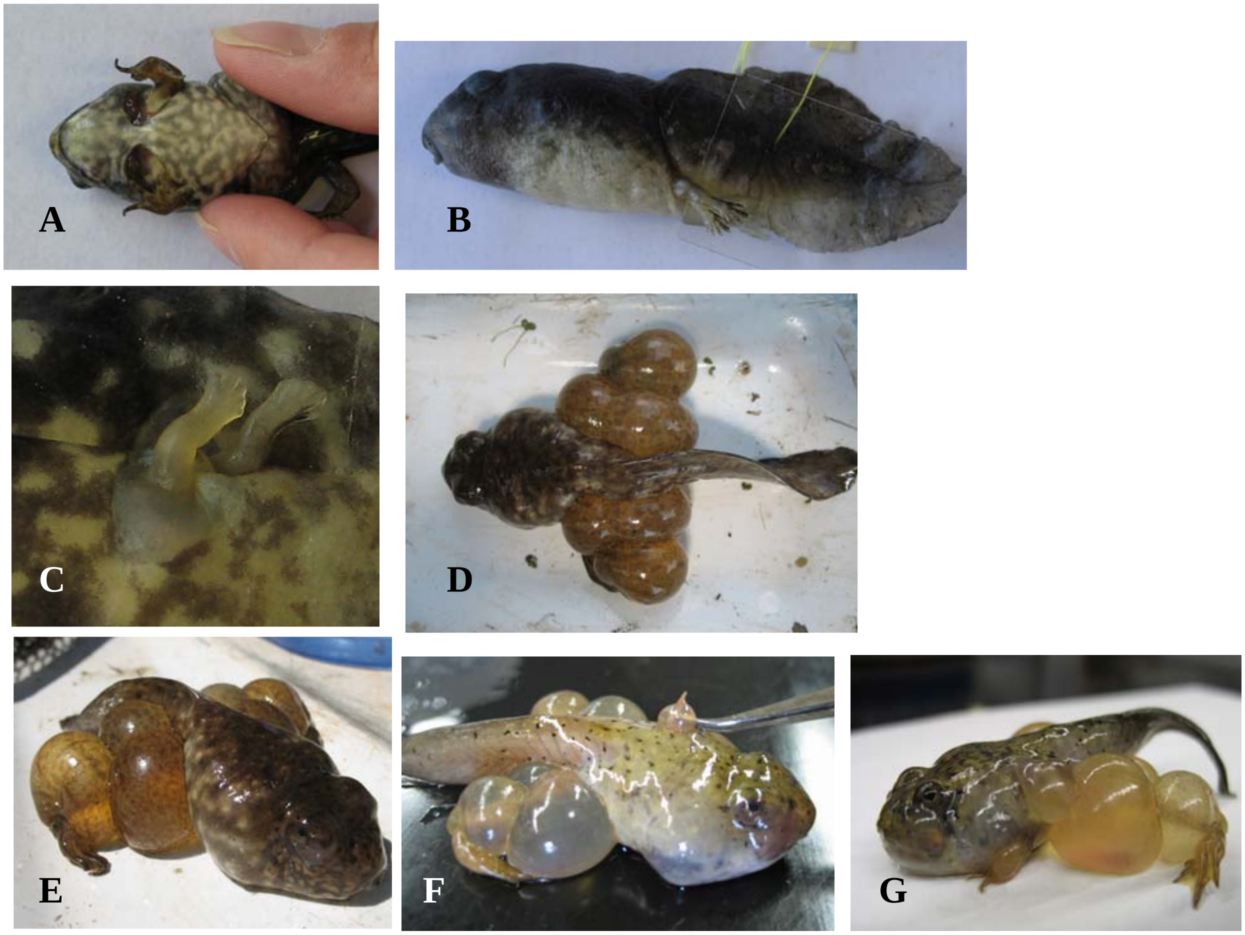

Figure 3.3b. Other external abnormalities observed among tadpoles at the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Pictured are (A) exposed lungs at arm holes, (B) extra appendage growing from hind limb, (C) extra set of hind legs growing from abdomen, (D & E) fluid-filled legs, (F) fluid-filled extra appendage growing from dorsal surface, and (G) fluid-filled extra appendage 2 days later. All animals are American bullfrogs, *Rana catesbeiana*.

Figure 3.3c. Other external abnormalities observed among tadpoles at the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Pictured are (A) extra limb growing out of tail (*Rana catesbeiana*), (B) axial deformation (*Rana catesbeiana*), (C) ventral discoloration (*Rana sphenocephala*), and (D) missing right eye (*Rana catesbeiana*).