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Patterns of Development and Abnormalities among Tadpoles in a Constructed Wetland Receiving Treated Wastewater

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Constructed wetlands are promoted for effectiveness at treating wastewater and potential value as wildlife habitat; however, wildlife performance studies in treated wastewater wetlands are limited. We used repeated surveys of larval amphibians along three wetland systems and four reference sites to test the hypothesis that bullfrog tadpoles exposed to direct inputs of treated wastewater develop slower, show a higher frequency of abnormalities, and are smaller at metamorphosis compared to tadpoles from reference ponds. Bullfrog tadpoles from wastewater wetlands were similar in size at metamorphosis compared to tadpoles from reference sites; however, they did show a much higher frequency of abnormalities including severe edema, scoliosis, and extreme calcinosis of soft tissues. Calcinosis was novel to the literature on amphibian abnormalities, the most frequent abnormality, and restricted exclusively to treatment wetlands. Within the constructed wetlands, tadpole development was slower and the frequency of scoliosis and calcinosis was higher in those cells receiving direct inputs of treated wastewater. Our results suggest that portions of constructed wetlands that directly receive treated wastewater may be poor amphibian habitat.

Introduction

Rapidly growing human populations in urban areas are simultaneously increasing demands for natural resources and reducing habitat available for wildlife. In regions such as the southeastern United States, many municipalities are struggling to meet demands for water and to treat increasing amounts of wastewater, which could lead to the return of inadequately treated wastewater to rivers and lakes (*1*). Expanding urban areas and natural resource extraction also create progressively smaller, more isolated, and more degraded habitats for wildlife (*2*, *3*). In the United States, urbanization is identified as a major factor in the endangerment of native species (*4*, *5*). Even local extirpations of common or cosmopolitan species have been reported in urban environments as a result of habitat degradation (*6*). Obviously, there is great interest in solutions that alleviate both resource demands and human impacts on wildlife.

Constructed wetlands are increasingly promoted for their effectiveness at treating wastewater and potential value as wildlife habitat in urban areas. Constructed wetlands can be used as a tertiary treatment to remove nutrients and other pollutants from wastewater treated by a conventional wastewater treatment plant before waters are discharged into rivers, lakes, or reservoirs (*7*). Wetland treatment can also provide a lower-cost alternative to conventional secondary treatment or advanced secondary treatment processes, mainly because forced aeration is not needed when using wetland treatment systems (*8*), and wetlands also do not require advanced technology or highly skilled personnel to operate (*9*−*11*). Constructed wetlands may also provide potentially valuable habitat for wildlife in urban and suburban areas where such habitats are otherwise declining or rare (*12*). The U.S. Environmental Protection Agency has promoted the value of constructed wastewater wetlands as wildlife habitat (*13*). Superficially, constructed wetlands appear attractive to wildlife; however, information on performance of wildlife in wetlands that receive treated wastewater is lacking (*12*, *14*). Animals inhabiting constructed wetlands could be exposed to unnaturally high concentrations of nutrients or other pollutants (*15*), a higher occurrence of invasive species, or regular maintenance activities that may be inadvertently harmful. For example, Laposata and Dunson (*15*) report reduced numbers of amphibian egg masses and larval amphibian survival in wetlands receiving treated wastewater. The temporary vernal pools examined in their study are inundated by spray irrigation of treated wastewater onto forested and agricultural lands resulting in runoff into natural wetlands and the creation of additional ephemeral surface wetlands. Their study does not address the direct discharge of high loads of treated wastewater into constructed or natural wetlands. If the Lasposata and Dunson (*15*) results can be extended to wetlands that receive direct inputs of treated wastewater, then pollutants in treated wastewater could limit the value of constructed wetlands as wildlife habitat and would argue against the discharge of treated wastewater into natural wetlands.

The objectives of this study were to evaluate the "performance" of tadpoles in a constructed wastewater treatment wetland. Specifically, we compared the development rates, frequencies of developmental abnormalities, 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. We 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. We chose larval bullfrogs because research at our focal treatment wetlands showed that bullfrogs are the dominant larval amphibian at the site and the only species found in all wetlands throughout the complex (*16*). We focused on development rates and size at metamorphosis because both are considered key metrics for predicting the effects of larval environments on amphibian populations (*17*, *18*). We know from year-round monitoring that wetlands receiving direct inputs of treated wastewater are significantly warmer during

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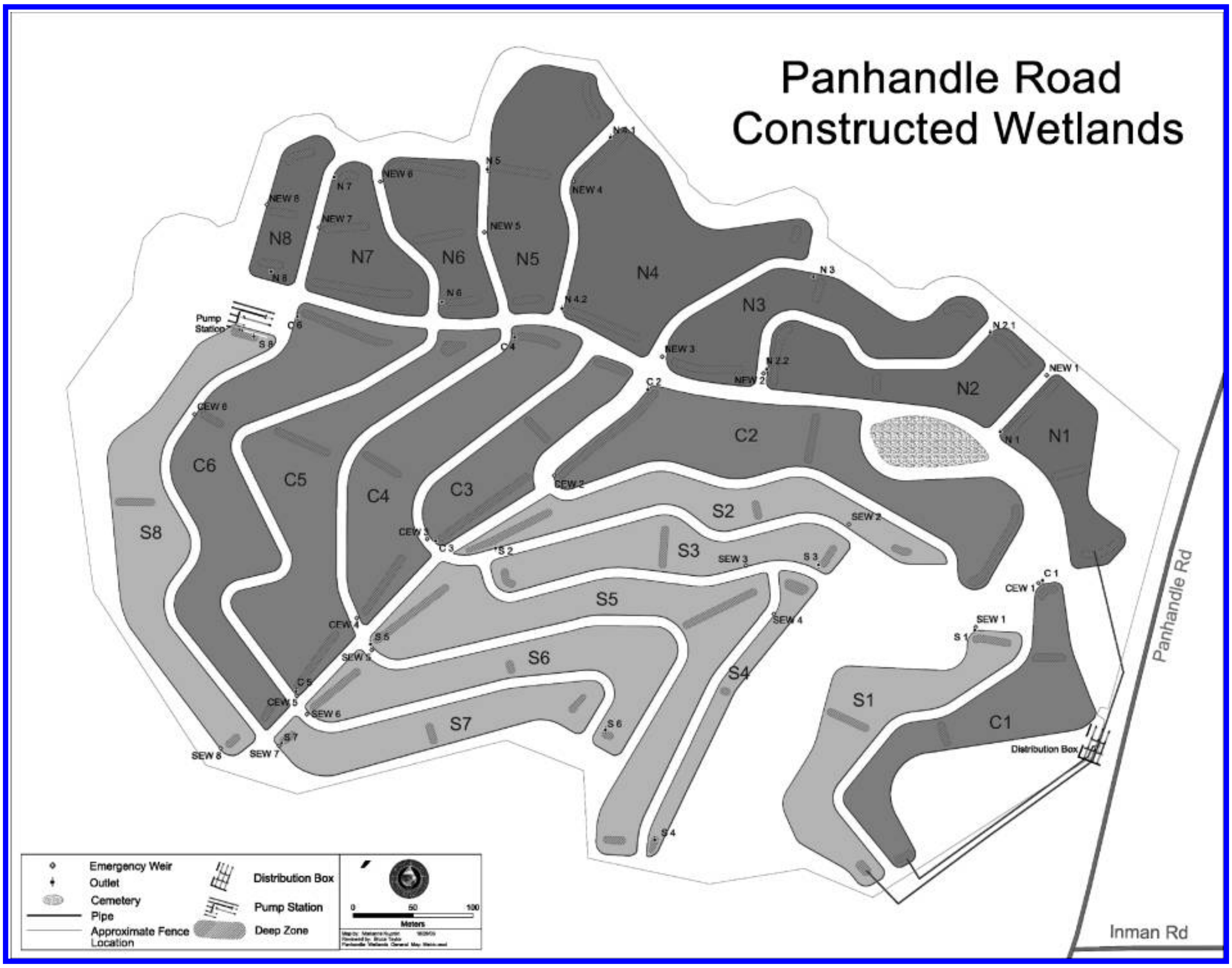

FIGURE 1. Map of the Clayton County Water Authority, Panhandle Road Constructed Wetlands. Includes three separate systems (N = north, C = central, and S = south) and their associated cells (cell number, e.g., C2, refers to its sequence in the system; water enters cells numbered 1 and flows in sequence through cells 2, 3, etc... .). Figure source, Clayton County Water Authority, Clayton County, GA.

the cool season months and have higher nutrient concentrations (see study sites description in next section). Therefore, if concentrations of nutrients and other pollutants are not detrimental, we expect bullfrog tadpoles exposed to direct inputs of treated wastewater will develop faster, show similar rates of abnormalities, and be larger at metamorphosis compared to tadpoles in reference ponds or from wastewater wetlands farther along the system that do not receive treated wastewater directly. However, if treated wastewater contains harmful concentrations of pollutants, we expect bullfrog tadpoles exposed to direct inputs of treated wastewater will develop slower, show higher rates of abnormalities, and be smaller in size at metamorphosis.

Experimental Section

Study Sites. Our study took place within the Panhandle Road Constructed Wetlands (PRCW) in the southern portion of Clayton County, a populous residential suburb immediately south of Atlanta, Georgia. PRCW is fed treated wastewater from the Shoal Creek Wastewater Reclamation Facility (WRF). Daily flow at the Shoal Creek WRF averages 1.8 MGD, most of which is residential or commercial flow with little industrial flow. There is one industrial flow in the basin in the form of leachate from a municipal landfill that composes <1000 gallons per day. The PRCW were constructed on land that contained several piles [described as totaling a dump truck load] of carbide materials located downslope and outside the area where wetlands were constructed. Replicate samples of the materials were submitted by CCWA for analysis and CCWA was advised that the materials presented no toxicological issues. The materials were composed of calcium and magnesium compounds notably calcium carbonate and magnesium carbonate.

Water from the Shoal Creek WRF is pumped to a single distribution box at PRCW that distributes the water into three parallel systems: North, Central, and South (Figure 1). Once discharged into each system, water is not exchanged among systems. Therefore, the PRCW is ideal for this research because it provides three replicate sets of wetlands. The systems flow from east to west along an elevation drop of approximately 30 m before discharging into a 400-acre reservoir, which then releases water south, either into the Flint River or into the reservoir of a water production plant. 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, the planting pattern of emergent vegetation was based on the direction of flow between each deep zone.

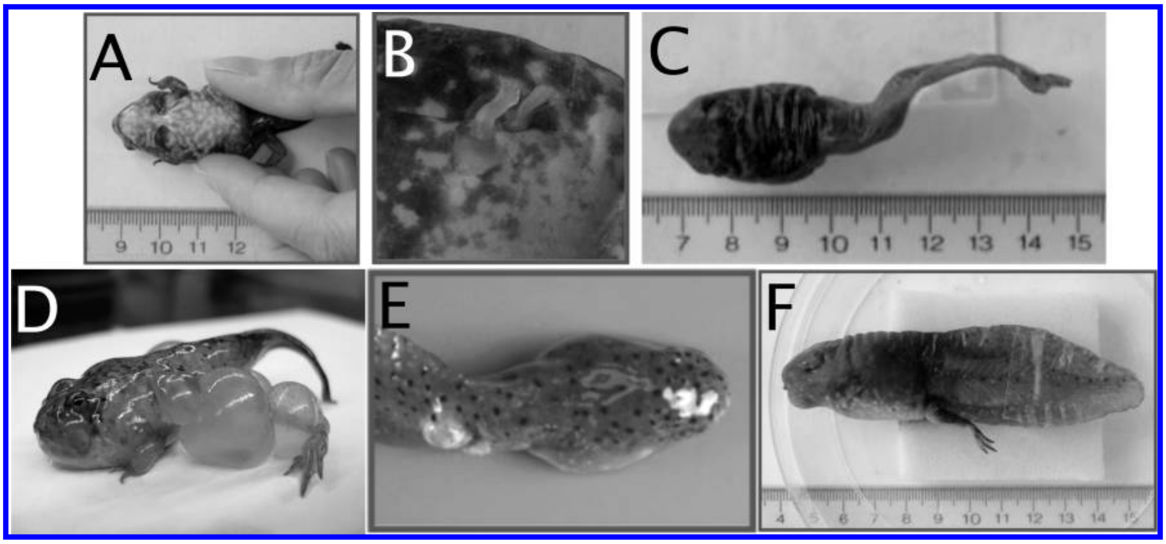

FIGURE 2. Visible abnormalities detected among tadpoles collected from PRCW and reference wetlands: (A) open skin from emergence of forelimbs; (B) extra limbs and pelvic girdle; (C) scoliosis; (D) severe edema of limbs and torso; (E) tail muscle nodules from calcinosis; and (F) gular [throat] nodules from calcinosis.

The CCWA collects daily samples of wastewater as it enters the treatent plant and leaves the wastewater treatment wetlands. Additionally, CCWA collects samples of wastewater entering the treatment plant (WRF), wastewater leaving WRF but not yet entering the wetlands treatment system, and wastewater leaving the PRCW (where the flow lines combine) once a week. Three consitutents are routinely analyzed for all of these samples: pH, total phosphorus, and ammonium-N. The CCWA provided these data for 1 year prior to and extending 1 year beyond our study period (2005−2007). Between January 2005 and December 2007, pH of WRF effluent generally ranged between 6.6 and 7.0 with one two-week period in June 2006 when pH ranged from 5.7 to 6.2. The pH of PRCW effluent was generally about 0.5 units greater than WRF effluent, generally ranging from 6.9 to 7.4. Between January 2005 and December 2007, total phosphorus in WRF averaged 1.0 mg/L and ranged from <0.1 to 2.5 mg/L; ammonium-N averaged 1.0 mg/L and ranged from 0.2 to 8.8 mg/L. Between January 2005 and December 2007 total phosphorus in PRCW effluent averaged 0.8 mg/L and ranged from <0.1 to 1.8 mg/L; ammonium-N averaged <0.1 mg/and ranged from <0.1 mg/L to 0.3 mg/L. It should be noted that these data were collected as a requirement of the National Pollutant Discharge Elimination System permit issued by the State of Georgia, Department of Natural Resources, Environmental Protection Division and do not correspond to locations where tadpoles were collected. However, they illustrate an increase in pH and a reduction in nutrient concentrations as water flowed through the PRCW.

For comparison purposes, we sampled four reference ponds in Athens-Clarke County, Georgia, about 70 miles east of Clayton County: three in the Whitehall Experimental Forest, and 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 all ponds were known to contain high densities of larval bullfrogs. Reference ponds had deeper open water zones (>3 m) and a smaller proportion of the pond contained emergent vegetation compared to PRCW wetlands. Reference ponds did have dense emergent vegetation near the inflow, and forming a narrow band around the pond perimeters, and central deep zone. Emergent vegetation was predominantly *Scirpus* spp. and *Typha latifolia*.

Tadpole Sampling. Between January and July 2006, we conducted repeated sampling for 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, we performed dipnet sweeps for 21 person-minutes in each cell. Tadpoles were killed on site by immersion in 1% neutral-buffered tricaine methanesulfonate (MS-222) and preserved in 10% neutral buffered formalin. In the lab, we weighed each animal, determined the Gosner stage (*19*), and took dorsal and lateral photos of each tadpole using a Canon Power Shot G6 digital camera mounted onto a stage. Program Image J (version 1.38, http://rsbweb.nih.gov/ij/) was used to measure body length of each tadpole from the tip of the snout to the midinsertion point on the tail, which was typically in vertical alignment with the vent (*20*). Because the relationship between Gosner stage and mass is unimodal for tadpoles and declines significantly between metamorphic Gosner stages 40 and 46, we could not use mass as a 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. Therefore, we used body length for all tadpoles of Gosner stage 39 as our measure of size at metamorphosis.

In addition to standard body measurements, tadpoles were inspected for externally visible abnormalities including scoliosis of the tail, edema, missing or duplicated limbs, extra limbs, and calcinosis (Figure 2). Note, calcinosis was a novel abnormality discovered as part of this study and a description of the lesions is available in Keel et al. (*21*). Affected tadpoles have tan or light-yellow nodules in the tail or gular region that elevate the skin (Figure 2). Additional mineralized nodules and tissues not visible from external surfaces are often present, but most afflicted tadpoles show externally visible nodules (Ferreira et al. unpublished data). The nodules consist of 100% apatite, a salt of calcium phosphate [$Ca_5(PO_4)_3(OH)$] (*21*). Scoliosis typically involved abnormal mineralization of the caudal vertebrae and could share a common pathogenesis with calcinosis.

Water Quality Measurements. We opportunistically collected basic water quality data during the tadpole sampling reported in this paper. We measured water temperature, total nitrogen, and total phosophorus in all first, third, and fifth cells, and reference sites. During each tadpole sampling period, we used a bulb thermometer to measure water temperature at 0.5 m depth at 3 three locations within each study wetland. We also collected duplicate, 30-mL water samples from each study wetland. Water samples were stored in opaque bottles in a darkened refrigerator at ~3 °C until

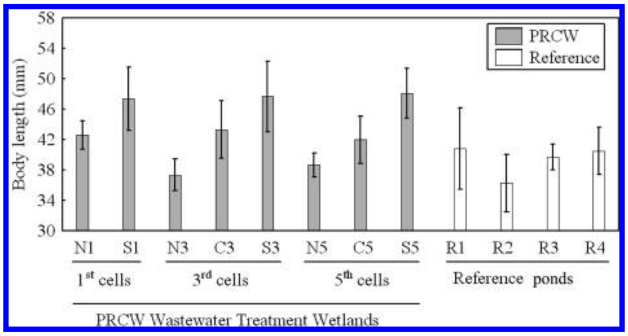

FIGURE 3. Mean body lengths of *Rana catesbeiana*, Gosner stages ≥39, from the Clayton County Water Authority, Panhandle Road Constructed Wetlands and four reference ponds in 2006. No bullfrog metamorphs were captured in Cell C1. Error bars show 95% confidence intervals.

analyzed by persulfate digest and continuous flow colorimetric analyzer for total orthophosphate and nitrate concentrations by the Stable Isotope/Soil Biology Laboratory of the University of Georgia (http://swpa.uga.edu/). Details of analyses are available on the laboratory's Web site.

Statistical Analysis. We 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 PRCW cells closer to the wastewater discharge point, the mean Gosner stage of tadpoles for each cell on each date was calculated and then used in 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, Tulsa, OK).

Results and Discussion

We collected 1023 bullfrog tadpoles among wetlands, 833 among the 9 PRCW wetlands and 190 among the 4 reference ponds. Overall tadpoles were more abundant in first cells at PRCW. We collected 264, 79, and 33 bullfrog tadpoles from the first cells of the north, central, and south systems, respectively. We collected 147, 111, and 51 bullfrog tadpoles from the third cells of the north, central, and south systems, respectively. We collected 64, 52, and 32 from the fifth cells of the north, central, and south systems, respectively. We failed to capture any metamorphic bullfrog tadpoles (Gosner stage 40+) from the first cell of the central system (C1), and cell 3 in the Central system (C3) was drained for maintenance shortly after sampling started. As a result, we could not include the central system in our analysis of development rates; however, we did include data from the central system in our analyses of abnormality frequencies.

Bullfrog Tadpole Size at Metamorphosis. Mean metamorphic body length of bullfrogs, collected in June and July, were larger at PRCW compared to those from reference ponds (MS = 296.200, $F_{1,147} = 13.402$, $P < 0.001$; Figure 3). There were no significant differences in metamorphic body length among any reference ponds (Figure 3). Metamorphic body length varied significantly among cells within the PRCW complex (MS = 141.400, $F_{10,147} = 6.397$, $P < 0.001$; Figure 3); however, this variation in metamorph body size within PRCW was not related to proximity to the discharge of treated wastewater. That is, metamorph size was not greater or smaller among first cells compared to third or fifth cells (Figure 3). Instead, most of the variation in body size was attributable to the different systems of cells within PRCW. Mean metamorph size was smaller in cells from the North system, intermediate in the Central system, and largest in the South system (Figure 3), and 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 3). The differences in metamorph size among North, Central, and South systems are consistent with the inverse pattern of tadpole abundance among those systems. The South system had fewer tadpoles but they were larger at metamorphosis. This may indicated negative density-dependent effects on tadpole size at metamorphosis.

Bullfrog Tadpole Development Rates. Within the North and South PRCW systems, the proximity of cells to the treated wastewater discharge point had a significant effect on tadpole development rates (MS = 23.850, $F_{2,20} = 4.107$, $P = 0.032$; Figure 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 fifth cells, which were most distant from the discharge point, than in the first cells that directly received the treated wastewater (cell × date interaction: MS = 23.921, $F_{2,20} = 4.119$, $P = 0.032$). By mid-June, most tadpoles in the fifth cells were metamorphic (Gosner stage >39), and most tadpoles were metamorphic in the third

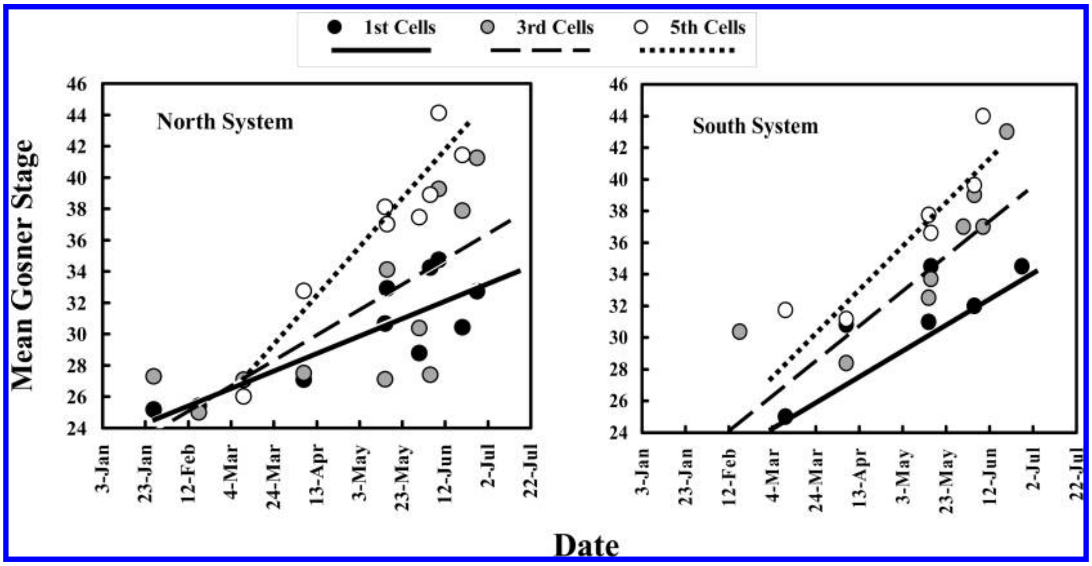

FIGURE 4. Mean Gosner stage of bullfrog tadpoles through time of the North and South systems of the Clayton County Water Authority, Panhandle Road Constructed Wetlands in 2006.

TABLE 1. Distribution of Abnormalities Observed among Bullfrog Tadpoles Collected from Nine Wetland Cells Receiving Treated Wastewater at the Panhandle Road Constructed Wetlands Complex (PRCW) and Four Reference Ponds

	PRCW cells			reference ponds
abnormality	1st[a] (*n* = 376)	3rd (*n* = 310)	5th (*n* = 147)	(*n* = 190)
malformed/extra limbs	4 [1%]	0	1 [<1%]	3 [2%][b]
open limb slits	1 [<1%]	0	3 [2%]	0
missing eyes	2 [<1%]	1 [<1%]	0	0
edema	19 [5%]	11 [4%]	9 [6%]	0
scoliosis	37 [10%]	14 [5%]	3 [2%]	0
calcinosis	108 [29%]	2 [<1%]	0	0

[a] 1st cells are those that receive direct inputs of treated wastewater; water has transited through two and four wetland cells before arriving at the 3rd and 5th cells respectively (see Figure 1). Reference ponds do not receive treated wastewater.
[b] Bracketed values are the percentage of tadpoles within the wetland category that exhibited the abnormality.

cells by early July. By contrast, few tadpoles from first cells were metamorphic by late July (Figure 4).

Frequencies of Bullfrog Tadpole Abnormalities. We found 177 bullfrog tadpoles exhibited at least one externally visible abnormality. Nearly all tadpoles with visible abnormalities, 174 out of 177 (98%), were found at PRCW. Only 3 abnormal tadpoles were found among references sites. All three abnormal tadpoles at reference sites exhibited limb malformations, while abnormalities observed at PRCW included five cases of a malformed limb, four cases of "open wounds" all of which were failures of the slits in the body cavity to close after forelimb emergence, edema [often severe, see Figure 2] of the vent and limbs, scoliosis, and calcinosis of the tail and gular regions (Table 1). The distribution of abnormal tadpoles was highly nonrandom between PRCW and reference sites and among first, third, and fifth wetland cells within the PRCW complex. A large majority of abnormal tadpoles, 137 of 177 (75%) were collected from first wetland cells at PRCW, which receive direct inputs of treated wastewater. In particular, 69% and 98% of tadpoles exhibiting scoliosis and calcinosis respectively were found in first wetland cells at PRCW. Calcinosis was detected in all three first cells and was seen in 29% of bullfrog tadpoles collected from first cells (Table 1). Calcinosis was also detected in two leopard frog tadpoles (*R. sphenocephala*), both also from first cells at PRCW. The frequency of scoliosis among PRCW tadpoles declined gradually from first to third to fifth wetland cells. The frequency of calcinosis showed a much more dramatic pattern, dropping from 28% to <1% between first and third cells, and no calcinosis was detected in tadpoles from the fifth cells.

The patterns of slower development and higher frequency of calcinosis and scoliosis among bullfrog tadpoles from first cells raise concerns about the effects of direct exposure to treated wastewater on larval amphibians. We note that the cause of the reduced development, calcinosis, and scoliosis are not known, and we do not know whether the same agents are responsible for each pattern. We have very limited data on differences between the treated wastewater discharged into the first cells of the PRCW and the water farther along the systems and at reference sites where we do not see tadpole calcinosis. Based on information provided by CCWA, we know that effluent from the Shoal Creek WRF during the study period was higher in ammonium-N. We also found that mean total N and total P concentrations were higher in first and third cells at PRCW compared to fifth cells at PRCW and reference sites (*16*). During the study period, mean total N concentration among first cells ranged from 1.5 to 4.3 mg/L, which was within the range of ammonium-N concentrations reported by CCWA for the effluent of the Shoal Creek WRF. Mean total N concentration among fifth cells was 1.4−2.1 mg/L, which was lower than that of the first cells. In February 2006, mean total N concentrations among third cells was 2.6 mg/L, which was intermediate to first and fifth cells; however, mean total N concentrations among third cells from March through July 2006 were <1.5 mg/L and were similar to concentrations in fifth cells (*16*). Mean total N among reference sites was <1 mg/L. We also found that February 2006 water temperatures were 6 °C warmer in first cells compared to third and fifth cells. By March, first cells water temperatures were 4 °C warmer than third and fifth cells, and by April first cells water temperatures were 2 °C warmer than third and fifth cells. Water temperatures were similar among all cells and reference sites from June through July 2006.

The effects of nitrogen pollution on amphibians have been studied extensively in the lab and field (*22*), and results suggest that the effects of inorganic nitrogen pollution can vary among species and populations. The majority of studies including several on *Rana* spp. at concentrations similar to those observed at our study sites report no effects of nitrogen on development rates of tadpoles (*23*−*26*); however, a few studies do report delayed development at sublethal concentrations. Additionally, Krishnamuthry et al. (*27*) reported "bent tails" and "swollen sac-like" bodies among *Nyctibatrachus major* and *Fejervarya limnocharis* exposed to 1.0 mg/L nitrate for 7−21 days, and Hecnar (*28*) reported increased bent tails and swollen bodies among *R. clamitans* tadpoles after 5−8 days in ~3 mg/L ammonium nitrate compared to tadpoles in control water. The authors also reported some tadpoles developing head deformities and bulges on the body; however, the authors did not provide details on these abnormalities or provide data on the proportion of tadpoles with abnormalities among different ammonium-nitrate treatments. We feel that development of these symptoms after only 5−8 days is unlikely to be the result of deposition of calcium salts. Therefore, it is possible that the patterns of scoliosis we observed are the result of high nitrogen in first cells receiving direct inputs of treated wastewater; however, it is unlikely that nitrate is responsible for the patterns of calcinosis or delayed development in those cells. Because water temperatures in first cells were similar to water temperatures at reference sites where we did not observe calcinosis, we do not believe temperature is directly responsible for calcinosis; however, we cannot rule out the possibility that temperature interacts with a constituent of treated wastewater to induce calcinosis.

We think it is most likely that constituents of treated wastewater entering PRCW, other than nitrogen pollution, are affecting tadpole hormonal regulation of calcium. Residential and commercial treated wastewater can contain high concentrations of contaminants that might affect aquatic organisms including pharmaceuticals and household products (*29, 30*). Increases in parathyroid hormone, prolactin, estrogen, and cholecalciferol (25-OH-Vitamin D3) have all been shown to increase uptake of calcium by tadpoles (*31*). Baksi (*32*) showed increased plasma Ca^{2+} and cholecalciferol levels through exogenous administration of estrogen to leopard frog (*R. pipiens*) tadpoles, and Baldwin and Bentley (*33*) showed that increased cholecalciferol increased Ca^{2+} uptake across the gills of bullfrog tadpoles. We found that premetamorphic (Gosner stage <40) bullfrog tadpoles from first wetland cells that exhibited calcinosis had measurable levels (~6 nmol/L) of cholecalciferol (25-OH-Vitamin D3); however, premetamorphic bullfrog tadpoles from reference sites did not have detectable levels of cholecalciferol (*21*). Metamorphic bullfrogs from reference sites have detectable levels of cholecalciferol (7 nmol/L), but this is still lower than levels (10−12 nmol/L) detected in metamorphic bullfrog tadpoles from first wetland cells at PRCW (*21*). We suspect that elevated cholecalciferol among bullfrog tadpoles from first cells is the proximate cause of calcinosis. Tadpoles may be accumulating cholecalciferol from the environment or an exogenous endocrine disruptor such as an estrogenic compound is stimulating endogenous cholecalciferol production.

These patterns of development and abnormalities raise concerns about the effects of direct exposure to treated wastewater on larval amphibian habitats. Despite the high rates of tadpole abnormalities in the constructed wetlands complex, the near complete isolation of calcinosis to wetland cells receiving direct inputs of treated wastewater means that the causative agent is rapidly remediated by transit of the treated wastewater through the wetlands complex. In other words, wastewater constituents that could be harmful to wildlife persist through standard industrial treatment, but appear remedied by passage through constructed wetlands. Wetlands may accomplish what industrial treatment cannot because industrial treatment is designed to target known constituents in water. On the other hand, the bacterial and plant communities within wetlands can break down a wide array of compounds without complex design or high energy or chemical costs.

Like scoliosis and calcinosis, edema was significantly more common among tadpoles collected at PRCW compared to reference sites; however, the prevalence of edema was not related to cell proximity to discharge of treated wastewater (Table 1). Tadpole edema can be symptomatic of a number of factors including infection. We have found bullfrog tadpoles at PRCW infected with *Batrochytrium dendrobatidis* (*Bd*) and viral inclusion bodies consistent with a Ranavirus (Iridoviridae). *Bd* is a major causative agent of tropical and montane amphibian declines, but is not reported to cause edema and is not known to have caused any amphibian population die-offs in the southeastern U.S. (*34*). Ranaviruses are also a major causative agent of global amphibian declines, are known to cause edema, and have been linked to amphibian die-offs in the southeastern U.S. (*35*). Ranavirus infection rates in agricultural wetlands are positively associated with increased nitrogen pollution, which may compromise amphibian immune systems or increase pathogen prevalence (*35*). Similar to agricultural systems, the high concentrations of nitrogen in wastewater may increase the prevalence of amphibian diseases within wastewater treatment wetlands.

There are three important limitations to our study that make the results preliminary. First, our study deals only with one site during one year. However, we observed the same patterns of calcinosis at PRCW in follow-up samples in 2007 and 2008 (J. Maerz, A. Ferreira, and E. Reed, unpublished data). Second, we do not have data on the complete array of constituents of wastewater at our study sites. Collection and analysis of water samples for a broad spectrum of contaminants is extremely costly and well beyond the scope of our resources. Given the evidence of elevated cholecalciferol in bullfrog tadpoles from first cells and the connection between cholecalciferol and estrogen and calcium regulation in tadpoles, we suggest that future work target levels of cholecalciferol and estrogenic compounds in water and sediments. Finally, our study addresses a single amphibian species. Other amphibian species breed at PRCW and the reference sites, and tadpoles of those species are encountered at a higher rate in third and fifth cells but are uncommon in first cells (*16*). Why bullfrogs persist at high densities in first cells while other species do not is unknown. Bullfrogs may be competitively dominant under the conditions in first cells or simply more tolerant of the conditions in first cells (*25*). Some studies suggest *Hyla* spp. and *Bufo* spp. may be more sensitive to ammonium concentrations (*22*), which could explain their low occurrence in first cells. Though leopard frog tadpoles were uncommon in first cells, we did detect calcinosis in two leopard frog tadpoles from first cells. This suggests that this species is also susceptible to this abnormality.

Constructed wetlands show great potential to efficiently deal with water resource problems facing urban communities; however, concluding that constructed wetlands also have the added benefit of increasing wildlife habitat is premature and certainly in need of more critical investigation (*12*). Caution should be taken in statements regarding the value of these wetlands for wildlife based strictly on the observation that they are used by large numbers of species. Ours is only the second study we are aware of to investigate the potential effects of treated wastewater on amphibians, and both studies have found evidence of harm. We should be concerned about the potential impacts on amphibian populations of wastewater wetlands and other wetlands that collect waters with high concentrations of nutrients or other pollutants (*29, 30, 36*). In areas where natural wetlands have disappeared, wetlands constructed to treat stormwater or treated wastewater may represent a large proportion of amphibian breeding sites. If those sites negatively affect the health of amphibians, those sites may function as population sinks. We stress that our study does not demonstrate any negative population-level effects on amphibian populations; therefore, using this study as evidence of negative population effects is inappropriate. Rather, this study should stimulate further research into the performance of amphibians in constructed wetlands and whether those wetlands are having positive or negative effects on amphibian populations.

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Literature Cited

(1) Kaseva, M. E. Performance of a sub-surface flow constructed wetland in polishing pre-treated wastewater - a tropical case study. *Water Res.* **2004**, *38*, 681–687.

(2) Foley, J. A.; DeFries, R.; Asner, G. P.; Barford, C.; Bonan, G.; Carpenter, S. R.; Chapin, F. S.; Coe, M. T.; Daily, G. C.; Gibbs, H. K.; Helkowski, J. H.; Holloway, T.; Howard, E. A.; Kucharik, C. J.; Monfreda, C.; Patz, J. A.; Prentice, I. C.; Ramankutty, N.; Snyder, P. K. Global consequences of land use. *Science* **2005**, *309*, 570–574.

(3) Wilcox, B. A.; Murphy, D. D. Conservation Strategy - the effects of fragmentation on extinction. *Am. Nat.* **1985**, *125*, 879–887.

(4) Czech, B.; Krausman, P. R.; Devers, P. K. Economic associations among causes of species endangerment in the United States. *Bioscience* **2000**, *50*, 593–601.

(5) *Millennium Ecosystem Assessment. Ecosystems and human well-being: biodiversity synthesis*; World Resources Institute: Washington, DC, 2005.

(6) Urban, M. C.; Skelly, D. K.; Burchsted, D.; Price, W.; Lowry, S. Stream communities across a rural-urban landscape gradient. *Diversity.Distrib.* **2006**, *12*, 337–350.

(7) Greenway, M. The role of constructed wetlands in secondary effluent treatment and water reuse in subtropical and arid Australia. *Ecol. Eng.* **2005**, *25*, 501–509.

(8) Gersberg, R. M.; Elkins, B. V.; Lyon, S. R.; Goldman, C. R. Role of aquatic plants in wastewater treatment by artificial wetlands. *Water Res.* **1986**, *20*, 363–368.

(9) Al-Omari, A.; Fayyad, M. Treatment of domestic wastewater by subsurface flow constructed wetlands in Jordan. *Desalination* **2003**, *155*, 27–39.

(10) Hiley, P. D. The reality of sewage treatment using wetlands. *Water Sci. Technol.* **1995**, *32*, 329–338.

(11) Juwarkar, A. S.; Oke, B.; Juwarkar, A.; Patnaik, S. M. Domestic wastewater treatment through constructed wetland in India. *Water Sci. Technol.* **1995**, *32*, 291–294.

(12) Knight, R. L.; Clarke, R. A.; Bastian, R. K. Surface flow (SF) treatment wetlands as a habitat for wildlife and humans. *Water Sci. Technol.* **2001**, *44*, 27–37.

(13) U.S. Environmental Protection Agency. *Constructed Wetlands for Wastewater Treatment and Wildlife Habitat: 17 Case Studies*; U.S. Environmental Protection Agency: Washington, DC, 1993.

(14) Knight, R. L. Wildlife habitat and public use benefits of treatment wetlands. *Water Sci. Technol.* **1997**, *35*, 35–43.

(15) Laposata, M. M.; Dunson, W. A. Effects of spray-irrigated wastewater effluent on temporary pond-breeding amphibians. *Ecotoxicol. Environ. Saf.* **2000**, *46*, 192–201.

(16) Ruiz, A. M. An Assessment of a Constructed Wastewater Treatment Wetland Complex as Urban Amphibian Habitat. Masters Thesis. D. B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA, 2007.

(17) Alford, R. A. Ecology: resource use, competition, and predation. In *Tadpoles: the Biology of Anuran Larvae*; McDiarmid, R. W., Altig, R., Eds.; The University of Chicago Press: Chicago, 1999; pp 240−278.

(18) Berven, K. A. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). *Ecology* **1990**, *71*, 1599–1608.

(19) Gosner, N. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **1960**, *16*, 183–190.

(20) Van Buskirk, J.; McCollum, S. A. Influence of tail shape on tadpole swimming performance. *J. Exp. Biol.* **2000**, *203*, 2149–2158.

(21) Keel, M. K.; Ruiz, A. M.; Fisk, A. T.; Rumbeiha, W.; Davis, A. K.; Maerz, J. C. Soft-Tissue Mineralization of Bullfrog Larvae (*Rana catesbeiana*) at a Wastewater Treatment Facility. *J. Vet. Diagn. Invest.* **2010**, *22*, 655−659.

(22) Rouse, J. D.; Bishop, C. A.; Struger, J. Nitrogen pollution: An assessment of its threat to amphibian survival. *Environ. Health Perspect.* **1999**, *107*, 799–803.

(23) Allran, J. W.; Karasov, W. H. Effects of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Environ. Toxicol. Chem.* **2000**, *19*, 2850–2855.

(24) Johansson, M.; Räsänen, K.; Merilä, J. Comparison of nitrate tolerance between different populations of the common frog *Rana temporaria*. *Aquat. Toxicol.* **2001**, *54*, 1–14.

(25) Smith, G. R.; Temple, K. G.; Vaala, D. A.; Dingfelder, H. A. Effects of nitrate on the tadpoles of two ranids (*Rana catesbeiana* and *R. clamitans*). *Arch. Environ. Contam. Toxicol.* **2005**, *49*, 559–562.

(26) Griffis-Kyle, K. L.; Ritchie, M. E. Amphibian, survival, growth and development in response to mineral nitrogen exposure and predator cues in the field: an experimental approach. *Oecologia* **2007**, *152*, 633–642.

(27) Krishnamurthy, S. V.; Meenakumari, D.; Gurushankara, H. P.; Vasudev, V. Nitrate-induced morphological anomalies in the tadpoles of *Nyctibactrachus major* and *Fejervarya limnocharis* (Anura: Ranidae). *Turkish J. Zool.* **2008**, *32*, 239–244.

(28) Hecnar, S. J. Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. *Environ. Toxicol. Chem.* **1995**, *12*, 2131–2137.

(29) Smith, G. R.; Burgett, A. A. Effects of three organic wastewater contaminants on American toad, *Bufo americanus*, tadpoles. *Ecotoxicology* **2005**, *14*, 477–482.

(30) Gros, M.; Petrovic, M.; Barcelo, D. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro River Basin (Northeast Spain). *Environ. Toxicol. Chem.* **2007**, *26*, 1553–1562.

(31) Stifler, D. F. Amphibian calcium metabolism. *J. Exp. Biol.* **1993**, *184*, 47–61.

(32) Baksi, S. N.; Kenny, A. D.; Galli-Gallardo, S. M.; Pang, P. K. T. Vitamin D metabolism in bullfrogs and Japanese quail: Effects of estradiol and prolactin. *Gen. Comp. Endrocrinol.* **1978**, *35*, 258–262.

(33) Baldwin, G. F.; Bentley, P. J. Calcium metabolism in bullfrog tadpoles (*Rana catesbeiana*). *J. Exp. Biol.* **1980**, *88*, 357–365.

(34) Rothermel, B. B.; Walls, S. C.; Mitchell, J. C.; Dodd, C. K., Jr.; Irwin, L. K.; Green, D. E.; Vazquez, V. M.; Petranka, J. W.; Stevenson, D. J. Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Dis. Aquat. Org.* **2008**, *82*, 3–18.

(35) Gray, M. J.; Miller, D. L.; Schmutzer, A. C.; Baldwin, C. A. *Frog virus 3* prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, USA. *Dis. Aquat. Org.* **1997**, *77*, 97–103.

(36) Massal, L. R.; Snodgrass, J. W.; Casey, R. E. Nitrogen pollution of stormwater ponds: Potential for toxic effects on amphibian embryos and larvae. *Appl. Herpetol.* **2007**, *4*, 19–29.

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