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ENVIRONMENTAL CONTAMINATION AND UNUSUAL SNAKE MORTALITY IN AN URBAN NATIONAL WILDLIFE REFUGE

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Abstract*.—National Wildlife Refuges (NWRs) collectively provide habitat for > 2,000 vertebrate species in the United States, including about 250 reptile and amphibian species. Although legally protected, wildlife populations in NWRs can be threatened by disease and human activities. To address these potential threats in southeast Louisiana, we conducted morbidity and mortality surveys for reptiles and amphibians in Bayou Sauvage (BS), Big Branch Marsh, and Bogue Chitto NWRs (958 search-hours, 10 sites total). We observed minimal morbidity/mortality at all sites except Haul Trail (BS-NWR), where dead snakes (Colubrinae, Dipsadinae, Natricinae, and Crotalinae species) were encountered at a high frequency (9.4-fold above background). Among intact carcasses, cause of death was undetermined (n = 9) or attributed to various pathogens (n = 5). To further investigate possible underlying causes of mortality, we conducted water exposure challenges (with embryos of Fowler's Toad, *Anaxyrus fowleri*) and soil contaminant analyses using samples from BS-NWR (n = six and 11 sites, respectively). Survival was reduced 92% or 48% among embryos exposed to water from Recovery Road (located 250 m from a landfill) or Haul Trail, respectively, versus the remaining sites. Soil analyses indicated at least seven potential sources of contamination, including the landfill, illegal dumping, pesticide migration, vehicle emissions, and leaked oil from a pipeline vent near Haul Trail. Although further research is needed to determine whether the observed mortality and contamination are related, our collective findings indicate that human activities may threaten wildlife populations in BS-NWR. More broadly, our study highlights the need for research and monitoring in NWRs.

Key Words.—amphibian; conservation; die off; disease; heavy metal; hydrocarbon; pollution; reptile

INTRODUCTION

With over 60 million ha (about 150 million ac), the National Wildlife Refuge (NWR) System is the largest conservation-focused network of lands in the U.S. and encompasses a greater diversity of ecosystems than any other protected area (Fischman 2002). Protected landscapes are crucial for herpetological conservation in the U.S. because habitat loss is the primary driver of reptile and amphibian declines in this country (Wilcove et al. 1998). In addition to protecting habitat for about 250 reptile or amphibian species and about 2,000 other vertebrate species (U.S. Fish and Wildlife Service [USFWS] 2013), the NWR System provides important social and economic benefits (Carver and Caudill 2019). Collectively, these lands are visited by about 53.6 million people each year and generate a combined $4.3 billion in sales, tax revenue, and employment income (Carver and Caudill 2019). Urban refuges increase nearby home values by 3–9% (Taylor et al. 2012) and, with a majority of Americans living in cities (U.S. Census Bureau 2015), help connect people with nature. Wetland refuges, in particular, provide important ecosystem services, including flood control (Bohannan et al. 2009) and water quality improvement (Rea et al. 2015). Yet, despite their well-established benefits, NWRs face many anthropogenic threats, the cumulative effects of which are unknown.

Wildlife populations within NWRs and other protected areas are threatened by factors that span socio-political boundaries, including disease and climate change (Griffith et al. 2009). Yet these populations may also face more localized threats, stemming from both legal and illegal activities. From 1985–1987, approximately 1,000 ducks in Kesterson NWR (Merced County, California, USA) died from selenium toxicity after exposure to contaminated water from agricultural lands (Ohlendorf 2011), prompting a rapid contamination assessment of the NWR System. Nearly 20% of refuges were reported as contaminated, but the majority of these determinations were based on observations or knowledge by refuge personnel, without any corresponding sampling data (U.S. Government Accountability Office 1987). In addition to agriculture, oil and natural gas development represent a potential source of contaminants, particularly for refuges located in Alaska and the Gulf Coast of the U.S. (Ramirez and Mosley 2015). Oil and natural gas extraction occur on refuge lands where mineral rights are owned by private entities or local governments (U.S. Government Accountability Office 2003; Ramirez and Mosley 2015). Of the 599 NWR units, about one-third contain hydrocarbon wells or pipelines (Ramirez and Mosley 2015). In 2015, 5,002 hydrocarbon wells were located in 107 NWR units (including 1,665 active wells in 45 units), and > 2,100 km of pipeline bisected these lands (Ramirez and Mosley 2015). The degree of oversight for oil and gas activities on NWRs varies widely, due to differences in authority, knowledge, and resources among refuge managers (U.S. Government Accountability Office 2003). Environmental contamination related to hydrocarbon extraction has been documented at multiple refuges, primarily in the Gulf Coast (USFWS 2014a,b,c).

Understanding how reptiles, amphibians, and other wildlife are affected by contamination represents a major challenge for effective management of public lands, given the lack of contaminant sampling data in most areas and the limited toxicological research in most taxa. In regard to reptiles, direct evidence for adverse effects of contaminant exposure within a NWR comes from a series of studies at Merritt Island NWR (Titusville, Florida, USA), an area exposed to toxins from the nearby Kennedy Space Center (Bowden et al. 2014). Heavy metals and perfluorinated alkyl acids were detected in blood/tissue from American Alligators (*Alligator mississippiensis*) in the refuge and linked to changes in metabolic and endocrine markers (Hamlin et al. 2010; Gunderson et al. 2016). Other studies have documented contaminant exposure among turtle populations in NWRs, including Loggerhead Sea Turtles (*Caretta caretta*; Cobb and Wood 1997), Common Snapping Turtles (*Chelydra serpentina*) and Painted Turtles (*Chrysemys picta*; Bell et al. 2006). Among other taxa sampled in NWRs, evidence for contaminant exposure has been found in fish (Welsh and Maughan 1994), birds (e.g., Finkelstein et al. 2003; Straub et al. 2007; Warren and Cutting 2011), mice (Allen and Otis 1998), and bats (O'Shea et al. 2001). Collectively, these studies highlight the need to better understand the extent and consequences of environmental contamination in NWRs.

As the largest (about 10,000 ha) urban wildlife refuge in the eastern U.S., Bayou Sauvage NWR (Orleans Parish, Louisiana) has substantial ecological and economic value. More than 300 bird species use the refuge, including 27 species of regional conservation interest (Bohannan et al. 2009). While the herpetological community has not been previously inventoried, the refuge is located in a regional hotspot of reptile and amphibian diversity (Jenkins et al. 2015). Composed mostly of marsh and open water, this refuge provides essential flood control for New Orleans and encompasses some of the last remaining wetlands in the area (Bohannan et al. 2009; Fig. 1). Yet, Bayou Sauvage NWR is heavily impacted by human activities;

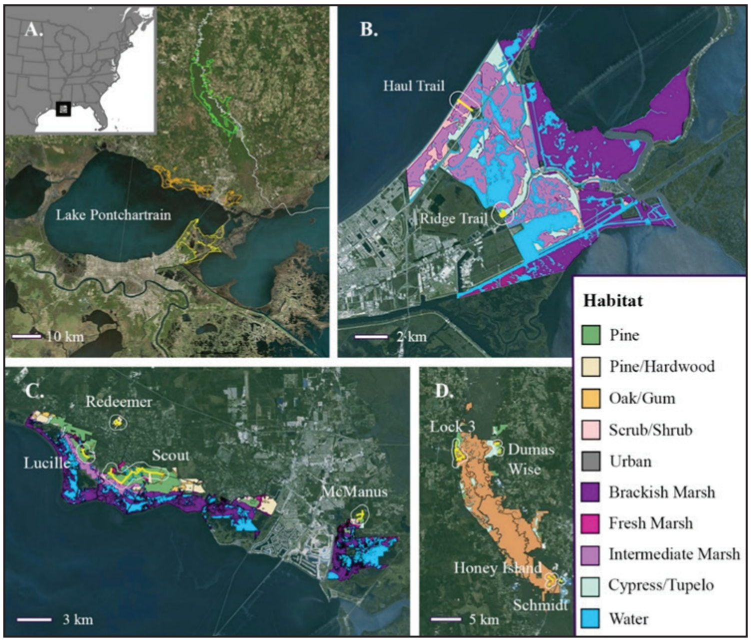

Figure 1. (A) Broad-scale and (B) detailed maps of Bayou Sauvage National Wildlife Refuge (NWR), (C) Big Branch Marsh NWR, and (D) Bogue Chitto NWR in Louisiana, USA. Search transects (yellow lines) within a 500-m buffer (white outline) were grouped together. Panel A inset illustrates the relative location of the study area (black square). (Habitat data shapefile provided by the U.S. Fish and Wildlife Service, and basemap from Esri, Redlands, California, USA).

approximately 60% of the refuge is impounded by a hurricane protection levee, making it susceptible to subsidence and saltwater intrusion (Bohannan et al. 2009). Furthermore, multiple potential sources of contaminants occur in or near the refuge, including an unlined landfill, illegal dumping, interstate (I-10) traffic, and oil/gas infrastructure (Bohannan et al. 2009).

Given the exceptional combination of anthropogenic threats and ecological benefits associated with Bayou Sauvage NWR, we evaluated reptile and amphibian morbidity and mortality in this refuge relative to two reference areas, chosen for their proximity to Bayou Sauvage: Bogue Chitto NWR and Big Branch Marsh NWR (Fig. 1). Both refuges are less affected by anthropogenic activity compared to Bayou Sauvage NWR (Breaux et al. 2007, 2011). Our two-part approach began with broad-scale visual surveys for diseased or dead amphibians and reptiles, followed by a targeted investigation of Bayou Sauvage NWR that included pathological evaluation of intact carcasses, a water exposure challenge using Fowler's Toad embryos (*Anaxyrus fowleri*), and soil contaminant analyses. Our objectives were to detect unknown risks (if present) to reptile or amphibian communities in the study area and investigate potential causes of observed morbidity/mortality, including environmental contamination.

Materials and Methods

Broad-scale morbidity/mortality surveys.—We conducted visual encounter surveys for dead or diseased reptiles and amphibians (in conjunction with a larger effort to establish species inventories) at Bayou Sauvage NWR (Orleans Parish, Louisiana, USA), Big Branch Marsh NWR (St. Tammany Parish, Louisiana, USA) and Bogue Chitto NWR (Washington Parish, Louisiana, and Pearl River County, Mississippi, USA). A single, experienced field biologist led all surveys, with 0–5 volunteer assistants, depending on their availability. Although most of the volunteers had minimal field research experience, all were similarly capable of detecting a carcass on a transect, particularly given that all transects were grassy paths, boardwalks, or roads. We selected transects (n = 10) based on accessibility (Supplementary Information Tables S1 and S2; Fig. 1) and visited them once every 2–3 weeks from 1 February 2015 to 15 January 2016, except for periods when daytime temperatures were below about 8° C or above about 37° C, given the lower likelihood of detecting amphibians or reptiles. We conducted surveys during the morning (0700–1159, 19–35% search effort per refuge), afternoon (1200–1759, 39–56% effort), and night (1800–0130, 26–30% effort).

Given the limited terrestrial habitat present, we focused Bayou Sauvage NWR surveys on two transects separated by 6 km: Haul Trail and Ridge Trail (Fig. 1). Haul Trail is a paved, about 5 m wide, 1 km long, former bicycle trail that transects intermediate marsh and is occasionally used for levee maintenance, but rarely visited by the public. Ridge Trail is a popular boardwalk located along a natural ridge, surrounded by intermediate marsh and the remnants of a Cypress Swamp that has been degraded by impoundment, Hurricane Katrina, and invasive species (primarily Chinese Tallow, *Triadica sebifera*, feral Hogs, *Sus scrofa*, and Nutria, *Myocastor coypus*).

We conducted surveys at Big Branch Marsh NWR (about 7,300 ha) at four transects with varying levels of public use, each separated by ≥ 2.4 km (Fig. 1). Lucille Trail transects Pine/Hardwood Forest, surrounded by intermediate marsh. McManus Trail crosses through scrub and Pine Flatwoods. Redeemer is the site of the Southeast Louisiana Refuge Complex Headquarters, surrounded by upland Pine Forest. Scout Trail transects intermediate marsh and Pine Flatwoods.

We conducted surveys at Bogue Chitto NWR (about 15,000 ha) at four transects with varying levels of public use, each separated by ≥ 5.3 km (except for Schmidt, separated from Honey Island by 0.8 km and an interstate highway; Fig. 1). Dumas Wise Trail crosses through Cypress/Tupelo Swamp and upland Oak/Pine Forest. Honey Island Trail is bordered by bottomland Oak/Gum Forest and Cypress/Tupelo Swamp. Lock 3 Trail parallels the Pearl River Canal through upland Pine Forest. Schmidt is a popular boardwalk that transects Cypress/Tupelo Swamp.

Targeted follow-up morbidity/mortality surveys.—Given the unusual mortality of snakes we observed, we conducted additional visual encounter surveys at Haul Trail (Bayou Sauvage) 3–4 times/week from 15 April 2016 to 31 December 2016, except from 27 June to 28 September to avoid disturbing alligator nests. We installed a box trap (0.9 × 0.9 × 0.3 m) equipped with a water dish and reservoir 1 m from the trail on 26 April 2016, with silt fencing (15 × 61 cm) extending parallel to the trail (Burgdorf et al. 2005). We checked the trap once every 3–5 d through 31 December 2016. We set three crawfish traps (51 cm × 1 m; Lee Fisher International, Tampa, Florida, USA) in water at each of two locations (separated by 300 m) along Haul Trail and checked these every 24–48 h for 192 trap-nights from 1 October to 20 December 2016 (traps were not deployed earlier in the study due to budget constraints). Outside of these survey periods, we visited the transect opportunistically, with 14 total surveys (0–3 per month) in early 2016 and throughout 2017. We conducted occasional opportunistic site visits in 2018, after the completion of the study, and noted the presence of any observed carcasses. We collected and chilled (n = 1, Accession #27573-001) or froze (n = 17), depending on post-mortem condition and submission timing, intact snake carcasses until postmortem examination.

***Necropsies*.**—We shipped all intact carcasses on ice packs to the National Wildlife Health Center (NWHC) of the U.S. Geological Survey for necropsy (Table 1, Supplementary Information Table S3). For all snakes subject to postmortem examination (n = 14), we recorded sex, age, body condition, postmortem state, and external and internal gross observations. We fixed samples of all organs in 10% neutral buffered formalin and submitted them to the Wisconsin Veterinary Diagnostic Laboratory (Madison, Wisconsin, USA) for routine processing. We embedded samples in paraffin wax, sectioned them at about 5 µm, and stained them with hematoxylin and eosin for histopathologic evaluation. We also collected fresh tissues for ancillary tests performed at NWHC, including microbial cultures (nine snakes), Polymerase Chain Reaction (PCR) for the pathogen (*Ophidiomyces ophiodiicola*) that causes Snake Fungal Disease (five snakes; Bohuski et al. 2015), parasite identification by direct examination, Giemsa-stained impression smears, Sheather's or acid fast exams (four snakes), and virus isolation in Russel's Viper spleen tumor or Vero cells (four snakes). Frozen samples, stored in Whirl-Pak bags, from five snakes were extracted via QuEChERS (https://nucleus.iaea.org/sites/fcris/Shared%20Documents/SOP/AOAC_2007_01.pdf) and screened for organic toxicological compounds via gas chromatography with a mass selective detector (GC-MS) using the Wiley Mass Spectral Library, 9th Edition (John Wiley & Sons, Inc., Hoboken, New Jersey, USA) at Michigan State University (Lansing, Michigan, USA). These samples consisted of: (1) pooled skin, muscle, and bone of three Glossy Swampsnakes (*Liodytes rigida*) collected 3 November 2015; (2) pooled skin, muscle, and bone of a Western Mudsnake (*Farancia abacura reinwardtii*) collected 6 December 2015; and (3) the liver of a Western Mudsnake collected 14 January 2017.

***Water exposure challenges*.**—To assess environmental factors as a potential cause of snake mortality at Bayou Sauvage NWR, we conducted water exposure trials using embryos from a model amphibian species, the Fowler's Toad. We obtained a single water sample from each of six locations in the refuge: Haul Trail South (HTS), Michoud Road (MIC; a site of frequent illegal dumping), Recovery Road North (RRN; about 250 m from an unlined landfill), Crabbing Bridge (CRB; a popular fishing site), Ridge Trail North (RTN), and Ridge Trail South (RTS; along a public boardwalk; Fig. 2). Although located near (< 1 km) a junkyard and landfill, RTN and RTS are hydrologically isolated from these potential contaminant sources by a natural ridge (Fig. 2). We collected water samples (about 19 L each) using new polyethylene containers, then transported on ice and vacuum-filtered them to remove pathogens and suspended solids (0.45 µm membranes; Pall Corporation, New York, New York, USA).

The embryos were produced as a single clutch by wild-caught Fowler's Toads at the Memphis Zoo following hormone stimulation on 29 March 2017 (male: 7.5 IU hCG/g body weight; female: 2.5 IU hCG/g body weight at -96 h and -24 h, followed by 12.5 IU hCG). Oviposition occurred overnight, and we considered midnight to be time 0 (i.e., fertilization). The next morning (15 h post-injection), we transferred embryos to polyethylene cups containing 400 mL of aged tap water (n = 50 embryos/cup). Embryos remained in aged tap water for 9 h during transport of the water samples. We equilibrated water samples

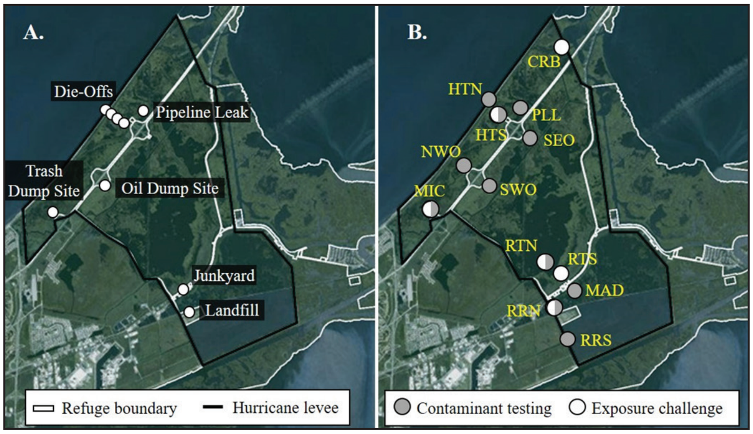

FIGURE 2. (A) Potential contaminant sources and (B) samples collected for sediment contaminant analysis or an exposure challenge using toad embryos and site water from Bayou Sauvage National Wildlife Refuge, Louisiana, USA. Site codes are Michoud Road (MIC), NW Overpass (NWO), SW Overpass (SWO), Haul Trail North (HTN), Haul Trail South (HTS), SE Overpass (SEO), Pipeline Leak (PLL), Crabbing Bridge (CRB), Ridge Trail North (RTN), Ridge Trail South (RTS), Madere Launch (MAD), Recovery Road North (RRN), Recovery Road South (RRS). (Basemap from Esri, Redlands, California, USA).

(stored at 4° C) to ambient temperature (19.8°–22.0° C) and added samples to embryos (three cups/site) through 25%, 25%, and 75% water changes at 24, 36, and 60 h post-fertilization, respectively. We assessed embryo status at 15× magnification (LaDage et al. 2012) within a 2.5-h window centered at 10, 34, 58, and 82 h post fertilization. Observers were blind to treatments.

Sediment analyses.—To evaluate potential environmental contamination in Bayou Sauvage NWR, we collected surface sediment samples (about 75 ml) on 17–18 April 2017 from 10 sites located 0–1 km from possible contaminant sources (Fig. 2). We collected an 11th sample on 18 July 2017 at a hydrocarbon pipeline leak (i.e., oil condensate escaping through a gas vent) that we discovered on the same date, 0.6 km east of Haul Trail (Fig. 2). We collected samples using new, laboratory-grade, plastic containers, transported them on ice to the Mississippi State Chemical Lab (Starkville, Mississippi, USA), stored them at -20° C, and analyzed them during 24 April to 31 May 2017 or 31 July to 1 September 2017 (11th sample only) for a broad group of organic and inorganic contaminants (Supplementary Information Table S4). Due to budget constraints, we only analyzed the pipeline sample for polycyclic aromatic hydrocarbons (PAHs) and heavy metals. We extracted subsamples via QuEChERS and analyzed these by gas chromatography and mass spectrometry (GC-MS) for PAHs (Environmental Protection Agency [EPA] 1998c) or a liquid chromatograph (LC) coupled to ultraviolet and fluorescent detectors for polychlorinated biphenyls (PCBs) and glyphosate (Cowell et al. 1986; EPA 1986). We quantified Sulfmeturon-methyl using a modified acid QuEChERS technique and LC coupled to a triple quadrupole mass spectrometer. We quantified heavy metals and inorganic compounds by microwave-assisted acid digestion (EPA 1998a) and inductively coupled plasma mass spectrometry (ICP-MS; EPA 1994) or a Milestone DMA-80 Analyzer (Hg only; EPA 1998b).

Statistical analyses and mapping.—We conducted all statistical analyses in R (R Core Team 2017), and considered results significant at $P \leq 0.05$. We evaluated differences in carcass frequencies among transects using a Generalized Linear Model with a negative binomial distribution and Tukey's Test (Hothorn et al. 2008). Given the low number of carcasses encountered overall, we combined data for all transects except Haul Trail. We analyzed differences in embryo survival among treatments using Kruskall-Wallace Rank Sum and Dunn tests in the dunn.test package of R (Dinno 2017). To increase statistical power, we combined treatments with ≥ 94% mean embryo survival into a single control group. We chose this cutoff given the large gap between 94% and the next lowest value (51%). We evaluated the relation between water quality (pH, salinity, and total dissolved solids) and embryo survival with a Kendall Rank Correlation using the Kendall package of R (McLeod 2011). Because the normal (i.e., baseline) levels of inorganic compounds at Bayou Sauvage are unknown, we defined elevated concentrations of inorganic compounds as those exceeding 95% confidence limits, calculated through an iterative process by removing values greater than mean ± 1.96 standard deviations until no outliers remained (Supplementary Information Table S5). We mapped our findings using ArcGIS (Esri, Redlands, California, USA).

RESULTS

Morbidity/mortality surveys at three NWRs.—We conducted 327 site visits and 958 person-hours of visual surveys, with 30%, 44%, and 26% of effort at Bayou Sauvage, Big Branch Marsh, and Bogue Chitto NWRs, respectively (Supplementary Information Table S2). All species observed at Bayou Sauvage NWR were also found at Big Branch Marsh or Bogue Chitto NWRs. We commonly observed dead snakes at Haul Trail (Bayou Sauvage NWR), with ≥ one carcass detected during 33 of 113 surveys (29%). Among all other sites, we observed three snake carcasses: a Southern Black Racer (*Coluber constrictor priapus*) in a vernal pond and two ribbonsnakes (*Thamnophis* sp.), one being predated by a hawk and the other likely killed by a mower, based on apparent injuries. The probability of encountering a snake carcass at Haul Trail was 9.4-fold greater than all other sites combined ($Z = -5.59$, $P < 0.001$; Fig. 3). The only other reptile mortality that we detected was an American Alligator and a Mississippi Green Watersnake (*Nerodia cyclopion*) at Bayou Sauvage NWR and a Ringed Map Turtle (*Graptemys oculifera*) at Bogue Chitto NWR, all outside the survey area. Excluding roadkill, we observed no amphibian carcasses.

Targeted morbidity/mortality surveys at Haul Trail.—Gross abnormalities were present in three of 204 (1.5%) live snakes at Haul Trail: a severely-emaciated Western Mudsnake, an emaciated Northern Cottonmouth (*Agkistrodon piscivorus*), and a lethargic Black-Masked Racer (*Coluber constrictor latrunculus*). The latter had a 3-cm-long section of shrunken, discolored skin on the ventrum, indicative of a severe burn (Supplementary Information Fig. S1). Overall, we observed 49 snake carcasses at Haul Trail, representing seven species (Table 1). Three of these carcasses were too decomposed for identification. Most of the identifiable carcasses represented fossorial (74%), versus terrestrial (17%), or semiaquatic (9%) species (Fig. 3; Supplementary Information Table S3). We

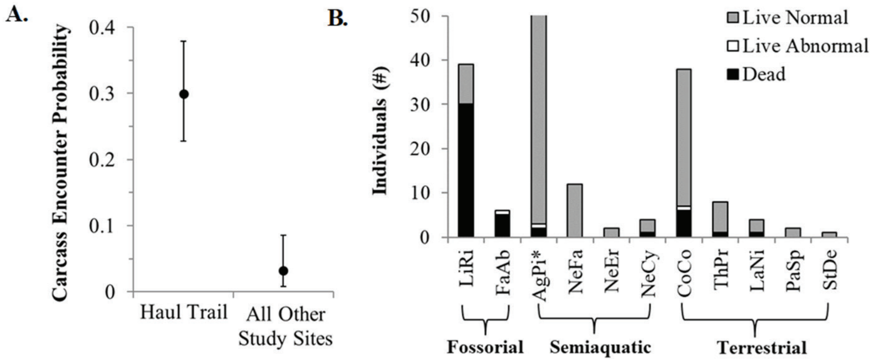

Figure 3. (A) Increased (Z = -5.59, $P < 0.001$) carcass encounter probability and (B) species observations at Haul Trail, Bayou Sauvage National Wildlife Refuge, Louisiana, USA. Species codes are Glossy Swampsnake (*Liodytes rigida;* LiRi), Western Mudsnake (*Farancia abacura reinwardtii*; FaAb), Northern Cottonmouth (*Agkistrodon piscivorus;* AgPi), Southern Watersnake (*Nerodia fasciata;* NeFa), Plain-bellied Watersnake (*Nerodia erythrogaster; NeEr*), Mississippi Green Watersnake Snake (*Nerodia cyclopion*; NeCy), Black-masked Racer (*Coluber constrictor latrunculus*; CoCo), Western Ribbonsnake (*Thamnophis proximus*; ThPr), Eastern Black Kingsnake (*Lampropeltis nigra*; LaNi), Gray Ratsnake (*Pantherophis spiloides*; PaSp), Dekay's Brownsnake (*Storeria dekayi*; StDe). The asterisk is for the total of 108 live, normal Northern Cottonmouths (smaller Y-axis scale shown for comparisons).

observed carcasses throughout the study period, with the first detection on 22 May 2015 and the most recent on 12 November 2017 (a small number of site visits conducted opportunistically in spring and fall 2018 documented additional carcasses; data not included). Over the study period, the number of carcasses detected per month correlated positively with numbers of site visits (r = 0.69, P = 0.013) and live snakes (r = 0.75, P = 0.005; Supplementary Information Fig. S2). Two carcasses showed signs of predation and were therefore not collected for necropsy: a Black-Masked Racer that was consumed by a conspecific and a mucus-covered Mississippi Green Watersnake adjacent to a live Cottonmouth (suspected regurgitation).

Necropsies.—Of the 49 carcasses detected, we determined 18 to be suitable for necropsy (i.e., adequate post-mortem condition and death not caused by injury/predation; Table 1, Supplementary Information Table S3). Four of these carcasses (Glossy Swampsnakes) were not examined because six conspecifics from the same mortality event were necropsied (preserving the opportunity to evaluate these four carcasses in the future, when more diagnostic tools may be available). We conducted postmortem examinations on the remaining 14 carcasses, including four females, three males, and seven snakes for which the sex was not determined (Table 1, Supplementary Information Table S3). Ages included hatch year (n = 1), second year (n = 1), immature (n = 8) and adult (n = 4). Body condition ranged from excellent (n = 3) to good (n = 6) to fair (n = 2) to poor (n = 1) to emaciated (n = 2). Postmortem state varied from excellent (n = 1), to good (n = 2) to fair (n = 10), and we did not record it for one snake. Culture was positive for *Salmonella* sp. in six cases. PCR for *Ophidiomyces* sp. was positive in one of the three carcasses tested. Virus isolation was negative for all tested cases (n = 4). A subset of carcasses in both groups (determined versus undetermined cause of death) was infected with various classes of parasites, including *Cryptosporidium, Hepatozoa*, and multiple phyla of helminths (Table 1). Toxicological screenings of three tissue samples (representing pooled skin, muscle, and bone from four snakes and liver from a fifth snake) were negative for toxic organic compounds that can be detected by GC-MS. We could not identify the cause of death in nine of the 14 carcasses we examined, including one that showed pathology indicative of a viral infection (Table 1). Among the remaining five carcasses, we attributed three deaths to salmonellosis, one to protozoal infection, and one to bacterial septicemia (Table 1).

Water exposure challenges.—All embryos were hatched or dead by 82 h post-fertilization. At 10 h and 34 h post-fertilization, survival was high (≥ 96%) in every replicate and was similar among treatment groups ($\chi2 \leq 1.60$, df = 5, $P \geq 0.449$; Supplementary Information Fig. S6). At 58 h, survival was reduced in embryos exposed to water from HTS (mean = 78.0 ± [standard error] 10.3%) and RRN (43.3 ± 21.8%) compared to reference sites (99.3 ± 4.7%; z ≥ -2.30, $P \leq 0.011$; Supplementary Information Fig. S6). The same pattern was observed at 82 h, with reduced survival in embryos exposed to water from HTS (50.7 ± 25.3%) or RRN (7.3 ± 4.1%) compared to reference sites (98.7 ± 6.2%; z ≥ -2.44, $P \leq 0.007$; Supplementary Information Fig. S6). At each time point, survival was similar among reference sites (≥ 94%; $\chi^2 \leq 4.52$, df = 5, $P \geq 0.210$). Survival was unrelated to water pH, conductivity, total dissolved solids, or salinity ($\tau \leq -0.20$, $P \geq 0.209$; Supplementary Information Fig. S6).

Table 1. Summary of significant necropsy, diagnostic and histopathologic findings of snake carcasses collected at Haul Trail in Bayou Sauvage National Wildlife Refuge. For each species, the number of individuals necropsied out of the total number found dead are: Glossy Swampsnake (*Liodytes rigida*; 7/30), Western Mudsnake (*Farancia abacura reinwardtii;* 2/5), Western Ribbonsnake (*Thamnophis proximus;* 1/1), Black-masked Racer (*Coluber constrictor latrunculus*; 3/6), Northern Cottonmouth (*Agkistrodon piscivorous*; 1/2), Mississippi Green Watersnake (*Nerodia cyclopion*; 0/1), Eastern Black Kingsnake (*Lampropeltis nigra*; 0/1), unidentified (0/3).

Case and Accession(s)	Collection Date	Species*	Cause of Death	Significant Findings
26922-001-006	3–5 November 2015	Glossy Swampsnake (n = 6)	Undetermined	Minimal skin infection consistent with snake fungal disease in one carcass. PCR and fungal culture negative for *Ophidiomyces*.
26922-011	6 December 2015	Western Mudsnake (n = 1)	Salmonellosis	*Salmonella* sp. III_38:(k):z35; Granulomatous colitis and bacterial septicemia
26922-012	17 January 2016	Western Ribbonsnake (n = 1)	Protozoal infection	Heavy protozoan infection (possibly *Hepatozoon* sp.) of liver; 3-cm segment of blackened skin and muscle on ventrum, consistent with thermal/chemical burn.
27197-001	19 April 2016	Glossy Swampsnake (n = 1)	Undetermined	Hepatic necrosis, interstitial pneumonia, interstitial nephritis; suspected viral infection; emaciation.
27573-001	3 October 2016	Black-masked Racer (n = 1)	Undetermined	*Salmonella* sp. III_21:z10:e,n,x,z15 culture only; *Cryptosporidium* infection of stomach, myositis, few intestinal parasites (*Kalicephalus* sp., *Coccidia* sp.), emaciation.
27595-002	19 October 2016	Black-masked Racer (n = 1)	Bacterial septicemia with severe, acute hepatic infarctions	Hepatic necrosis with infarctions*, Edwardsiella tarda* and *Yarrowia lipolytica* isolated from liver, *Salmonella* sp. III38:(k):- culture only; *Ophidiomyces* positive by PCR but no associated fungal skin lesions (suspected environmental contamination); helminth (acanthocephalana, cestoda, trematoda, and nematoda) infection of lung and intestines; protozoal infection in blood vessels.
27595-003	23 October 2016	Northern Cottonmouth (n = 1)	Undetermined	*Cryptosporidium* infection of stomach, widespread protozoa infection (suspect *Hepatozoon* sp.), possible *Salmonella* sp. III_21:z10:e,n,x,z15 infection of liver (may be artifact of decomposition); *Ophidiomyces* positive by PCR but no associated skin lesions (suspected environmental contamination); emaciation.
27655-001	11 December 2016	Black-masked Racer (n = 1)	Salmonellosis	Necrotizing hepatitis, colitis, proctitis, and coelomitis; *Salmonella enterica* serovar Infantis III_47:r:z53 isolated from liver and colon; minor esophagitis.
27695-001	14 January 2017	Mud Snake (n = 1)	Salmonellosis	*Salmonella* sp. III_60:r:z; Hepatic and intestinal necrosis, low numbers of intestinal nematodes (*Capillarid*, *Gnathostoma* sp.) and cestodes; cutaneous scars with no inflammation or microorganisms**.**

Sediment analyses.—At least one contaminant class (PAHs, pesticide, PCBs, and elevated heavy metal or other inorganic compound) was detected in all 11 sediment samples, including the two samples collected from Haul Trail (i.e., the die-off site; Table 2). Polycyclic aromatic hydrocarbons (PAHs) were present in six samples, with the highest concentration at the pipeline leak (Fig. 4, Table 2, Supplementary Information Table S6). Glyphosate, a pesticide not used by refuge managers, was detected at four sites (Table 2, Supplementary Information Table S6). Sulfometuron methyl was not detected at any site. Polychlorinated biphenyls (PCBs) were only detected at SW Overpass (Table 2, Supplementary Information Table S6). Elevated levels of at least one inorganic compound was detected at every site (Fig. 4; Supplementary Information Table S7).

Discussion

We documented a complex pattern of contamination in Bayou Sauvage NWR and ongoing mortality among a taxonomically diverse group of snakes. To our knowledge, this is the first report of mass mortality in a free-ranging snake population not caused by snake fungal disease. The die-offs were highly unusual, being localized to a single transect, occurring over

Table 2. Sample characteristics and dry-weight contaminant concentrations of Bayou Sauvage National Wildlife Refuge sediment. The abbreviation ND = not detected. An asterisk (*) denotes carcinogenic substances: Benzo[a]anthracene, Benzo[a]pyrene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Chrysene; Non-carcinogenic: Acenaphthene, Anthracene, Fluoranthene, Fluorene, Naphthalene, Phenanthrene, and Pyrene (EPA 2018). Two asterisks (**) are for compounds reported when concentrations exceeded mean ± 1.96 standard deviations, using standard symbols.

Location	Distance (km) from Die-Offs	Moisture (%)	PAHs (ppb)*		PCBs, Heavy Metals**, Glyphosate, and Inorganic Compounds** (ppm)
			Total	Carcinogenic	
Haul Trail North	0.0	54.2	16	16	Tl (0.5), K (3,987), NO_2^- (11.1)
Haul Trail South	0.0	86.3	ND	ND	NO_3^- (274), SO_4^{2-} (968.7)
Pipeline Leak	0.6	63.1	1,624	194	Be (0.4)
Southeast Overpass	0.8	85.6	87	87	Cd (2.7), Pb (215)
Northwest Overpass	2.3	81.2	ND	ND	NO_3^- (20.1), Se (4.5), glyphosate (0.97)
Southwest Overpass	2.5	67.8	628	234	Cd (2.3), Cr (46.8), Hg (0.5), Mn (518), Pb (363), PCB 138 (0.01), NO_2^- (10.1), glyphosate (0.46)
Michoud Boulevard	4.5	89.8	ND	ND	SO_4^{2-} (4,391)
Ridge Trail North	6.2	60.5	ND	ND	As (44.0), Cr (39.0), Tl (0.5)
Madere Launch	6.8	71.0	141	71	Cl^- (11,628), Na (4,622), SO_4^2 (33,102)
Recovery Road North	7.2	63.0	770	113	SO_4^{2-} (1,660), glyphosate (0.17)
Recovery Road South	8.4	50.7	ND	ND	Ca (39,926), glyphosate (0.20)

a prolonged (≥ 2.5 y) period, and being associated with multiple diseases that are rarely reported in free-ranging snakes. Furthermore, infectious disease was not implicated in all deaths, indicating the contribution of other factor(s). Sediment contamination (identified at the die-off location and at other nearby locations), the natural history of affected species (i.e., the disproportionate representation of fossorial species in carcasses, considering the preferential binding of most contaminants to soil), and results of an experimental exposure indicate the need for further investigation of contamination as a possible factor in the die-offs. We documented multiple classes of contaminants at sites up to 8 km from observed mortality, indicating a larger scope of potential ecological effects than reported here.

The frequency of snake carcasses at Haul Trail was substantially greater than other survey sites, yet the population-level effects of this finding are unclear. The lack of amphibian carcasses at Haul Trail may be due to low detectability because live amphibians were rarely observed but were often heard in the surrounding marsh. With almost no prior information about the herpetological community in Bayou Sauvage NWR, it is difficult to determine how the underlying cause(s) of mortality may have affected reptile or amphibian populations. Inferences about the baseline herpetological assemblage are complicated by the extensive anthropogenic impacts to the area. Regardless, there is potential for population-level impacts from the die-offs, given the high frequency of carcasses (about nine-fold above background), the extended timeframe of carcass detection, and the taxonomic diversity of the carcasses.

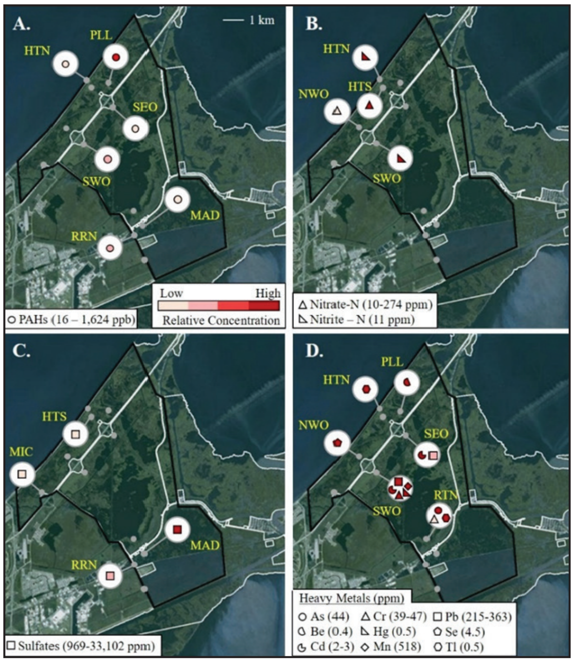

Figure 4. (A) Relative concentrations of polycyclic aromatic hydrocarbons (PAHs), (B) nitrates and nitrites, (C) sulfates, and (D) heavy metals in sediment samples (gray circles) from the Bayou Sauvage National Wildlife Refuge, Louisiana, USA. Inorganic compounds are reported when concentration > mean ± 1.96 standard deviations (SD; see methods). For each contaminant type, relative concentration was determined by equally subdividing the total range of values, starting at the minimum detection limit (PAHs) or the mean ± 1.96 SD (inorganic compounds). Some compound classes are omitted for clarity (see Table 3 for the full list of compounds). (Basemap from Esri, Redlands, California, USA).

Although we could not determine the cause of death for most (90%) carcasses due to decomposition (n = 31) or lack of diagnostic findings (n = 13), we detected multiple diseases that are rarely reported in wild snake populations, specifically, cryptosporidiosis, salmonellosis, and edwardsiellosis. While cryptosporidiosis and salmonellosis are relatively common causes of mortality among captive reptiles (Upton et al. 1989; Whiley et al. 2017), these diseases are infrequently reported in free-ranging snakes. Healthy snakes are typically asymptomatic carriers of *Salmonella* but may succumb to disease when immunosuppressed (Chiodini 1982). We found only one previous report of edwardsiellosis (a major disease of fish) in any snake, a captive Grass Snake (*Natrix natrix*; Kobolkuti et al. 2013). Across a subset of carcasses, we detected various parasites that have been previously documented in free-ranging snakes (e.g., Coccidia, Hepatozoa, and helminths; Telford et al. 2001; Davis et al. 2012). Yet, in contrast to the diversity of infectious agents identified in dead snakes from Haul Trail, we found no evidence of morbidity or disease mortality at nine other transects (31.5 km total) collectively surveyed for 708 person-hours.

Although ophidiomycosis (i.e., snake fungal disease) has been documented in diverse snake species across the eastern U.S. (Allender et al. 2015) and has been implicated in the decline of Timber Rattlesnake (*Crotalus horridus*) populations (Clark et al. 2011), this disease did not play a role in the snake mortality reported here. Dermatitis is consistently associated with ophidiomycosis in pit vipers (Allender et al. 2015) but was not observed in the sole pit viper carcass (a Northern Cottonmouth) that we encountered that was suitable for necropsy. While the manifestation of ophidiomycosis is more variable in North American colubrids (reviewed in Allender et al. 2015), we did not observe any of the common signs of this disease (i.e., significant skin lesions, subcutaneous nodules, pneumonia, or ocular infections) among the colubrid carcasses (three Black-masked Racers), nor, with one exception, among any of the remaining 10 carcasses (Dipsadinae and Natricinae species) that were suitable for postmortem examination. The exception was a juvenile Glossy Swampsnake that exhibited a minor skin infection consistent with ophidiomycosis; however, this observed disease was not sufficiently severe to be considered a cause of death.

One potential explanation for the localized mortality at Haul Trail was that snakes were immunosuppressed and therefore susceptible to ubiquitous pathogens. Many of the contaminants detected within 800 m of Haul Trail are known to suppress immune function, including nitrates (Ustyugova et al. 2002), heavy metals (Koller 1980), and hydrocarbons (Bayha et al. 2017). While we have no definitive evidence of contaminant exposure among the snakes submitted for toxicological analysis, it is noteworthy that the suspected burns observed on two snakes might have been caused by exposure to a caustic chemical or oil condensate (which can reach high temperatures) leaking from a vent in a nearby gas pipeline that was previously used to transport oil. Within 2 d of the leak being reported in July 2017, the pipeline company installed barrels to collect the leaking oil condensate; however, it is unknown whether similar leaks occur at other vents along this pipeline within Bayou Sauvage NWR. Importantly, the negative GC-MS screenings do not rule out contaminant exposure because they would not have detected inorganic compounds, including nitrates and nitrites, which were detected at high concentrations near the carcass locations. Furthermore, contaminants may have been present in untested organs (e.g., kidney or other tissues known to accumulate toxins; Sereshk and Bakhtiari 2014), particularly because two of the three samples tested consisted only of skin, muscle, and bone (due to the limited amount of tissue available for testing).

Environmental findings, the natural history of affected species, and experimental studies indicate a possible link between contamination and snake mortality at Bayou Sauvage NWR. Contaminant levels at multiple locations near (2–800 m) the die-offs exceeded established thresholds for adverse effects in model vertebrate species (nitrate: Camargo et al. 2005; nitrite: Rouse et al. 1999; PAHs: Cherr et al. 2017; lead: Dave and Xiu 1991; sulfate: Wang et al. 2016). Furthermore, fossorial species represented 75% of snake carcasses (but < 5% of live encounters) and may face greater exposure risk because many contaminants preferentially bind to sediment (Schuette 1998). Finally, survival of toad embryos exposed to water from Haul Trail was reduced 48% versus controls. Notably, increased mortality also was observed in embryos exposed to water from RRN, where high PAH concentrations were detected. We considered infectious disease and natural differences in water quality as alternative explanations for embryo mortality; however, we filter-sterilized water samples and embryo survivorship was unrelated to water pH, total dissolved solids, or salinity. Although filtration would not have removed viruses, we observed no clinical signs of ranavirus, the only virus known to cause mortality in anuran embryos (Green and Converse 2005). Thus, our findings of reduced survivorship in toad embryos indicate environmental contamination at levels sufficient to cause direct mortality. Unfortunately, contaminant analyses of the water samples used in this experiment was not performed due to budget constraints and histopathologic evaluation of toad embryos was not performed, given the rapid decomposition of these tissues.

Sediment analyses indicated seven distinct sources of contamination in Bayou Sauvage NWR: (1) a hydrocarbon pipeline leak discovered in July 2017; (2) oil dumping near Interstate-10; (3) oil dumping near Madere Marsh; (4) an unlined landfill; (5) pesticide migration; (6) vehicle exhaust; and (7) unidentified nitrate/nitrite and sulfate inputs. As expected, high concentrations of short-lived PAHs were detected at the pipeline leak (Stogiannidis and Laane 2015). A second source of PAH contamination was identified near a defunct Interstate-10 exit (SWO), and illegal oil dumping has been documented in this general area (Pon Dixon, pers. comm.). Illegal dumping has been suspected at a junkyard near Madere Marsh (Pon Dixon, pers. comm.) and could explain the elevated PAHs at that site, which is hydrologically isolated from the above locations. The unlined landfill at Recovery Road represents a fourth potential contaminant source, given the unique PAH profile detected 250 m from the landfill perimeter. Glyphosate, a pesticide not used by refuge staff, was present at multiple locations and represented a fifth contaminant source. The presence of a PAH associated with vehicle exhaust (benzo[b]fluoranthene) at Haul Trail and SE Overpass (SEO) indicated contamination from Interstate-10 (Stogiannidis and Laane 2015). Finally, high levels of nitrates (NO_3^-) and sulfates (SO_4^{2-}) at multiple locations north of Interstate-10 implicated another, unknown contaminant source. These compounds are associated with sewage facilities, agricultural runoff, and industrial effluent (Baldwin and Fraser 2009; EPA 2017). Collectively, our findings indicate a complex pattern of environmental contamination in the refuge.

Our study revealed that Bayou Sauvage, a National Wildlife Refuge of remarkable ecological and economic importance, is contaminated with compounds that have well-established toxicological and carcinogenic properties (Mumatz and George 1995; EPA 2018). Additional research is needed to understand the potential consequences of this contamination, including the possible relationship to observed snake mortality. Our collective findings raise concerns for human health because multiple areas of the refuge are popular fishing sites (Bohannan et al. 2009). The U.S. National Wildlife Refuge System Improvement Act (U.S. House 105th Congress 1997) protects the ecological integrity of NWRs. Yet, effective implementation of this law is often challenged by inadequate funding and limited baseline information (Matson 2004). In particular, reptiles have been inventoried in only about 20% of NWRs (Matson 2004) but are experiencing widespread declines (Gibbons et al. 2000). As a major resource for ecological conservation in the U.S. (Fischman 2002), the NWR system would benefit from a comprehensive inventory and regular monitoring. Such efforts could help prevent and mitigate environmental stressors that threaten to undermine the ecological integrity of the NWR system.

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