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Evaluating Potential Mechanisms for Altered Amphibian Performance in Treated Wastewater

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Abstract.—**Constructed wetlands (CWs) are an attractive solution for wastewater treatment due to their potential capacity to treat wastewater and support native species. These dual purpose CWs, however, can attract wildlife and expose them to aquatic environments high in nutrients and harmful contaminants. Although studies indicate tradeoffs between larval anuran growth and long-term adult survival following development in wastewater, it is unclear what specific mechanism is responsible for these effects. We suggest that behavior could be a mediating process that both responds to the environment and contributes to demography. We evaluated the behavioral responses of Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles exposed and reared in pond water versus wastewater treatment CWs to identify if behavior was affected by exposure or development in wastewater and to explore two potential causes of behavioral change: increased turbidity and a common contaminant, diphenhydramine. Specifically, we monitored activity, exploration, and predator avoidance. Tadpoles in wastewater were slower to begin moving, traveled shorter distances, and exhibited the shortest burst distances. We did not observe differences between tadpole origin that indicated exposure to wastewater was sufficient to alter behavior independent of developmental processes. Neither of our potential mechanisms for these effects were sufficient to explain the magnitude of behavioral change in treated wastewater. As wastewater can host unique chemical and physical conditions, including contaminant mixtures, learning more about how behavior responds to specific conditions of wastewater may help identify mechanisms to explain why frogs exhibit changes in behavior, growth, and survival following development in wastewater.**

Key Words.—anuran; behavior; contaminant; effluent; pharmaceuticals; tadpole; turbidity

Introduction

Wetland habitat loss represents a significant challenge to the maintenance of freshwater biodiversity (Gibbs 1993; Cardinale et al. 2012; Hu et al. 2017). Freshwater habitats, particularly in the southeastern U.S., are recognized as ecosystems experiencing significant declines in the prevalence and distribution of freshwater species (Brooks et al. 2002; Dudgeon et al. 2006; Strayer and Dudgeon 2010). Land conversion and pollution by human waste disposal has eliminated or degraded 33–50%, or approximately 30 million km2 of wetland habitat (Russi et al. 2013; Hu et al. 2017). Constructed wetlands (CWs) are an effective mitigation strategy to combat habitat loss through the replication of topography, substrate, plant community, and flow regimes of natural wetlands (Kadlec and Knight 2004). Confirming the Field of Dreams hypothesis (if you build habitat, animals will come; Palmer et al. 1997), CWs can improve the regional diversity and abundance of avian, fish, and anuran communities reliant on wetland habitat (Soulliere and Monfils 1996; Snell-Rood and Cristol 2003; Balcombe et al. 2005; Fleming-Singer and Horne 2006; Lacki et al. 1992). Additionally, resource managers propose CWs as a low cost, minimal maintenance solution for polishing treated effluent to address deficiencies in traditional municipal wastewater treatment, namely the removal of pharmaceuticals and personal care products (PPCPs; Kivaisi 2002; Verhoeven et al. 2006). As a tertiary wastewater treatment system, CWs provide improvements to wastewater management by reducing concentrations of nitrogen and phosphorus in discharge, transforming and sequestering PPCPs, and reducing the chemical and biological oxygen demand in receiving waters (Rousseau et al. 2004; Fleming-Singer and Horne 2006; Scholz et al. 2007; Kadlec and Wallace 2008; Hsu et al. 2011).

Unfortunately, the implementation of CWs for wastewater treatment may degrade their ability to offer high quality wildlife habitat (Helfield and Diamond 1997; Pankratz et al. 2007), as excess nutrients and bioactive PPCPs sequestered in natural environments can interact with the physiological systems of native organisms (Koplin et al. 2002; Ebele et al. 2017; Richmond et al. 2017). Such contaminants, when absorbed or transformed by the wetlands, can remobilize into wetland food webs (Hammer and Bastian 1989; Devito and Dillon 2011) resulting in different community structure (Strand and Weisner 2011), altered competitive interactions (Kross and Richter 2016), and contaminant bioaccumulation (Barber et al. 2006) relative to natural wetlands. Many anurans are particularly vulnerable to the negative impacts of PPCP exposure due to obligate aquatic life stages and semi-permeable skin (Werner 1986; Alford and Harris 1988; Chelgren et al. 2006). Furthermore, adult amphibians in CWs will consume similarly exposed invertebrates, increasing the potential for biomagnification of contaminants and transport into terrestrial food webs (Valdés et al. 2014; Lanctôt et al. 2016; Burket et al. 2018). The possible population-level consequences of exposure to treated wastewater include changes in demography including smaller clutch sizes, higher larval survival, lower juvenile survival and growth, shifts in adult sex ratios (Laposata and Dunson 2000; Ruiz et al. 2010; Smith and Burgett 2012; Zeitler et al. 2021), as well as developmental abnormalities, such as malformed/extra limbs, open limb slits, missing eyes, edema, scoliosis, and calcinosis (Keel et al. 2010; Ruiz et al. 2010). These patterns suggest that CWs might not serve simultaneous functions in treating wastewater and providing high-quality wildlife habitat (Zeitler et al. 2021).

Animal behavior is a key link between internal physiological processes and the environment, with implications for individual and population-level fitness (Wong and Candolin 2015; Martin et al. 2017). Recent studies (Zeitler et al. 2019; Zeitler et al. 2021) on the morphological development of anurans in treated effluent from CWs found that, by most metrics of larval anural success, these tadpoles performed better than those raised in local pond water. Post-metamorphic morphological evaluations, however, revealed longer limbs but narrower heads in these individuals, which has been shown in other studies to be associated with reduced foraging success (Emerson 1985; Emerson and Bramble 1993; Tejedo et al. 2010). Therefore, this success at metamorphosis did not translate to the terrestrial environment at the juvenile stage (Zeitler et al. 2021). Research also shows that PPCPs can negatively affect the behavior of an organism, inhibiting antipredator responses (Martin et al. 2017), reducing foraging, and impairing locomotive ability (Barry 2014). For example, diphenhydramine occurs in large concentrations within wastewater treatment CWs and has behavior-altering physiological effects on aquatic organisms, including depressed muscle ability, augmented muscle twitches, analgesic effects, and reduced neuromuscular transmission, all of which can alter individual movement and responses to stimuli (Takiuchi 1964; Abdel-Aziz and Bakry 1973; Katyama and Tasaka 1985; Stevens 2012). Determining if and how treated wastewater may modify behaviors may identify a potential mechanism for altered developmental processes in wastewater, and provide insight into possible ecological side effects of development in treated wastewater, such as limited expression of anti-predator behaviors.

Wastewater differs from pond water in multiple ways that could contribute to altered tadpole behaviors. First, high concentrations of nitrogen and phosphorus stimulate algal blooms that increase turbidity and create high availability of food resources (Lewitus et al. 2008). Algal overgrowth may reduce foraging activity and refuge seeking behaviors (Zamor and Grossman 2007; Ostrand 2016). Although larval anurans often use chemical cues for predator detection, increased turbidity may inhibit detection of visual predator cues (Shingles et al. 2005; Ferrari et al. 2010; Zabierek and Gabor 2016). Secondly, treated wastewater includes PPCPs known to interfere with physiological movement and response processes by impairing the neural processing of cues, inhibiting the signaling pathways between the brain and musculature, and affecting muscle contractions or inducing hyperactivity (Kiesecker 2002; Palenske et al. 2010; Egea-Serrano et al. 2012; Johnson et al. 2019; Sievers et al. 2019). Furthermore, increased size at metamorphosis for larval anurans developing in treated wastewater is likely an interactive effect of increased resource availability and reduced activity or energy expenditures (Scheffers and Pszkowski 2015; Drayer and Richter 2016). Reduced activity in wastewater treatment CWs could also reduce the probability of encountering a predator, improving survival rates (Ham et al. 1995; Bridges 1999; Junges et al 2012).

The overall objective of our study was to evaluate the extent of tadpole exploratory behavior and predator response in wastewater. Because development in wastewater confers long-term effects (Zeitler et al. 2021), we also sought to determine if any behavioral shifts were due to development in wastewater or simply due to exposure. Specifically, we addressed the three following questions: (1) how does wastewater affect tadpole exploration, movement, and antipredator behaviors, (2) are affected behaviors associated with development in treated wastewater, and (3) why does wastewater affect behavior? We tested for two potential mechanisms behind altered behavior in wastewater including high turbidity and environmentally relevant concentrations of diphenhydramine. We selected diphenhydramine as one possible PPCP that could affect anuran behavior because it was one of 16 tested compounds that persisted after 5 mo in *ex-situ* mesocosms used in a previous study of anuran development (Zeitler et al. 2021). Diphenhydramine can act as an anuran analgesic, affects ecosystem processes, and regularly occurs in discharged effluent (Bartelt-Hunt et al. 2009; Stevens 2012; Burket et al. 2020; Robson et al. 2020). We expected that wastewater would reduce exploratory behaviors and inhibit antipredator behavior, and that this effect would be similar to effects observed in water with high turbidity and/or environmentally relevant concentrations of diphenhydramine. Secondly, we hypothesized that development of tadpoles in wastewater would be necessary to induce a behavioral difference and predicted that short term exposure to wastewater would not elicit behavioral changes.

Materials and Methods

Study system.—The municipal water cycle of Sewanee, Tennessee, USA, begins in a rain-fed pond before treatment and dissemination to the town. Returned wastewater undergoes traditional primary and secondary treatment. An experimental treatment operation pumps treated effluent from the secondary treatment lagoon into a three-cell wetland complex with native vegetation (described in Zeitler et al. 2018; Fig. 1). We collected wastewater at the outflow of the wastewater treatment CWs and pond water from an easily accessible rain-fed pond on the campus of the University of the South, Sewanee, Tennessee. We selected Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles for our experiments because they rapidly colonized and bred within the wastewater treatment CWs, and we used them in prior studies of this system (Zeitler et al. 2018; Zeitler et al. 2021; Fig. 1). We collected tadpoles for the two experiments described below from the same rain-filled pond where we collected water and from the end of the wastewater treatment cycle in the CWs. The CWs have no natural watershed and depend entirely on inputs of treated wastewater. The rain-fed pond has forested upland habitat surrounding the pond (> 50 m from the shoreline), but its complete watershed is not fully protected. The rain-filled pond has limited residential development within the watershed, and a hiking trail is mowed around its perimeter. Although contaminants have not been tested at this location, a nearby pond with similar surrounding land-use had low concentrations of pesticides known to impact amphibians (e.g., atrazine; Zeitler et al. 2021). At each site, we captured fish alongside tadpoles, but the dominant fish observed in the rain-filled pond was Bluegill (*Lepomis macrochirus*) whereas Western Mosquitofish (*Gambusia affinis*) dominated the wastewater treatment facility.

Figure 1. Treated effluent moves through three consecutive cells of a constructed wetland (CW) complex planted with native vegetation and colonized by native wildlife such as the Southern Leopard Frog (*Lithobates sphenocephalus*; inset) observed in this study at Sewanee, Tennessee, USA. The shallow second cell of the wastewater treatment CW is pictured here. (Photographed by Saunders Drukker).

Developmental origin and behaviors.—We designed a repeated-measures experiment using tadpoles captured from a rain-fed pond and from the wastewater treatment CWs. We collected 30 tadpoles between stages 26 and 30 (Gosner 1960) in July 2018 from the locations described above. We used a repeated measures approach and completed behavioral observations within 14 d of capture. One individual did not complete all treatments in the experiment leaving 29 and 30 trials per treatment. We tested tadpoles in a random order in pond water and wastewater and in the presence or absence of a caged predator for a total of four randomly ordered trials per individual. We chose *L. macrochirus* as the predator species because it is a common species in the small rain-fed ponds and regularly used to evaluate anuran antipredator behavior (e.g., Bridges and Gutzke 1997; Eklöv and Werner 2000; Smith et al. 2007).

Test enclosures were 37.85 L aquaria marked with a 1 × 1 cm grid on the bottom. We filled them with 9.5 L of water from the treatment source. We placed tadpoles into the tanks and monitored them for 30 min from a distance of 1 m. All aquaria had 10 × 10 × 10 cm cages at one end of the enclosure that housed a single L. macrochirus. We only used L. macrochirus with a total length < 5 cm for our study. The cage was constructed with 1 × 1 mm window screen to allow for transmission of visual and chemical cues. We introduced tadpoles to the aquarium on the opposite side from the cage. We replaced treatment water daily, and we washed aquaria with a bleach solution between treatments. We housed tadpoles in 37.85 L aquaria with 19 L of water collected from their capture location. We placed a bubbler in the aquaria, and we refreshed water halfway through our trials. We fed tadpoles Rabbit Chow (main ingredients: alfalfa, wheat, soybean, corn; 18% protein, 1.6% fat, 14.5–19% fiber) *ad libitum* when not being used in behavioral trials. Trials began approximately 24 h after capture to eliminate any post-capture behavioral differences (Hoffacker et al. 2018).

Response variables included latency to move, number of movements, and exploration. We defined latency as the time to emerge from an enclosure. We allowed tadpoles to acclimate to the enclosure for 5 min in a 35 cm^2 enclosure. At the end of the acclimation period, we opened a 4 × 4 cm gap to allow the tadpoles to leave the enclosure, which was when we began our observations. If tadpoles had not emerged after 10 min, we removed the enclosure from the test aquarium and excluded these individuals from analyses of latency. We quantified exploration as the total number of 1 × 1 cm boxes entered by the tadpole head. We also quantified the number of movements as the number of times that movement occurred. We counted a new movement after the tadpole was still (i.e., not entering or exiting any new grid boxes). Because some individuals had long movement latencies, we time-corrected the exploration and number of movement metrics to maintain independence among our response metrics. We used this approach because 10–13% of the variance in exploration and the number of movements were predicted by latency. If individuals had high latencies, we observed them for less time in the open container. Therefore, we divided exploration and number of movements by the time remaining after they emerged from cover to minimize the implicit relationship among the response variables.

To evaluate whether tadpoles exhibited behavioral differences after exposure to wastewater or after development in wastewater, we performed a Linear Mixed Model for each of our three behavioral response variables: latency to emerge, exploration, and number of movements. First, we evaluated correlations among independent variables, which resulted in us removing the number of movements response variable from our analysis. The number of movements was correlated with the exploration (r^2 = 0.75, P < 0.001) and latency to move (r^2 = 0.02, P = 0.019), which were unassociated with one another (r^2 < 0.001, P = 0.804). Predictor variables included the waterbody from which we captured tadpoles, the water source in which they were tested, the presence or absence of a predator, and interactions among these predictors. To account for repeated testing of individuals, we included individual identifiers as a random effect in each model. We performed all analyses in R, and Linear Mixed Models were performed with the lme4 package (Bates et al. 2015; R Development Core Team 2019). We assessed significance of tests with a = 0.05.

Mechanisms of altered behaviors.—We evaluated the behavioral responses of tadpoles captured from a rain-fed pond exposed to pond water, treated wastewater, pond water with clay added to increase turbidity, and pond water with added diphenhydramine. Using the same testing enclosure described above, we quantified latency, exploration, and burst distance for each individual. We time-corrected exploration for the time remaining after the tadpoles left the acclimation enclosure observing them for 20 min. At the end of the observation period, we measured burst distance by pinching the tail of the tadpole with forceps and quantifying the distance it traveled before resting again.

We created turbidity and diphenhydramine treatments using pond water we collected from the rain-filled pond where we collected tadpoles. To this water, we added clay or diphenhydramine to create the water for each treatment. We collected clay locally and added it to pond water until it reached 10 NTU, which was the mean turbidity of water collected from the wastewater treatment CWs. We dissolved diphenhydramine powder in pond water to create a solution of 85 ngL-1 representing the mean concentration of diphenhydramine found among the three cells of the local wastewater treatment CWs (Wright 2019). We added 9.5 L of pond water, pond water + clay, pond water + diphenhydramine, and wastewater collected from the discharge of the wastewater treatment CWs to aquaria for behavioral testing.

We collected 125 tadpoles from a rain-filled pond and housed them in an *ex situ* 350 L mesocosm filled with rainwater, a 1 L introduction of pond water, and 1 kg of dried oak-hickory leaf litter. At least 24 h prior to testing, we captured tadpoles from the mesocosm and housed them in an aquarium in the lab under the conditions described above. After observing their behavior in a single treatment, we released them at their capture location. We observed 30 tadpoles per treatment that were Gosner stages 26–30 (Gosner 1960). We completed all observations within 7 d in November 2019.

To evaluate our hypotheses regarding behavioral differences in tadpoles between wastewater and natural ponds, we used ANOVAs to assess how latency to emerge, exploration, and burst distance varied among treatments with pond water, wastewater, pond water with higher turbidity, and pond water with added diphenhydramine. Exploration and burst distance were correlated (r^2 = 0.05, P = 0.007), but neither was associated with latency (r^2 = 0.003, P = *0.565;* r^2 = 0.009, P = 0.174, respectively). In this instance, we decided to evaluate both outcomes because exploration was directly comparable with the first experiment, and burst distance also provides information about startle reflexes not previously measured. Although these three response variables were non-normally distributed (W > 0.72), one-way ANOVAs are robust to violations of the assumption of normality (Schmider et al. 2010). We used a Bartlett's test to assess homoscedacity and found that all three response variables met this assumption for ANOVA (K2 = 3.64–4.61, P = 0.204–0.303). For these analyses, we evaluated post hoc, pairwise comparisons using Tukey's Honest Significant Difference tests.

Results

Only one individual did not emerge from the refuge; we excluded it from the analysis of latency. Predator

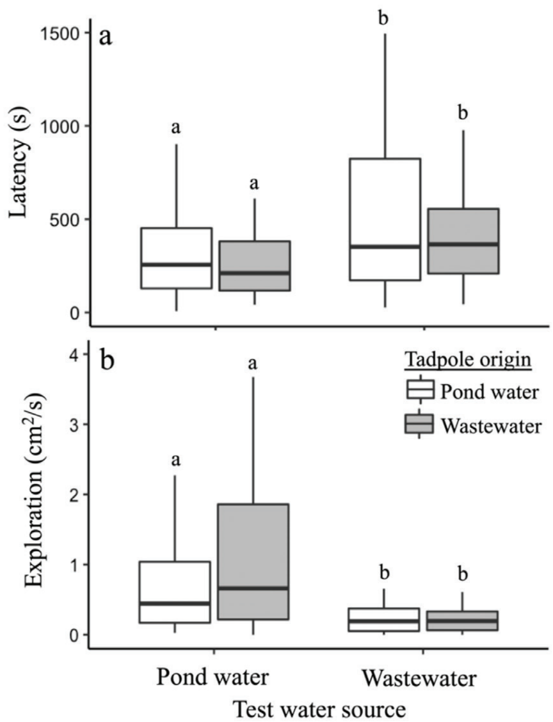

Figure 2. Quartiles of latency to emerge from shelter (a) and exploration (b) by tadpoles of the Southern Leopard Frog (*Lithobates sphenocephalus*) collected from a local pond and wastewater treatment facility at Sewanee, Tennessee, USA, when placed in local pond water or wastewater. Overall, tadpoles were slower to move and moved less distance when tested in wastewater regardless of where they were captured. Letters indicate significant differences from post-hoc tests (Tukey's Honest Significant Differences).

Table 1. Linear Mixed Models evaluating effects of predator (presence or absence), tadpole origin (pond water or wastewater treatment constructed wetland), and water source (pond water or wastewater treatment constructed wetland) on the latency to emerge from refuge and exploration of a novel enclosure by tadpoles of the Southern Leopard Frog (*Lithobates sphenocephalus*). Models included a random effect of individual, and we excluded the number of movements from analyses because it was correlated with exploration ($r^2 = 0.75$, $P < 0.001$) and latency to move ($r^2 = 0.02$, $P = 0.019$). The abbreviation df = degrees of freedom.

Response Variable/Factor	*F*-value	df	*P*-value
Latency			
Predator	2.01	1,87	0.158
Origin	0.27	1,87	0.601
Water	15.85	1,87	< 0.001
Predator × Origin	0.02	1,87	0.887
Predator × Water	2.73	1,87	0.101
Origin × Water	0.03	1,87	0.870
Predator × Origin × Water	0.49	1,87	0.485
Exploration			
Predator	1.19	1,88	0.278
Origin	0.85	1,88	0.358
Water	25.21	1,88	< 0.001
Predator × Origin	0.40	1,88	0.530
Predator × Water	0.10	1,88	0.750
Origin × Water	0.04	1,88	0.852
Predator × Origin × Water	0.14	1,88	0.712

presence did not influence latency to emerge (Table 1) nor did tadpole source location (Table 1; Fig. 2). Latency to emerge was greater when we tested tadpoles in wastewater relative to pond water regardless of their capture location (Table 1; Fig. 2). No interactions among predictors were significant (Table 1). We observed a similar pattern for exploration, as tadpoles moved more when in pond water relative to treated wastewater. The presence of a predator and tadpole origin did not influence exploration (Table 1; Fig. 2). No interactions among predictors were significant (Table 1).

Equivalent numbers of individuals across all treatments did not emerge from refuge during the latency trials, and thus, we removed 25 individuals from the analysis of latency. Latency to emerge was unassociated with treatment ($F_{3,92} = 1.55$, $P = 0.207$; Fig. 3), but exploration was associated with treatment ($F_{3,117} = 3.31$, $P = 0.023$; Fig. 3). Overall, exploration was less in wastewater relative to any other treatment (Fig. 3). Post hoc comparisons revealed that the only significant difference was between pond water with clay and wastewater treatments (Table 2), but there may be a marginal difference between wastewater and pond water with diphenhydramine treatments as well (Table 2; Fig. 3). Burst distance was also different among treatments ($F_{3,117} = 5.36$, $P = 0.002$; Fig. 3). Similar to exploration, burst distance was lowest among individuals tested in wastewater. Low burst distance in wastewater was significantly different only relative to pond water with clay and pond water treatments (Table 2; Fig. 3).

Discussion

Our data suggest wastewater directly affected the exploratory and movement behaviors of *L. sphenocephalus* tadpoles but did not change anti-predator behaviors. Tadpoles tested in wastewater were slower to begin moving, traveled less area, and exhibited the shortest burst distances. These findings are consistent with other research that shows decreased activity and lethargy in tadpoles after exposure to compounds in wastewater (Fraker and Smith 2004; Smith and Burgett 2005). Tadpoles from the wastewater treatment CWs were *L. macrochirus* naïve. Lack of prior experience

Table 2. Significance (*P*-values) of pairwise post-hoc tests using Tukey's Honest Significant Difference evaluating water treatments (pond water, turbidity, diphenhydramine, and wastewater) on tadpoles of the Southern Leopard Frog (*Lithobates sphenocephalus*) in terms of latency to emerge from refuge, exploration, and burst distance. The abbreviation DI = diphenhydramine.

Treatment	Pond water	Pond water + clay	Pond water + DI	Wastewater
Latency				
Pond water	—	0.411	0.324	0.991
Pond water + clay		—	0.999	0.559
Pond water + DI			—	0.473
Wastewater				—
Exploration				
Pond water	—	0.31	0.613	0.654
Pond water + clay		—	0.957	0.025
Pond water + DI			—	0.09
Wastewater				—
Burst distance				
Pond water	—	0.957	0.273	0.002
Pond water + clay		—	0.568	0.011
Pond water + DI			—	0.253
Wastewater				—

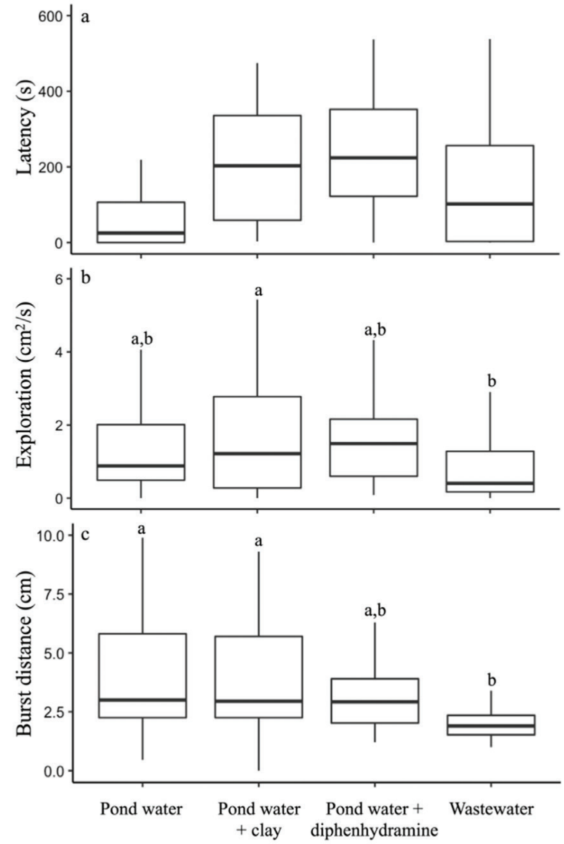

Figure 3. Quartiles of latency to (a) emerge, (b) exploration, and (c) burst distances of tadpoles of the Southern Leopard Frog (*Lithobates sphenocephalus*) when tested in four different water treatments. For response variables with a significant effect of treatment, letters indicate statistically significant differences from post-hoc tests (Tukey's Honest Significant Differences).

may have inhibited the common antipredator behaviors that tadpoles typically exhibit in the presence of *L. macrochirus* (e.g., Bridges and Gutzke 1997; Eklöv and Werner 2000; Smith et al. 2007); however, both sets of tadpoles failed to alter their behavior in the presence of this fish. The small *L. macrochirus* may not have been large enough before tadpoles to consider them a significant threat or the lack of water movement in the tank may have prevented accurate detection of predator risk on the far side of the aquaria (Eklöv and Werner 2000). Contrary to our prediction, short term occupancy in wastewater was enough to shift tadpole movement behaviors, suggesting that reduced movements are not a result of changes in developmental processes but rather immediate responses to conditions in the wastewater. Finally, our results show that neither turbidity nor diphenhydramine fully account for the behavioral differences we observed in *L. sphenocephalus* tadpoles exposed to wastewater.

Lethargy and reduced exploration are common responses of anurans to contaminant exposure and can lead to changes in related behaviors including swimming speed and escape responses (Jung and Jagoe 1995; Lavorato et al. 2013; Sievers et al. 2018; Sievers et al. 2019). Contaminants can directly and indirectly result in shifting behaviors. With short term exposure to some contaminants, shifts in oxygen transport, immune response, enzymatic activity, and even DNA damage can affect individuals at the most basic level (Kiesecker 2002; Egea-Serrano et al. 2012). Shifts in cardiac and metabolic function can induce fatigue that can manifest itself in limited movement and burst distances observed in this study (Palenske et al. 2010; Cheng and Farrell 2007; Costa et al. 2007; Johnson et al. 2019). Alternatively, some contaminants can indirectly compromise the sensitivity of an individual to external stimuli (Tierney et al. 2010; Moore et al. 2015; Sievers et al. 2018). For example, shifts in oxygen and conductivity associated with high concentrations of nitrogenous compounds can result in behavioral changes that prioritize responses to these conditions over others (Camarago and Alonso 2006). Notably, these behavioral effects could result from short-term exposure rather than chronic exposure during development in wastewater.

Chronic fatigue of tadpoles could also result in behavioral shifts that affect population processes through reduction in growth and survival (Laposata and Dunson 2000; Fraker and Smith 2004; Sievers et al. 2019; Zeitler et al. 2021). Although we did not observe differences in behavior of tadpoles relative to a predator, lethargy that manifests as reduced startle distance decreases the capacity to escape predators, thereby increasing predation risk (Azevedo-Ramos et al. 1992; Verrell 2000; Sievers et al. 2018; Sievers et al. 2019). Another experiment, however, observed lower survival of tadpoles in the absence of predators suggesting that other mechanisms may be necessary to explain lower survival of tadpoles in wastewater (Zeitler et al. 2021). Lethargy could alter energy dynamics by minimizing both energy needs and foraging activity (Horat and Semlitsch 1994; Dayton and Fitzgerald 2001; Krishnamurthy and Smith 2011). Furthermore, sublethal stress induced by contaminants can contribute to higher parasite loads (Koprivnikar et al. 2007; Kiesecker 2002), reduced immunocompetency (Gendron et al. 2003) and developmental and hormonal abnormalities of tadpoles, some of which can also inhibit movement like scoliosis, edema, and axial defects (McDaniel et al. 2004; Ruiz et al. 2010; Slaby et al. 2019; Wesner et al. 2020). Most of these effects, however, would require longer term exposure of tadpoles than they experienced in our study.

Lethargy is consistent with the effects of diphenhydramine on amphibian behavior. As a histamine antagonist, diphenhydramine minimizes inflammatory responses and induces drowsiness; however, diphenhydramine alone did not replicate behavioral changes in wastewater. Another compound, alone or in combination with diphenhydramine, may be necessary to induce the behavioral changes we observed (Crain et al. 2008; Hale et al. 2017; Sievers et al. 2019). For example, medications to treat high blood pressure like valsartan and propranolol also found in wastewater are known to induce lethargy and fatigue by slowing heart rates in the Great Basin Spadefoot (*Scaphiopus intermontanus*; Hillman et al. 1982). Likewise, feeding behaviors and reproductive success can change in response to these and other compounds (Shao et al. 2006; Oskarsson et al. 2014; Ding et al. 2015; Capolupo et al. 2018; Matus et al. 2018). Although identifying single compounds resulting in behavioral change would be helpful, most PPCPs occur in mixtures of chemicals, meaning that any shifts in behavior in wastewater could be difficult to attribute to any one compound (Crain et al. 2008; Cizmas et al. 2015; Hale et al. 2017; Sievers et al. 2019).

Increasing the turbidity common to wastewater was not associated with shifts in tadpole behavior in wastewater, and in fact, turbidity increases activity in prey species like tadpoles (Van de Muetter et al. 2005; Chivers et al. 2013). Like most studies investigating turbidity, we used silt and clay to simulate turbidity whereas turbidity in wastewater is typically a result of overgrowth of algae and cyanobacteria (Smith 1990; Bilotta and Brazier 2008; Martins et al. 2011). Clay and cyanobacteria can also produce interactions that could overwhelm their role in impacting turbidity. For example, clay as a negatively charged compound can bind to positively charged ions and can destabilize nanoparticles potentially influencing how tadpoles sense their environment (Zhou et al. 2012; Pal and Marschner 2016). Alternately, cyanobacteria regularly produce neurotoxins that impede larval anuran swimming frequency and speed and increase their vulnerability to parasites (Oberemm et al. 1999; Mastin et al. 2002; Webb and Crain 2006; Kotut et al. 2010; Buss et al. 2019). Cyanobacteria and algae are also food resources for tadpoles that could change their response to turbidity relative to an inorganic substance. Finally, it is possible that conditions of the pond water used in our study but otherwise typically absent in wastewater (e.g., chemical cues from large predatory fish), could minimize the effects of turbidity (Petranka et al. 1987; Mirza et al. 2006; Ferrari et al. 2008).

Exploratory behaviors in our study were clearly different in wastewater although we were unable to identify a causal mechanism. We also determined that these behavioral changes were the result of exposure to, rather than development in, wastewater. We recommend more studies to better understand the behavioral and population level consequences of exposure to PPCPs. Although this study focused on the effects of wastewater on larval behavior, it is possible that any life stage could be susceptible to lethargy induced by exposure to wastewater. Similarly, different species can also exhibit differences in how they respond to contaminants (Relyea 2009), and it is unclear whether these behavioral effects persist beyond the acute exposure to wastewater. Although these data exhibit consistent effects of exposure to treated wastewater, it also highlights significant research needs to understand how wastewater treatment CWs will affect amphibians. Anurans rapidly colonize and breed in wastewater treatment CWs (Ruiz et al. 2010; Zeitler et al. 2018), but poor larval development regularly results in reduced adult fitness (Werner 1986). The presence of wastewater treatment CWs, therefore, may negatively contribute to population-level success of local amphibians.

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