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Research article

Carryover effects minimized the positive effects of treated wastewater on anuran development

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ABSTRACT

Constructed wetlands (CWs) are a potential solution for wastewater treatment due to their capacity to support native species and provide tertiary wastewater treatment. However, CWs can expose wildlife communities to excess nutrients and harmful contaminants, affecting their development, morphology, and behavior. To examine how wastewater CWs may affect wildlife, we raised Southern leopard frogs, *Lithobates sphenocephalus,* in wastewater from conventional secondary lagoon and tertiary CW treatments for comparison with pondwater along with the presence and absence of a common plant invader to these systems – common duckweed (*Lemna minor*) - and monitored their juvenile development for potential carryover effects into the terrestrial environment. The tertiary CW treatment did not change demographic or morphological outcomes relative to conventional wastewater treatment in our study. Individuals emerging from both wastewater treatments demonstrated lower terrestrial survival rates than those emerging from pondwater throughout the experiment though experiment-wide survival rates were equivalent among treatments. Individuals from wastewater treatments transformed at larger sizes relative to those in pondwater, but this advantage was minimized in the terrestrial environment. Individuals that developed with duckweed had consistent but marginally better performance in both environments. Our results suggest a potential trade-off between short-term benefits of development in treated effluent and long-term consequences on overall fitness. Overall, we demonstrate that CWs for the purpose of wastewater treatment may not be suitable replicates for wildlife habitat and could have consequences for local population dynamics.

1. Introduction

In the past century, constructed wetlands (CWs) have been designed to facilitate wastewater treatment and potentially treat wastewater for reuse as a strategy for mitigating water scarcity (Tchobanoglous and Burton 1991; Sayadi et al., 2012; Qu et al., 2013). Like conventional wastewater treatment systems, CWs reduce pollutants such as excess nitrogen (N) and phosphorus (P) (Vymazal 2010). By mimicking biogeochemical processes of naturally occurring wetlands, CWs remove a host of inorganic and organic compounds that otherwise make treated wastewater unusable (Stottmeister et al., 2003; Vymazal 2008). Unlike conventional systems, CWs exhibit some success in removing pharmaceuticals and personal care products (PPCPs) including endocrine disrupting chemicals (EDCs), anti-inflammatory drugs, contraceptives, antibiotics, and neuroactive compounds (Hussain et al., 2012; Reyes-Contreras et al., 2012; Carranza-Diaz et al., 2014; Hijosa-Valsero et al., 2016; Papaevangelou et al., 2016; Li et al., 2017; Maine et al., 2017; Vymazal et al., 2017; Wojciechowska et al., 2017). However, removal rates are highly variable, and PPCP derivatives may remain after CW treatments (Halling-Sorensen et al., 1997; Oulton et al., 2010; Richmond et al., 2017). Simultaneously, CWs encourage the colonization and development of native wetland plant and animal communities, yet studies suggest that excess N and PPCPs can harm wildlife communities (Spieles and Mitsch 2000; DiMauro and Hunter 2002; Porej and Hetherington 2005; Verhoeven et al., 2006; Denton and Richter 2013; Drayer and Richter 2016). Little is known about potential effects of compound interaction or long-term impacts on wildlife using CWs as habitat (Gopal 1999; Hsu et al., 2011).

As ubiquitous wetland inhabitants with traits that make them indicators of environmental change, amphibians represent an ideal model system for studying the impact of elevated N and PPCPs in wastewater treatment wetlands on wildlife (Henry 2000; Degarady and Halbrook 2006). Wetland-breeding amphibians have aquatic life stages and permeable skin that often require both embryo and larval development

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in water, exposing the most sensitive life stages to PPCPs and other contaminants (Werner 1986; Alford and Harris 1988; Laposata and Dunson 2000; Chelgren et al., 2006). As consumers of wetland biofilms, amphibians ingest microbial organisms that metabolize nutrients and PPCPs (Ding et al., 2015; Du et al., 2015; Kastanek et al., 2018; Burket et al., 2019). Adult amphibians consume invertebrates, many of which will have developed and emerged from treatment wetlands with similar diets as tadpoles, potentially biomagnifying contaminants (Asku 2005; Valdés et al., 2014; Lanctôt et al., 2016; Grabicova et al., 2017). Amphibians function at intermediate trophic positions and link aquatic and terrestrial habitats allowing contaminants that bioaccumulate like pesticides and PPCPs to enter adjacent food webs with potential cascading effects (Flecker et al., 1999; Wilson et al., 2003; Ranvestel et al., 2004; Unrine et al., 2007; Regester et al., 2008; Colón-Gaud et al., 2009; Van Donk et al., 2016; Richmond et al., 2017; Parrish et al., 2019).

Wastewater CWs differ significantly from natural wetlands because they typically contain higher concentrations of organic matter, N, P and PPCPs (Scholz and Lee 2005; Brown et al., 2012; Reyes-Contreras et al., 2012; Ansola et al., 2014; Yi et al., 2017). Like most vertebrates, amphibians demonstrate developmental, physiological, morphological, and behavioral responses when exposed to high nitrate concentrations and a range of tested PPCPs (Hecnar, 1995; Rouse et al., 1999; Ortiz et al., 2004; Griffis-Kyle and Ritchie 2007; Marco and Ortiz-Santaliestra 2009). Studies have shown reduced feeding, as well as head and tail abnormalities in response to concentrations of nitrate as low as 3 mg/L (Rouse et al., 1999). Negative effects of N can directly impact anuran behavior and development, and indirectly decrease survival by promoting rapid development of aquatic predators through bottom-up trophic cascades (Johnston et al., 1990; Deegan and Peterson 1992; Robinson and Gessner 2000; Pearson and Connolly 2001; Gulis and Suberkropp 2003; Johnson et al., 2006). PPCP compounds, like steroids and the insecticide imidacloprid, may increase developmental rates of anurans, while others, such as estrogens, atrazine, and the antidepressant sertraline may result in heterochrony with growth of calcium nodules or accelerated and altered sexual maturation (Conners et al., 2010; Rohr and McCoy 2010; Carfagno and Fong 2014; Robinson et al., 2017). Behavioral responses as a result of PPCP exposure include shifts towards either hyper- or hypo-activity and changes to startle reflexes and feeding rates, all of which may affect an individual's maintenance requirements, potentially altering size and age at metamorphosis (Fraker and Smith 2004; Van Donk et al., 2016; Richmond et al., 2017; Zeitler et al., 2018; Parrish et al., 2019). In wastewater treatment facilities where PPCPs collect, anurans show high rates of developmental abnormalities and physiological disease including calcinosis and abnormalities in sexual development (Hayes et al., 2002; Keel et al., 2010; Ruiz et al., 2010; James and Semlitsch 2011; Park et al., 2014; Lambert et al., 2019).

Another factor that may affect wetland organisms is duckweed (*Lemna minor*), an aquatic plant that can quickly colonize eutrophic waters. Common duckweed can greatly transform aquatic habitats through bottom-up trophic interactions as well as abiotic changes such as shifts in pH and dissolved oxygen (Madoni 1991; Ceschin et al., 2019). By producing a thick vegetative mat at the water's surface, duckweed alters light penetration, heat and gas exchange (Madoni 1991; Ceschin et al., 2019), which can result in reduced organic matter decomposition, changes in microbial community composition, and lower plant taxa richness, suggesting that it has negative effects on animal communities (Ceschin et al., 2019). Duckweed, in combination with aquatic contaminants found in wastewater, may have population-scale effects on anuran fitness by affecting body size, survivorship, and reproductive success (Werner 1986; Smith 1987; Semlitsch et al., 1988; Morey and Reznick 2001).

Carryover effects exist in biphasic organisms where development in one ecosystem affects performance in another (Pahkala et al., 2001; Van Allen et al., 2010; Harrison et al., 2011; Earl and Semlitsch 2013). Amphibian larvae are commonly used for testing for carryover effects because tradeoffs exist between rapid larval development and survival in the aquatic environment and adult, terrestrial survival (Earl and Whiteman 2015; Székely et al., 2020). Although factors such as size at metamorphosis are commonly associated with adult fitness, aquatic contaminants can have latent effects that worsen post-metamorphosis, including impacts on sexual development (Reeder et al., 1998; Rohr et al., 2006; Boone et al., 2007; Storrs and Semlitsch 2008; Todd et al., 2011). Moreover, variables studied are often limited to aggregate responses of survival to or size at metamorphosis, despite demonstrated effects on other responses including head width, limb length, musculature, fat stores, locomotor performance and color deposition (Pahkala et al., 2001; Watkins 2001; Vonesh 2005; Richter-Boix et al., 2006; Scott et al., 2007; Van Allen et al., 2010; Ogilvy et al., 2012; Boes and Benard 2013). Leg length and head width are two easily quantifiable morphological traits with consequences for avoiding predation and successful foraging during resource scarcity (Székely et al., 2020). Attention to fine-scale responses may provide more mechanistic insights into long-term impacts of development in variable aquatic environments.

This study examined carryover effects of anuran development in wastewater to evaluate the potential of constructed wastewater treatment wetlands to support native wildlife populations. We evaluated metamorphic and post-metamorphic success of *Lithobates sphenocephalus* after development in pondwater, conventionally (lagoon) treated wastewater (secondary treatment), and conventionally treated wastewater after passing through a constructed wetland (tertiary treatment). Because wetland invasion by common duckweed often accompanies eutrophication, we also assessed whether the presence of *Lemna minor* could impact anuran development. We tracked population- and individual-level responses to development in the three water treatments with and without duckweed, including survival and morphology (body length, head width, leg length) and body condition in both the aquatic and terrestrial environment. Across all response metrics, we expected to observe negative impacts of treated wastewater on pre- and post-metamorphic development.

2. Materials and methods

2.1. Study system

The study took place in the Boiling Fork tributary of the Duck River draining the southern Cumberland Plateau, in the municipality of approximately 4000 people in Sewanee, Tennessee, USA. The water treatments in the first, aquatic phase of our study represent the beginning (pondwater from a rain-fed reservoir) and end (secondary- and tertiary-treated wastewater) of the municipal water cycle of Sewanee, Tennessee. Carryover effects were observed in a second, terrestrial phase of froglet growth and survival in upland oak-hickory forests common to the region. Our pondwater treatment was pumped directly from Lake O'Donnell, an 8.9 ha man-made reservoir that serves as the municipal water source. The reservoir has a nearly fully forested watershed and hosts similar plant and animal communities as other rain-fed basins in the area. Following treatment, water is distributed through the town and returned to our wastewater treatment facility where we used wastewater from two linked treatment systems - a conventional secondary treatment lagoon and a two-year-old experimental tertiary treatment constructed wetland.

Conventional treatment includes passing through screens to remove large solids (primary treatment) before flowing via gravity to the secondary treatment lagoons where it undergoes settling and microbial digestion. The effluent is split between two 5 ha (2.5 m deep) lagoons and slowly flows into a final lagoon, with a total treatment time of ~45 days. In practice, the effluent is treated with 5% hypochlorite solution before being sprayed onto the surrounding forest for discharge into zero order streams of the Boiling Fork watershed. Wastewater from the secondary treatment lagoon was pumped into the mesocosms before the chlorination step. In 2016, construction of a 0.20 ha experimental surface flow wetland complex was completed at the municipal wastewater treatment facility and used to test the efficacy of tertiary wastewater treatment in a CW. Wastewater from the secondary treatment lagoon is pumped through three sequential basins vegetated with native wetland plants. The basins vary in surface area, depth, and vegetative communities all designed to maximize nutrient retention and PPCP breakdown (for more details see Hopson et al., 2018 and Zeitler et al., 2018). Since establishment, the tertiary treatment wetlands have been effective at removing different forms of nitrogen and phosphorus though removal rates are seasonally dependent (Hopson et al., 2018). PPCP removal has been more variable with decomposition and recombination resulting in lower concentrations of PPCPs only in Basins 1 and 3 (Wright 2019). The CWs were rapidly colonized by anurans and *in-situ* observations indicated that reproduction and larval growth were occurring (Zeitler et al., 2018). Water representing tertiary water treatment came from the end of the wastewater treatment CW in Basin 3.

2.2. Study design

2.2.1. Aquatic pre-metamorphosis study

We designed a two-phase experiment allowing tadpoles to develop in different aquatic environments before being released into the terrestrial environment to observe carryover effects. For the aquatic environment, we designed a full factorial experiment with three different water sources and the presence or absence of common duckweed (*Lemna minor*). The three water sources (described above) included pondwater, secondary treated wastewater, and tertiary treated wastewater. Each treatment (water x duckweed) combination had 4 replicates for a total of 24 experimental *ex-situ* mesocosm units.

Our study organism was the southern leopard frog (*Lithobates sphenocephalus*), a species observed by Zeitler et al. (2018) to both breed and develop in the constructed wastewater treatment wetlands. This species breeds in early spring and metamorphoses after two to five months. On February 21, 2018, we collected 10 egg masses from a breeding aggregation at a rainfed reservoir similar to the reservoir where we collected pondwater, located 2.6 km from our study location. The egg masses were reared in a lab where they were placed individually into 10-gal aquaria, filled to a depth of 8 cm with rain-fed reservoir water, aerated with a bubbler, and kept in a 12:12 light to dark cycle at room temperature. Upon hatching, we removed the gelatinous egg mass and allowed the tadpoles to develop to a minimum of Gosner stage 20 (Gosner 1960; fewer than 4 days separated the mean hatching day of each egg mass). We haphazardly selected 5 tadpoles from each egg mass to form groups of 50 tadpoles that were randomly assigned to and released into *ex-situ* mesocosms on March 3, 2018.

The tadpoles were reared in *ex-situ,* 350-gal mesocosms that were assembled adjacent to the secondary wastewater treatment lagoon and the constructed wastewater treatment wetland complex. Water and duckweed treatments were randomly assigned to each mesocosm. On February 9, 2018, we filled each mesocosm with the appropriate water type to approximately ¾ full and added 1 kg of dried oak-hickory leaf litter collected from adjacent forest. After one week, we added 500 g (wet mass) of rinsed common duckweed collected from the municipal wastewater treatment facility. Each mesocosm was fitted with a standpipe to prevent water overflow, a 315-cm^2 mesh disk to allow metamorphic frogs to rest at the water surface, and a mesh covering that allowed sun and precipitation through but prevented predators like birds from entering the mesocosms. The mesocosms were allowed to settle for 22 days before introducing tadpoles.

To describe *ex-situ* mesocosm water characteristics, we assessed nitrogen and phosphorus fractions and a subset of PPCPs in February before introduction of water from these sources into the mesocosms and in May as tadpoles began to metamorphose. After 15 weeks, mesocosms had 4-12 times lower total N than at the beginning of the experiment, and total P was 2–6 times lower (Table 1; Hopson et al., 2018). However, patterns of the relative total N and P were secondary treated wastewater concentrations > tertiary treated wastewater > pondwater (Table 1; Hopson et al., 2018). Fractions of N retained their relative proportions through time, but variation in total P was not reflected in proportional changes in soluble reactive phosphorus (Table 1; Hopson et al., 2018). Overall concentrations of contaminants were lower in the mesocosms in May relative to February with below detection limit concentrations of atenolol, diethyltoluamide (DEET), methylphenidate, carbamazepine, norethindrone, norgestrel, medroxyprogesterone, and thiacloprid. Notably, DEET is typically detected in high concentrations in both wastewater sources but was low in our samples (Wright 2019). Of the remaining compounds, we observed little change between water introduction and mesocosm samples in acetaminophen, atrazine, and propranolol (Table 1). Sertraline in May was higher in both wastewater treatments relative to the beginning of the experiment, while caffeine, diphenhydramine, imidacloprid, and valsartan declined in proportion to their relative availability in treated wastewater. Conductivity was much lower in two wastewater treatment mesocosms in May relative to February, suggesting rainwater dilution of water treatments in the mesocosms (Table 1). However, decreasing concentrations of PPCPs over the spring and summer in the mesocosms is consistent with trends observed *in situ* (Wright 2019). Although it is unlikely that *ex-situ* mesocosms completely replicate *in-situ* conditions, it is likely that the relative differences among treatments will indicate shifts associated with *in-situ* differences.

The mesocosms were checked daily for metamorphic individuals captured by hand with dipnets. Individuals achieving Gosner stage 46 were considered to have completed metamorphosis and were captured for removal, measurements and marking (Gosner 1960). The froglets were kept moist in deli cups in the lab in the dark at room temperature for less than 48 h being released into terrestrial pens described below. During this time, they were lightly anesthetized in sodium bicarbonate buffered 1 gL^{-1} MS-222 until their righting reflex was impaired. We measured snout-vent length (SVL), mass, leg length of the right leg, and maximum head width and inspected them for any external abnormalities or injuries. The leg length was evaluated by positioning the right limb straight behind the individual and measuring from the cloaca to the longest toe tip. Individuals were also implanted with an 8 × 1.4 mm passive integrated transponder (PIT) tag (MiniHPT8; Biomark Inc., Boise, ID). Wounds from injection were not sealed or treated before being released, and we experienced a 0.84% tag loss rate. Because all future individuals were measured with their PIT tags, we took all measurements post PIT tag insertion and under light anesthesia for consistency of measurements (Hoffacker and Cecala 2018). We released the froglets after sunset to one of six randomly assigned terrestrial pens, ensuring individuals had more than 6 h to recover from anesthesia. On September 22, 2018, we emptied the mesocosms and removed any remaining tadpoles that were euthanized ($N = 4$, all from pondwater treatments). The number of metamorphic frogs removed from each mesocosm was tallied, and days to metamorphosis from mesocosm introduction was calculated for each individual.

2.2.2. Terrestrial post metamorphosis study

The terrestrial pens were installed 450 m from the mesocosms and surrounded by forest for more than 25 m. The pens were constructed by burying metal hardware cloth at least 10 cm deep leaving the walls of the pens 0.8 m tall. The mesh size was 6 × 6 mm to allow macroinvertebrates to pass between the inside and outside of the pens. The pens were arranged in two blocks of three pens that shared interior walls. Each pen was approximately 50 m^2, and through random assignment, 119–134 frogs were released into each pen for a maximum density of 2.4–2.7 frogs per m^2. We also placed two plastic irrigation trays (50 × 25 × 5 cm) filled with water into each pen because standing water was otherwise unavailable. Water in the trays was replenished as needed, and these trays also served as the locations where we released frogs into the terrestrial pens.

Metamorphic frogs were released into the pens from May–September

Table 1

To characterize the mesocosms, concentrations of PPCPs and fractions of N and P measured in samples collected directly from the two wastewater treatments in Feb 2018 prior to mesocosm filling and from mesocosm samples collected in May 2018 as tadpoles metamorphosed. The asterisk represents *in-situ* values that were only available from sampling in April 2018. "BDL" indicates measurements that were below the detection limits for a particular compound. All compounds were tested in triplicate with mean values reported. Means ± one standard error are presented for the four mesocosm replicates of each water and common duckweed (*Lemna minor*) treatments. *In-situ* samples were collected from the three water sources in February 2018 (immediately prior to mesocosm filling), and from the *ex-situ* mesocosms in May 2018 when the tadpoles began to metamorphose to describe the shift in water conditions during the period of tadpole development. *In-situ* samples were unavailable from the pondwater and were assumed to be below detection limits because they are filled with rainwater collected within a fully forested watershed. Seasonal shifts in *in-situ* conditions can be found in Hopson et al. (2018) and Wright (2019). Sixteen PPCPs, previously identified in wastewater at our site by Wright (2019), were extracted from the samples following a modification of EPA Method 1694 and analyzed by LC-MS/MS (Accela HPLC, TSQ Quantum Ultra mass spectrometer, Thermo Scientific, using methods described by Wright, 2018). Nitrite (NO_2^-), nitrate (NO_3^-), ammonia plus ammonium (NH_3+NH_4^+), total nitrogen (TN), reactive phosphate (orthophosphate PO_4), and total phosphorus (TP) were determined following protocols outlined in Hopson et al. (2018).

	In situ		*Ex situ* Mesocosms						Detection Limit
Water source	Secondary wastewater treatment	Tertiary wastewater treatment	Pondwater		Secondary wastewater treatment		Tertiary wastewater treatment		
Duckweed			Absent	Present	Absent	Present	Absent	Present	
Tested contaminants (ng/L)									
Acetaminophen	5.23	5.23	2.20 ± 1.39	1.38 ± 0.72	1.52 ± 1.52	2.85 ± 1.22	0.00	0.00	0.00
Atenolol	3.60	29.69	BDL	BDL	BDL	BDL	BDL	BDL	0.02
Atrazine	3.22	3.40	2.43 ± 0.23	2.62 ± 1.16	2.71 ± 0.30	1.25 ± 0.44	3.36 ± 0.56	1.08 ± 0.16	0.01
Caffeine	4919.63	96.37	5.05 ± 1.77	5.82 ± 0.80	14.67 ± 7.43	9.00 ± 0.91	5.29 ± 1.86	4.11 ± 0.60	0.59
Carbamazepine	2.04	2.31	BDL	BDL	0.10 ± 0.02	0.13 ± 0.02	0.13 ± 0.01	0.08 ± 0.04	0.04
DEET	126.05	82.02	BDL	BDL	1.10 ± 0.06	BDL	2.09 ± 0.84	BDL	0.32
Diphenhydramine	57.45	15.51	BDL	BDL	8.23 ± 2.22	3.51 ± 1.33	5.61 ± 1.58	1.97 ± 0.32	0.41
Imidacloprid	11.19	11.01	BDL	0.22 ± 0.05	0.46 ± 0.11	0.76 ± 0.22	0.71 ± 0.06	0.28 ± 0.24	0.03
Medroxyprogesterone	2.41	2.63	BDL	BDL	BDL	BDL	BDL	BDL	0.26
Methylphenidate	0.078	0.078	BDL	BDL	BDL	BDL	BDL	BDL	0.03
Norgestrel	24.87	24.87	BDL	BDL	0.24 ± 0.13	0.15 ± 0.05	0.23 ± 0.09	0.18 ± 0.02	0.06
Norethindrone	15.00	15.00	BDL	BDL	BDL	0.22 ± 0.05	0.29 ± 0.08	BDL	0.03
Propranolol	0.37	0.37	0.35 ± 0.13	BDL	0.50 ± 0.04	0.47 ± 0.10	0.54 ± 0.07	0.26 ± 0.06	0.06
Sertraline	0.56	0.56	BDL	1.68 ± 0.45	9.22 ± 5.35	1.3 ± 0.63	4.77 ± 1.58	3.09 ± 1.60	0.20
Thiacloprid	0.81	0.07	0.00	0.00	0.00	0.01 ± 0.01	0.02 ± 0.02	0.00	0.00
Valsartan	287.76	51.15	1.67 ± 1.80	2.70 ± 1.80	5.91 ± 4.02	5.28 ± 2.16	2.24 ± 0.31	4.13 ± 4.08	0.01
Tested nutrients (mg/L)									
Ammonium	4.53*	0.08*	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.004	0.03 ± 0.01	0.02 ± 0.009	0.03 ± 0.008	
Nitrate	0.22	0.49	0.03 ± 0.003	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.003	0.07 ± 0.02	0.04 ± 0.01	
Nitrite	0.02	0.07	0.007 ± 0.002	<0.001	0.006 ± 0.001	0.007 ± 0.001	0.03 ± 0.005	0.004 ± 0.001	
Total nitrogen	13.47	3.78	0.05 ± 0.004	0.06 ± 0.01	3.67 ± 3.26	0.28 ± 0.19	0.83 ± 0.21	0.53 ± 0.15	
Phosphate	2.47	2.54	0.04 ± 0.02	0.02 ± 0.01	0.04 ± 0.003	0.03 ± 0.004	0.11 ± 0.02	0.03 ± 0.007	
Total phosphorus	10.85	9.90	2.36 ± 0.29	2.42 ± 0.46	6.61 ± 0.96	6.44 ± 0.94	3.90 ± 0.50	3.92 ± 0.57	
Water quality									
Conductivity	239.87	234.30	12.89 ± 2.00	13.62 ± 1.79	42.03 ± 1.66	34.38 ± 2/97	40.55 ± 1.56	36.03 ± 1.60	
pH	7.54	7.26	6.89 ± 0.05	6.76 ± 0.16	7.25 ± 0.02	6.00 ± 0.05	7.28 ± 0.11	7.03 ± 0.11	

2018. Terrestrial recapture surveys began in July and continued monthly until October before the site experienced freezing nighttime temperatures. We resumed recapture surveys in February and April 2019 after frog emergence from overwintering refugia. Recapture surveys were performed over 48 h and included both daytime and nighttime surveys. Terrestrial juveniles were located using a Biomark HPR Plus Reader and BP portable antenna (Biomark Inc., Boise, ID), but only locations where live frogs could be found and captured were considered recaptures. Recaptured frogs were removed to the lab, anesthetized and measured using the same protocol as for metamorphic frogs (i.e. length, mass, right leg length, and head width), and released after surveys were complete.

2.3. Data analysis

We evaluated several individual variables to assess our hypotheses that water source and duckweed may influence short- and long-term outcomes of anuran development. Specifically, we evaluated SVL after complete resorption of the tail for body length (Gosner stage 46). Body condition was evaluated using the scaled mass index that has been proposed as a more accurate index of body fat in small vertebrates (Peig and Green 2009, 2010; MacCracken and Stebbings 2012). We followed the parameterization presented in Peig and Green (2009) to calculate the scaled mass index using SVL as the measurement of body size. Leg length and head width were standardized by the SVL (i.e. leg length or head width divided by SVL) to better evaluate potential differences in body proportions associated with feeding and locomotion.

We assessed the effects of water source and duckweed presence or absence and their interactions on survival and time to metamorphosis using mixed-effects Cox proportional hazard models with mesocosm identity as a clustering factor. For the survival model, we also included a variable indicating life stage (at the end of the aquatic life stage versus terrestrial life stages) and all potential interactions to assess whether the effects of water source and duckweed were consistent in the aquatic larval environment versus the juvenile terrestrial environment. A significant interaction between life stage and either of our predictors of water source and duckweed could indicate a carryover effect of the larval environment into the terrestrial environment. We assessed significance of individual factors using an analysis of deviance using Wald tests. All analyses were run in R (R Core Team 2017). Cox proportional hazard models were run using package survival (Therneau 2020) with post hoc tests using package emmeans (Lenth 2019).

For morphological outcomes, we evaluated how water source, duckweed presence, and life stage (at the end of the aquatic life stage versus terrestrial life stages) affected body length (SVL), body condition, size-corrected leg length, and size-corrected head width using a linear mixed effects model. We included random effects of mesocosm, terrestrial pen, month, and individual. All analyses were performed in R. Linear mixed models were performed using the package lme4 and evaluated using an analysis of deviance and Tukey post hoc analyses of significant factors using the package emmeans (Bates et al., 2004; Lenth 2019).

3. Results

We recovered 759 metamorphic *L. sphenocephalus* from all 24 mesocosms. The number of individuals recovered per mesocosm ranged from 10 to 44 individuals. Of the recovered froglets, we released 707 identifiable individuals into terrestrial pens due to mortality during transportation and PIT tag loss. We recaptured 198 individuals at least once, but only 36 individuals survived the winter with each water source represented by survivors (pondwater = 11, secondary treated wastewater = 12, tertiary treated wastewater = 13). We observed that 2.6% of individuals from secondary and tertiary treated wastewater had visible external abnormalities at metamorphosis, the most common of which was an inflamed upper jaw that protruded 2–3 mm beyond the lower jaw (N = 12). We also externally observed bony nodules of the long bones in the hind limbs through the transparent skin of the limbs (N = 8). While we never recaptured any of the individuals with the jaw abnormality in the terrestrial environment, we did recapture those with bony nodules that appeared, externally, to have resolved themselves. No abnormalities were observed in individuals reared in pondwater.

Survival to metamorphosis varied among water sources (Fig. 1; Table 2) but did not vary between the presence and absence of duckweed nor was there an interaction between water source and duckweed presence or absence (Table 2). We also observed a significant interaction between water and life stage. Survival was 14.2–22.6% higher in pondwater relative to secondary treated wastewater and tertiary treated wastewater ($z = 7.26$, $p < 0.001$; $z = 10.43$, $p < 0.001$ respectively), which were similar to one another (Fig. 1, $z = 1.47$, $p = 0.304$). Survival was 20.3–22.0% higher in the terrestrial environment overall relative to the aquatic environment with the biggest increase in survival observed for individuals reared in pondwater relative to all other water treatments ($z > 87.10$, $p < 0.001$; Fig. 1).

The first metamorphosed froglets were found 81 days after tadpole introduction to the mesocosms on May 23, 2018. The final froglets were removed from the mesocosms after 157 days on August 7, 2018. Time to metamorphosis was significantly different among water sources (Fig. 2; Table 2) and between the presence and absence of duckweed (Fig. 2; Table 2). We did not find a significant interaction between water source and duckweed (Table 2). Metamorphosis took 20–24 days longer in pondwater relative to secondary and tertiary treated wastewaters (Fig. 2, $z > 11.23$, $p < 0.001$). We observed no difference between the two wastewater treatments (Fig. 2, $z = 1.44$, $p = 0.299$). Transformation took 2.5 ± 0.66 days longer in the absence of duckweed (Table 2).

At metamorphosis, froglets ranged in snout-vent length from 15 to 40 mm (Fig. 3a) and in mass from 0.38 to 4.96 g. Length at metamorphosis differed among water sources and duckweed treatments but no interaction was observed between water and duckweed (Fig. 3a, Table 3). While length was significantly different among all water treatments (Fig. 3a; $t > 12.44$, $p < 0.001$), froglets from the secondary and tertiary treated wastewater were 7.65–10.87 mm longer than froglets that emerged from pondwater (Fig. 3a). Individuals from the tertiary treated wastewater were 1.91 ± 0.64 mm larger than individuals from the secondary treated wastewater at metamorphosis (Fig. 3a). Individuals in duckweed present treatments were 2.19 ± 0.53 mm larger than in duckweed absent treatments at metamorphosis (Fig. 3a). Snout-vent length increased into the terrestrial environment, and we observed a significant interaction between water source and life stage (Fig. 3b; Table 3). Terrestrial increases in length did not vary between secondary and tertiary treated wastewater (Fig. 3b, $t = 1.50$, $p = 0.670$). However, froglet size was 1.31–1.35 times larger for individuals reared in pondwater relative to treated wastewater (Fig. 3b; $t > 13.75$, $p < 0.001$). However, individuals from treated wastewater still retained larger body lengths than individuals from pondwater at the end of our experiment. Individuals from treatments with duckweed were 1.1 times larger across both life stages (Table 3).

Body condition of froglets was significantly affected by interactions between life stage and water source and life stage and duckweed presence. Body condition was higher in the terrestrial landscape than the aquatic landscape (Table 3), and body condition increased the most in tadpoles reared in treated wastewater relative to those from pondwater when they transitioned to the terrestrial landscape (Table 3). Body condition in the aquatic environment was 1.14–1.20 times higher in individuals reared in tertiary treated wastewater relative to those reared in pondwater (Fig. 3c; $t = 3.33$, $p = 0.022$), and this difference increased in the terrestrial environment with individuals reared in wastewater having a body condition 1.84 to 2.08 times greater than those reared in pondwater (Fig. 3d; $t > 20.84$, $p < 0.001$).

Size-corrected leg length was significantly affected by water source, an interaction between water source and duckweed and an interaction between duckweed and life stage (Table 3). Overall, leg length remained

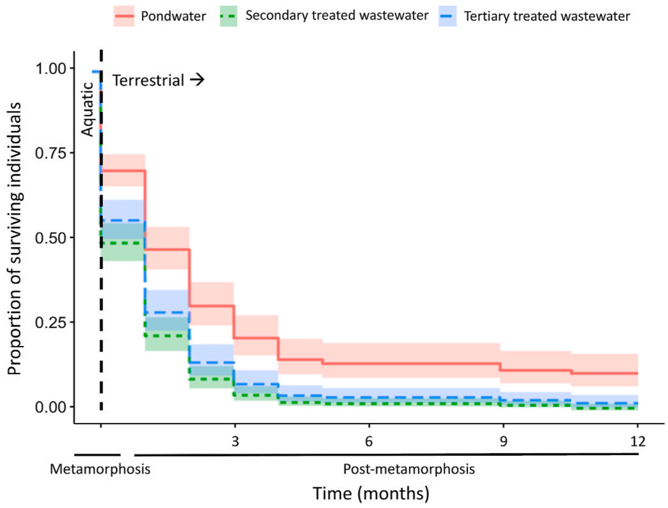

Fig. 1. Proportion of Southern leopard frogs (*Lithobates sphenocephalus*) surviving to metamorphosis in *ex-situ* aquatic mesocosms and post-metamorphic survival in terrestrial pens. Aquatic mesocosms held one of three water sources (pondwater and secondary and tertiary treated wastewater).

Table 2
Statistical results of Cox proportional hazard models evaluating the role of water source and common duckweed (*Lemna minor*) on survival and time to metamorphosis of Southern leopard frogs (*Lithobates sphenocephalus*) reared in mesocosms. Models for survival also included an additional fixed factor of life stage to evaluate differences in survival in the aquatic versus the terrestrial landscape.

	df	χ^2	p
Survival			
Water source	2	6.80	0.033
Duckweed	1	0.01	0.930
Life stage	1	8681.95	<0.001
Water source × duckweed	2	0.05	0.974
Water source × life stage	2	131.26	<0.001
Duckweed × life stage	1	1.10	0.294
Water source × duckweed × life stage	2	3.60	0.165
Time to metamorphosis			
Water source	2	134.13	<0.001
Duckweed	1	10.88	<0.001
Water source x duckweed	2	1.82	0.403

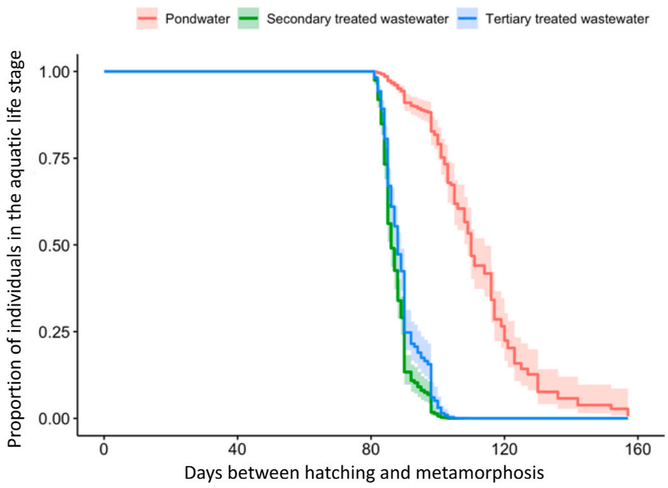

Fig. 2. Proportion of Southern leopard frogs (*Lithobates sphenocephalus*) individuals remaining as tadpoles in the aquatic environment while being reared in three different water sources in *ex-situ* mesocosms.

consistent in individuals reared in wastewater treatments regardless of duckweed treatment and was larger than individuals reared in pondwater with or without duckweed (Fig. 3ef; t > 4.44, p < 0.003). Leg length for individuals in pondwater with duckweed was 1.08 times larger than treatments without duckweed (Fig. 3ef; t = 3.583, p = 0.017). In the larval life stage in the aquatic environment, there was no difference between treatments with and without duckweed (Fig. 3e; t = 1.51, p = 0.448), but as juveniles in the terrestrial environment, leg length in individuals from treatments with duckweed was 1.03 times larger than those from treatments without duckweed (Fig. 3f; t = 2.99, p = 0.030).

Size-corrected head width was significantly associated with an interaction between water source and duckweed as well as between water and life stage (Table 3). In the presence of duckweed, head width was unassociated with water source (t > 0.515, p > 0.811), but in the

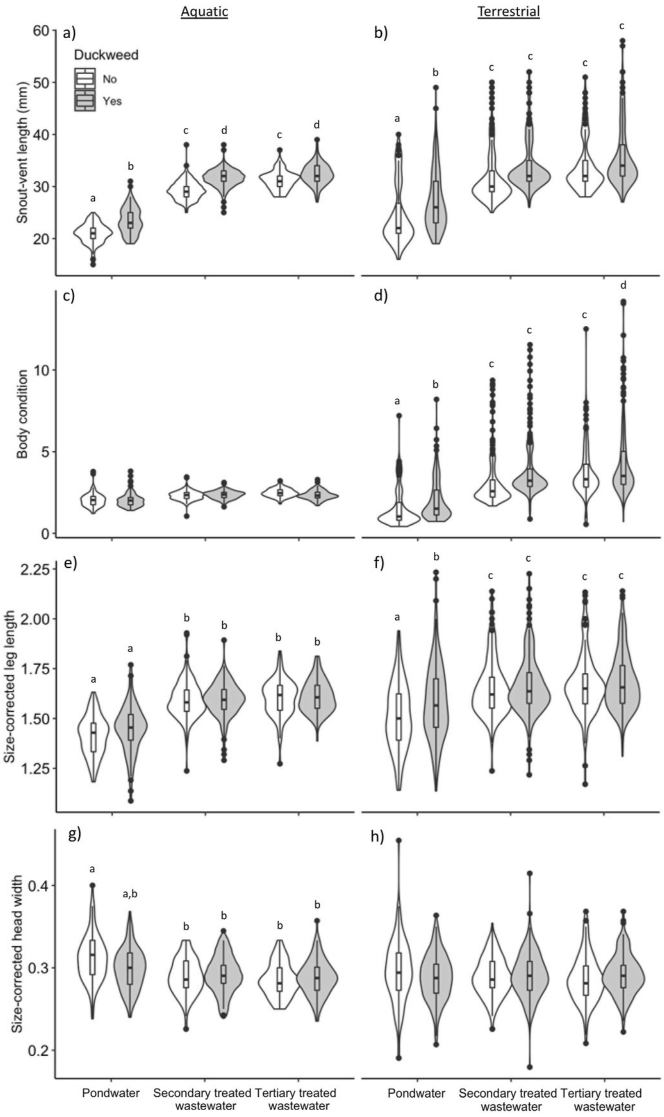

Fig. 3. Morphological measurements of Southern leopard frogs (*Lithobates sphenocephalus*) after being reared in *ex-situ* aquatic mesocosms with one of three water sources (pondwater and secondary or tertiary treated wastewater) and the absence or presence of common duckweed (*Lemna minor*) and in terrestrial pens post-metamorphosis. Morphology was quantified through snout-vent length (a, b), body condition calculated as the scaled mass index (c, d), leg length corrected for snout-vent length (e, f), and head width corrected for snout-vent length (g, h). Letters represent significantly different distributions.

Table 3
Statistical results of linear mixed models using an analysis of deviance evaluating the relationships between water source, common duckweed (*Lemna minor)*, and life stage on the morphology of pre- and post-metamorphic Southern leopard frogs (*Lithobates sphenocephalus*) reared in aquatic and terrestrial mesocosms. Leg length and head width were adjusted for body length.

	df	χ^2	p
Snout-vent length			
Water source	2	282.22	<0.001
Duckweed	1	39.74	<0.001
Life stage	1	1.45	0.229
Water source × duckweed	2	5.03	0.081
Water source × life stage	2	5.74	0.057
Duckweed × life stage	1	0.46	0.500
Water source × duckweed × life stage	2	2.70	0.259
Body condition			
Water source	2	301.23	<0.001
Duckweed	1	20.04	<0.001
Life stage	1	1.57	0.211
Water source × duckweed	2	0.26	0.880
Water source × life stage	2	580.56	<0.001
Duckweed × life stage	1	54.52	<0.001
Water source × duckweed × life stage	2	1.31	0.520
Size-corrected leg length			
Water source	2	103.87	<0.001
Duckweed	1	6.41	0.011
Life stage	1	1.83	0.176
Water source × duckweed	2	8.80	0.012
Water source × life stage	2	1.80	0.406
Duckweed × life stage	1	4.23	0.040
Water source × duckweed × life stage	2	5.50	0.064
Size-corrected head width			
Water source	2	14.51	<0.001
Duckweed	1	0.44	0.509
Life stage	1	0.04	0.833
Water source × duckweed	2	7.81	0.020
Water source × life stage	2	18.02	<0.001
Duckweed × life stage	1	1.25	0.263
Water source × duckweed × life stage	2	0.96	0.619

absence of duckweed, head width was 1.04–1.06 times larger in individuals reared in pondwater relative to the two wastewater treatments (Fig. 3gh; $t > 4.101$, $p < 0.006$). In the aquatic environment, individuals reared in pondwater had head widths that were 1.06–1.07 times larger than individuals reared in wastewater (Fig. 3g; $t > 4.575$; $p < 0.001$). When individuals transitioned to the terrestrial environment, differences between water treatments were eliminated (Fig. 3h; $t > 1.18$, $p > 0.558$).

4. Discussion

Overall, we observed few differences between secondary and tertiary treated wastewater, with froglets emerging from either wastewater source exhibiting larger morphometric measurements at metamorphosis than those reared in pondwater. However, these performance differences became smaller with time spent in the terrestrial environment. Tadpoles reared in wastewater took less time to transform and had lower survival than those in pondwater. We also observed a small but consistent improvement in performance when reared in treatments with common duckweed. Although we are unable to link consistent differences in performance with any single nutrient or compound found in treated wastewater, our results are likely conservative due to rainwater dilution of wastewater over the course of our experiment. We acknowledge that the effect sizes of differences may vary between our experimental mesocosm and *in-situ* conditions though they do replicate general patterns observed in tadpoles captured *in situ* (Skelly 2002; Zoeller and Vandenberg 2015; Zeitler et al., 2018). We also demonstrated complex interactions between morphometrics and demography as frogs move between aquatic and terrestrial environments. Regardless, these results suggest that wastewater treatment CWs may not serve as sustainable wildlife habitats and underscore the importance of carryover studies that follow post-metamorphic survival and growth.

We hypothesize that the morphological advantages we observed in metamorphic anurans from treated wastewater mesocosms, including larger body size, leg length and better body condition, were mainly due to the eutrophic state of the water sources. Nutrient enrichment, such as that from treated wastewater, increases microbial activity and leaf decomposition rates, which has also been linked to higher invertebrate abundance (Robinson and Gessner 2000; Pearson and Connolly 2001; Gulis and Suberkropp 2003). Higher microbial biomass and turnover in eutrophic habitats may also contribute to faster tadpole growth rates, something observed in other aquatic vertebrates though the composition of microbial communities can have complex effects (e.g. Taipale et al., 2018). This conclusion is also supported by the shortened time to metamorphosis and faster growth that we observed in treated wastewater (Johnston et al., 1990; Deegan and Peterson 1992; Johnson et al., 2006). Furthermore, in studies that manipulated food availability, researchers demonstrated results like those we observed in metamorphosing froglets from wastewater, demonstrating longer legs but narrower heads (Nathan and James 1972; Steinwascher and Travis 1983; Marian and Pandian 1985; Pfennig 1992; Richter-Boix et al., 2006; Gomez-Mestre et al., 2010). The increased resources available in nutrient-enriched wastewater may explain the trait-specific development that we initially observed in our metamorphic individuals.

Body size is often positively associated with fitness because anurans that metamorphose at larger sizes are larger at maturation and can produce more eggs, are less likely to starve, and more likely to avoid desiccation and predation (Tracey et al., 1993; Newman 1994; Semlitsch et al., 1988). For this reason, reduced survival and weaker terrestrial performance of froglets from treated wastewater were unexpected though not unprecedented. In a review study of carryover effects in amphibians, Earl and Whiteman (2015) note that there is a temporal component to environmental effects, meaning that fitness proxies that integrate a larger portion of the lifespan will be more accurate. Therefore, short-term outcomes are likely to exhibit larger magnitude effect sizes relative to long-term outcomes. Likewise, we cannot rule out that results from our terrestrial component may be the result of low recapture success and high mortality rates that may be unrelated to morphological parameters. Furthermore, our study may not have extended long enough to determine if this reversal of growth patterns persisted or simply reflected variation in aquatic versus terrestrial resource availability. However, several potential explanations exist for the minimization of effects between the aquatic and terrestrial environment, and it is likely that these mechanisms are non-exclusive and potentially synergistic.

Plasticity in anuran development allows them to take advantage of habitats with high temporal and spatial variability. Therefore, there is potential for anurans to alter developmental rates, allowing them to catch up in the terrestrial environment (Hector and Nakagawa 2012). Our study supports this mechanism, as we observed that growth rates of individuals reared in pondwater were accelerated in the terrestrial environment relative to individuals from treated wastewater. However, amphibians can also exhibit plastic developmental responses to conditions within these habitats, including resource availability and aquatic contaminants that may likewise affect post-metamorphic development. In contaminated environments, studies have demonstrated that rapid larval growth to escape poor aquatic conditions can come at the cost of slower development in specific body parts (Alvarez and Nicieza 2002; Fischer et al., 2004; Tejedo et al., 2010), higher metabolic rates, increased starvation potential, and weaker immune responses in juveniles (Stockhoff 1991; Gotthard et al., 1994; Gervasi and Foufopoulos 2008), which could potentially affect anurans developing in wastewater and contribute to reduced survival or growth rates. Studies examining this trade-off found that later-metamorphosing froglets, like those from pondwater, had a strong advantage in terms of terrestrial locomotor performance relative to those with an earlier metamorphosis (Ficetola and De Bernardi 2006). Furthermore, as larval intrinsic growth and development are prioritized, other fitness-related traits (*i.e.* locomotion, etc.) and resulting environmental and predation resilience are disadvantaged (Conover and Shultz 1995; Arendt et al., 2001; Fischer et al., 2004; Skelly 2004; Székely et al., 2020). It may be that conditions in the wastewater environments prompted early metamorphosis, juvenile growth patterns, and resulting survival patterns that we observed.

It is also possible that latent effects of aquatic contaminants or eutrophication may affect post-metamorphic development. Although we did not dissect individuals in this study to assess gonadal or other internal abnormalities, we did observe bony nodules that affected the long bones of their legs. If the nodules also affected their function, this change in function would not be observed in the aquatic environment but could affect predation and predator escape abilities in the terrestrial environment minimizing their success (Emerson 1978; Zug and Altig 1978; Gomez-Mestre et al., 2010; Tejedo et al., 2010). Similarly, preliminary research also suggests that skin microbiomes of individuals from treated wastewater differ considerably from those in pondwater (Zeitler Unpubl. Data). Recent studies have demonstrated that anuran microbiomes can secrete antimicrobial peptides and bioactive molecules, which can aid in resistance against predators, pathogens, and contaminants (Holden et al., 2015; Walke et al., 2015; Rebollar et al., 2018).

Eutrophication from treated wastewater is also advantageous for the growth of common duckweed, which has become a widespread plant nuisance in ponds and lakes where it forms a dense mat. In our study, duckweed formed a less dense and more diffuse cover, and the presence of common duckweed appears to confer consistent positive effects on frogs. However, whether these effects are biologically relevant is unknown. We hypothesize that this positive effect may be largely due to the addition of food resources with the presence of common duckweed. Though duckweed mats may limit light penetration, they provide a large surface area for algal growth at the surface of the water and contain high levels of protein and fat (Zirschky and Reed 1988). Finally, this physical mat of duckweed at the surface provides refuge from predator detection.

Despite observing differences in aquatic contaminant profiles between the *ex-situ* mesocosms and *in-situ* measurements, we observed the persistence of acetaminophen, atrazine, caffeine, diphenhydramine, imidacloprid, propranolol, sertraline, and valsartan (8 of 16 tested compounds) in mesocosms with treated wastewater. Although it is impossible to know which compound was responsible, or if interactions among compounds were responsible (Hayes et al., 2006), we did observe small proportions of individuals emerging from these treatments with developmental abnormalities of the leg long bones and upper mandible. Of the compounds listed above, studies on caffeine, propranolol, atrazine, acetaminophen, and sertraline have produced opposite outcomes from observed growth and survival in this study suggesting that these compounds were either at low enough concentrations to not affect amphibian development or their interactions with other compounds promoted opposing outcomes (Smith and Burgett 2005; Hayes et al., 2006; Owen et al., 2009; Connors et al., 2010; Rohr and McCoy 2010; Carfagno and Fong 2014). Little direct data is available for the remaining compounds to hypothesize what impacts they may have on anuran development. We recommend more research be conducted on the potential effects of PPCPs on amphibian development, but we caution that the reality of wastewater treatment is a cocktail of substances at low doses yielding non-monotonic dose responses and interactions among compounds that we could not control in this experiment. Furthermore, these compounds can have direct and indirect effects on factors important to amphibian success making it particularly difficult to identify the compounds with the largest effects (Rosi-Marshall et al., 2013; Pennington et al., 2016; Giang et al., 2018; Zolti et al., 2019). Finally, we recommend more testing for leakage of sewage lines and persistence of compounds in groundwater because we observed contamination of pondwater mesocosms with compounds like acetaminophen, caffeine, sertraline, and valsartan that may have come into our pondwater from sewage or from decomposition of duckweed collected from treated wastewater.

Although aquatic contaminants and nutrients can be documented to have negative, positive, or neutral effects on organisms, these compounds rarely affect our waterways individually (Ebele et al., 2017; Richmond et al., 2017). Instead, they occur as a mixture in which synergistic, additive, and antagonistic relationships exist among compounds and their effects on the ecosystem (Ebele et al., 2017; Richmond et al., 2017). Treated effluent returned to the environment is the principal way many of these contaminants enter the environment, and therefore, studies such as this evaluating cumulative effects are needed to contextualize studies on individual compounds. Individuals reared in pondwater transformed at smaller sizes but had higher overall survival than individuals reared in treated wastewater. The size advantage of individuals from treated wastewater was minimized as individuals developed in the terrestrial environment. The population-level impacts of development in treated wastewater will depend on the long-term balance between reproductive advantages of larger size versus lower larval and adult survival (Van Allen and Rudolf 2016). The effects we found to be associated with aquatic contaminants did not emerge until after certain developmental milestones, emphasizing the need for any future assessments to be extended throughout the focal organism's lifetime and the potential for tradeoffs between development in the aquatic and terrestrial environment. We also recommend further investigation of the behavior and broader impacts of PPCPs in the environment. Our observations focused on morphological abnormalities, however future studies may examine the behavioral and molecular effects on aquatic organisms. Additionally, assessing the population dynamics and trophic consequences of contaminant exposure can further inform our understanding of the impact and implementation of constructed wetlands. Though CWs may be effective at nutrient and contaminant removal (Vymazal et al., 2017; Hopson et al., 2018), our results demonstrate that this environmental technology could have potentially harmful effects on anurans and may not serve as a healthy wetland habitat for wildlife communities. Therefore, the establishment of wastewater treatment wetlands with the co-purpose of wildlife conservation should be approached with caution.

CRediT statement

Emma Zeitler: Conceptualization, Methodology, Investigation, Writing, Data curation, Funding acquisition. Kristen Cecala: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing, Visualization, Project administration, Supervision. Deborah McGrath: Conceptualization, Investigation, Writing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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