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DISTRIBUTION, DIET, AND PREVALENCE OF AMPHIBIAN CHYTRID FUNGUS IN NON-NATIVE AMERICAN BULLFROGS (*LITHOBATES CATESBEIANUS*) AT THE VALENTINE NATIONAL WILDLIFE REFUGE, NEBRASKA, USA

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ABSTRACT: American Bullfrogs (*Lithobates catesbeianus*) have been widely introduced beyond their native range in North America and can negatively affect organisms in wetland environments via a suite of mechanisms including interspecific interactions and disease transmission. Bullfrogs were introduced to the Valentine National Wildlife Refuge in the Sandhill Region of Nebraska, USA, yet little is known regarding their distribution, abundance, and potential effects on other vertebrates in the Refuge. Surveys in 1991-1992 documented bullfrogs in only one lake by the Refuge headquarters, and anecdotal historical observations indicated that bullfrogs were present primarily in lakes open to public fishing. In 2012, we determined the distribution of bullfrogs across the Refuge, examined their diets, and sampled them for the occurrence of the pathogenic fungus, *Batrachochytrium dendrobatidis*. We documented that bullfrogs were almost ubiquitous across the Refuge lakes and wetlands. From diet analyses, we observed that adult bullfrogs consumed several vertebrate species on the Refuge including: a Blanding's Turtle (*Emydoidea blandingii*), small bullfrogs, an unidentifiable frog species, and numerous invertebrates, including crayfish. The amphibian chytrid fungus was prevalent on the refuge with 73.7% of bullfrogs testing positive for the fungus in early June and 6.3% in late June-July. Preliminary data indicate that bullfrogs likely have already affected interspecific interactions with native amphibians via predation, competition, and/or disease transmission. Bullfrogs likely cannot be eradicated from the Refuge, but expanding the season of harvest of bullfrogs might reduce their abundance, which may benefit native amphibians and reptiles.

INTRODUCTION

American Bullfrogs (*Lithobates catesbeianus*; hereafter, bullfrogs) originally were native to southern and eastern North America, but the species has been widely introduced throughout western and northern parts of the United States (Bury and Whelan, 1984; Ficetola et al., 2007). The exact western limits of its native distribution are not well understood across the Great Plains. In Nebraska, for example, bullfrogs are suspected to be native only to eastern parts of the state in river-bottoms and river oxbows (McAuliffe, 1978; Fogell, 2010). Purposeful and accidental releases beyond its native range in North America have caused major changes to ecosystems worldwide where they now occur (Moyle, 1973; Kats and Ferrer, 2003; see Kraus, 2009). Some effects of

bullfrog introductions include consumption of other vertebrate species (Bury and Whelan, 1984; Krupa, 2002), interspecific competition (Kiesecker et al., 2001), and increased occurrence and transmission of the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*; Lawler et al., 1999; Bai et al., 2010). For example, Northern Leopard Frogs (*Lithobates pipiens*) appear to have been dramatically impacted by bullfrogs in western parts of North America (Hammerson, 1982; Johnson et al., 2011). Furthermore, in Ontario, Canada, Hecnar and McCloskey (1997) demonstrated that when bullfrogs were eradicated from an area, populations of other amphibians increased, in part, due to predator release. Bullfrogs have been included in the list of the 100 worst invasive species worldwide (Lowe et al., 2000; Crayon, 2009).

Understanding the potential role of non-native bullfrogs in the decline of native amphibians is important because native amphibians are often vital components in both terrestrial and aquatic ecosystems (Whiles et al., 2006).

In addition, many amphibian species are experiencing declines due to the emergence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Berger et al., 1998; Daszak et al., 1999; Skerratt et al., 2007). The amphibian chytrid fungus causes hyperkeratosis, a skin infection that creates erosions and, sometimes, ulcerations on the epidermis (Berger et al., 1998; Bosch et al., 2001). Death may ensue due to osmotic imbalance which leads to heart dysfunction (Voyles et al., 2009). Although many amphibians appear susceptible to the disease, bullfrogs normally do not show symptoms (Daszak et al., 2004) and are considered carriers, likely having a role in sustaining the fungus at sites and in transmission of the fungus to other species (Hanselmann et al., 2004; Garner et al., 2006). However, Gervasi et al. (2013) recently suggested that bullfrogs also can succumb to chytridiomycosis depending on the strain of the amphibian chytrid fungus that they are exposed to and the number of zoospores that are present at this exposure.

To date, little is known about the distribution or ecological effects of bullfrogs at the Valentine National Wildlife Refuge, Nebraska, USA (Corn et al., 1995; hereafter, the Refuge). In a herpetofaunal survey at the Refuge in 1991-1992, Corn et al. (1995) documented bullfrogs only at Hackberry Lake. In a comprehensive resurvey of the Refuge in 2012, we provide an update on the distribution of bullfrogs, in addition to investigating their diet and prevalence of the amphibian chytrid fungus in the species. Because bullfrogs are known to consume numerous prey species, including other species of frogs (Bury and Whelan, 1984), we were interested in examining whether bullfrogs directly affect native amphibians via predation at the Refuge, specifically the Northern Leopard Frog. Moreover, the amphibian chytrid fungus has recently been documented along the Platte River in south-central Nebraska, but no documentation exists in north-central parts of the state (Harner et al., 2011; Harner et al., 2013). Because the Refuge is located in the Sandhill Region of Nebraska, a rather pristine area of the state with little urban or suburban land use, we predicted that the fungus would be absent or have low prevalence in amphibian populations (Johnson et al., 2011).

MATERIALS AND METHODS

We surveyed for bullfrogs in June and July 2012 throughout the Valentine National Wildlife Refuge, Cherry County, in north-central Nebraska, USA, to determine their distribution. Survey sites included all major wetlands and lakes at the Refuge. We recorded visual sightings for bullfrogs with most observations occurring during the daytime. To better understand the historical abundance and distribution of bullfrogs on the Refuge, we interviewed Len McDaniel, a long-time biologist in the region and Wildlife Biologist at the Refuge from 1977-2001.

To sample for presence of the amphibian chytrid fungus, we captured bullfrogs on 2 June, 29 June, 24 July, and 26 July 2012. Bullfrogs ($n = 51$) were captured by hand at night, and we used a swabbing method to collect skin samples; 21 individuals were sampled from Pelican Lake, 8 from Duck Lake, 8 from West Long Lake, 7 from Watts Lake, 4 from Hackberry Lake, and 3 from Dewey Lake. Each bullfrog was swabbed with a nylon swab 20

times each on the belly, thighs, and feet (Fisherfinest Transport Swabs, Fisher Scientific Inc., Pittsburgh, PA, USA). Swabs were air dried and then stored on ice or in freezers until placed on dry ice and sent to the laboratory for testing. Contamination between frogs was limited by wearing vinyl gloves and changing them for each individual handled. We released most bullfrogs at sites of capture.

In the laboratory, we extracted DNA from the swab samples using Qiagen DNEasy spin columns and then ran a Taqman based fast qPCR (Quantitative Polymerase Chain Reaction) assay to determine the presence or absence of the chytrid fungus (Kerby et al., 2013). Samples were run in triplicate with both negative and positive controls per plate. Samples were designated as positive for the amphibian chytrid fungus if a minimum of 2/3 samples in each triplicate were positive. Samples with only a single positive result were rerun and determined to be positive if at least one of the subsequent triplicate was positive.

After swabbing bullfrogs in late July, 15 bullfrogs were collected for dietary analyses from Pelican and Dewey lakes. Larger bullfrogs (≥ 100 mm snout-vent length) were chosen to facilitate the potential for finding recently metamorphosed frogs in their stomach. Bullfrogs were placed in a container filled with a low dose (approximately 1%) Tricaine-S (Western Chemical Inc., Ferndale, WA, USA) in water for anesthetization, then, they were euthanized by exposing them to a lethal concentration of Tricaine-S. Twelve additional stomach samples were obtained from frogs captured by fisherman on Dewey Lake at the Refuge in August 2012. Frogs were placed in individually labeled water-proof bags and frozen for stomach analyses in the laboratory. We identified prey to lowest identifiable taxonomic determination and reported number of prey items in stomachs, number of frogs that consumed prey items, and percentage of frog stomachs with specific prey items.

RESULTS

Bullfrogs were observed in 33 of the 35 major lakes and marsh complexes throughout the refuge (Appendix 1), including all nine public fishing lakes. We did not observe bullfrogs inhabiting the two lakes farthest east on the Refuge (i.e., East Long and Crooked lakes) where human access is limited, although suitable frog habitat was available. Lakes with bullfrogs, and the two without bullfrogs, had emergent vegetation around their edges including bulrushes (*Scirpus* spp.), cattails (*Typha* spp.), and common reed (*Phragmites* spp.). We also documented bullfrogs in stock tanks with vegetation. At most lakes with bullfrogs, we failed to detect Northern Leopard Frogs during our brief surveys, but we observed both frog species on Pelican and Marsh lakes. Once we observed at least one bullfrog at a lake, we did not further search for leopard frogs, hence leopard frogs are likely to occur at more lakes throughout the refuge albeit in lower densities or are less obvious.

Len McDaniel reports having observed bullfrogs on the Refuge in 1963 and 1977-2001, with most bullfrogs west of U.S. Highway 83 in Watts, Hackberry, Dewey, West Long, Duck, Rice, and Pelican lakes, as well as, Sawyer Meadow. He observed occasional sightings on Marsh Lakes east of U.S. Highway 83.

Stomach analyses demonstrated that amphibians were among the wide variety of organisms consumed by bullfrogs (Table 1). Three bullfrogs had empty stomachs, four had an unidentifiable frog in their stomach, and one

Table 1. Stomach contents of 27 American Bullfrogs (*Lithobates catesbeianus*) from the Valentine National Wildlife Refuge in Cherry County, Nebraska, USA, July and August 2012. For higher taxonomic categories, summation of lower taxa within those categories is reported in parentheses.

Category	No. food items	No. stomachs with food items	Stomachs with food items (%)
Phylum Arthropoda	(114)	(21)	(78)
Class Insecta	(98)	(20)	(74)
Order Coleoptera	(47)	(16)	(59)
Unidentified Coleoptera	10	6	22
Family Cicindelidae (<i>Cicindela punctulata</i>)	1	1	4
Family Dytiscidae	2	1	4
Family Silphidae (<i>Necrophila americana</i>)	6	5	19
Family Staphylinidae	4	2	7
Family Curculionidae	2	2	7
Family Omphronidae	5	1	4
Family Carabidae (Unidentified)	14	8	30
Genus Elaphras	3	3	11
Order Odonata	(9)	(8)	(30)
Unidentified Odonota	2	2	7
Family Coenagrionidae	1	1	4
Suborder Anisoptera	1	1	4
Family Libellulidae	5	4	15
Order Hymenoptera	(9)	(8)	(30)
Unidentified Hymenoptera	3	3	11
Family Vespidae	4	4	15
Family Formicidae	1	1	4
Family Pompilidae	1	1	4
Order Diptera	(13)	(7)	(26)
Unidentified Diptera	8	4	15
Family Tipulidae	3	3	11
Suborder Brachycera	2	1	4
Order Ephemeroptera	1	1	4
Order Lepidoptera	2	2	7
Order Collembola	3	2	7
Order Hemiptera			
Family Belostomatidae	8	4	15
Order Orthoptera	(6)	(4)	(15)
Family Acrididae	1	1	4
Family Gryllidae	2	2	7
Family Gryllotalpidae	3	1	4
Class Arachnida	(6)	(4)	(15)
Order Araneae	5	3	11
Order Opiliones (Suborder Palpatores)	1	1	4
Class Malacostraca	(10)	(7)	(26)
Order Amphipoda	3	1	4
Order Decapoda	7	6	22
Phylum Mollusca	(9)	(5)	(19)
Class Gastropoda (Unidentified)	8	4	15
Family Planorbidae	1	1	4
Phylum Cordata	(7)	(6)	(22)
Class Amphibia	(6)	(5)	(19)
Order Anura			
Family Ranidae			
Unidentified Ranidae	4	4	15
<i>Lithobates catesbeianus</i>	2	1	4
Class Reptilia	(1)	(1)	(4)
Order Testudines			
Family Emydidae (<i>Emydoidea blandingii</i>)	1	1	4
Phylum Nematode/Nematomorpha	(4)	(1)	(4)

had two smaller bullfrogs in its stomach. In total, 134 total organisms from four different phyla were consumed by bullfrogs (Table 1), with chordates (i.e., bullfrogs, unknown frogs, and a Blanding's Turtle, *Emydoidea blandingii*) representing 5.2% of the diet and insects representing 73.1%. We detected at least 9 orders and 17 families of insects in bullfrog diets (Table 1). We observed that 22% of individuals contained a prey item in the phylum Chordata (Table 1). Of 51 bullfrogs sampled for the amphibian chytrid fungus, 16 (31.4%) tested positive for the fungus. In early June, 14 of 19 (73.7%) bullfrogs were positive for chytrid and from late June to late July, 2 of 32 (6.3%) bullfrogs tested positive.

DISCUSSION

We observed bullfrogs to be almost ubiquitous across the Refuge in major lakes and marsh complexes. Although there is only one previously published record of bullfrogs at the Refuge, occurring at Hackberry Lake (Corn et al., 1995), our conversations with a long-time resident and biologist of the region confirmed that the species had been observed at additional locations for decades. Countless bullfrogs were released throughout the state prior to and during the early 1940s by the Nebraska Game and Parks Commission (Hudson, 1942). We suspect that the "fishing lakes" on the Refuge likely were the original site of bullfrog stockings either by accident or intentionally. Considering bullfrogs are known to colonize new water sources ≤ 1 km by traveling over land (Snow and Witmer, 2010), and during wet years water flows between many of the Refuge lakes and wetlands (M. Nenneman, personal observation), the widespread occurrence of the species is not surprising. We suspect it is a matter of time before bullfrogs will be observed at East Long and Crooked lakes on eastern parts of the Refuge. Therefore, given the amount of time bullfrogs have occurred on the Refuge, connectivity of waterways, and relatively short distances between more isolated lakes, bullfrogs have demonstrated their ability to be invasive at this wildlife refuge in north-central Nebraska.

Many studies have reported that bullfrogs consume a variety of organisms (e.g., Bury and Whelan, 1984; Krupa, 2002). At the Refuge, insects were the most frequent prey item consumed by bullfrogs, which is similar to the findings of other studies where insects and crayfish were commonly consumed by bullfrogs (see Bury and Whelan, 1984). Many insects consumed by bullfrogs at the Refuge are associated with wetland, lake, and marshy habitats (Arnett, 1985). Small turtles occasionally are reported in the diet of bullfrogs (Bury and Whelan, 1984). Consumption of a Blanding's Turtle on the Refuge represents a Tier 1 species of concern in Nebraska (Schneider et al., 2011). We documented consumption of a total of six frogs in stomachs, two of which were bullfrogs, but we were unable to identify the remaining four frogs to species. Frogs commonly are consumed by bullfrogs (e.g., Bury and Whelan, 1984). We suspect that with more sampling, bullfrogs would be shown to directly consume native amphibians on the Refuge. The lack of many lakes and marshes with an abundance of both bullfrogs and northern leopard frogs suggests an interaction may be occurring between them. Although our preliminary information cannot ascertain a mechanism of interaction, the potential exists for both predation by bullfrogs and presence of bullfrogs resulting in the sustained presence of the amphibian chytrid fungus, increasing the likelihood of interspecific transmission of the pathogen.

Our observations of the presence of the amphibian chytrid in bullfrogs at the Refuge represent the first detection of the amphibian chytrid fungus in northern Nebraska and the third published locality in the state (Harner et al., 2011; Harner et al., 2013). The Refuge is situated on a rather pristine location in the Sandhill Region of Nebraska with minimal human disturbance, thus we expected that amphibian chytrid may not be present or could occur at a low prevalence (Johnson et al., 2011). Fishing is allowed in nine of the lakes, which may be linked to its presence because human activities are strongly suspected to aid the spread of the chytrid fungus (Schloegel et al., 2009). Sampling of bullfrogs museum/archival specimens in North America (Ouellet et al., 2005) and other countries (Weldon et al., 2004) has demonstrated that the introduction of this fungus may have occurred decades ago; the earliest finding in North America to date is from Saint-Pierre-de-Wakefield, Quebec in 1961 (Oullet et al., 2005). Thus, the fungus might have always been present from early human activities in the area such as the initial bullfrog stockings throughout Nebraska. Chytrid prevalence at the Refuge was 31.4%, similar to rates observed in south-central Nebraska (Harner et al., 2011; Harner et al., 2013). We predict that amphibian chytrid fungus likely occurs throughout Nebraska where bullfrogs are present.

Our data appear to support the climate-chytrid paradox (Pounds et al., 2006) at the Refuge. For most pathogens, as temperatures rise, disease outbreaks are triggered and many become more lethal or spread more rapidly; however, amphibian chytrid fungus is more pathogenic at lower temperatures (Berger et al., 2004). Our results suggested a pattern between prevalence rates of amphibian chytrid and temperature, with higher rates of chytrid documented during a cooler period of time. In early June, 73.7% of samples tested positive for chytrid, whereas from late June to late July, only 6.3% were positive. Average high temperature for the 30 days prior to the early June sampling date was 23.2°C, whereas average high temperature 30 days prior to 24 July was 37.0°C (temperatures reported at Valentine, Nebraska; wunderground.com). This study represents the third example of this relationship in Nebraska, comparing early sampling dates to those samples collected later in the season (K. Geluso, unpublished data; Harner et al., 2011; Harner et al., 2013). For methodological purposes, additional sampling for amphibian chytrid should be conducted during cooler seasons and not during the hottest months of the year to promote its detection in amphibian populations.

Although our study was not designed specifically to assess the effects of bullfrogs on Northern Leopard Frogs at the Refuge, we hypothesize that a negative interaction is occurring due to the paucity of Northern Leopard Frogs in our surveys. During nighttime sampling, we observed hundreds of bullfrogs whereas we only observed a few (< 5) Northern Leopard Frogs. Bullfrogs are likely affecting native amphibian species via both interspecific interactions and disease transmission. Although bullfrogs may be difficult to eradicate from the Refuge (see Snow and Witmer, 2010), especially given their current abundance and distribution, population control measures may benefit native species. Bullfrogs are currently a game species in Nebraska, with seasonal, size, and bag limits in place to prevent overharvesting (McAuliffe, 1978). In other areas with high bullfrog harvest rates, bullfrog numbers have decreased (Hudson, 1942; McAuliffe, 1978). Therefore, if regulations on bullfrog harvest were lessened or

eliminated in Nebraska, their abundance may decrease which could potentially benefit native amphibian and other vertebrate populations at the Refuge, as well as, across the state. However, incidental harvest of native frogs is a concern; hence elevated bullfrog harvest may warrant monitoring to assess effects on both native species and this invasive species. We propose that bullfrogs should be delisted as a game species across much of Nebraska where there were no native populations.

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APPENDIX 1. Localities of lakes on the Valentine National Wildlife Refuge, Cherry County, Nebraska, USA, where bullfrogs were observed and locations without bullfrogs in 2012. Latitudes and longitudes were determined via Google.earth.com

LAKES WITH BULLFROGS: 1. Baker Lake (42.4966°N, 100.6212°W); 2. Calf Camp Marsh (42.5263°N, 100.5587°W); 3. Campbell Lake (42.4687°N, 100.5793°W); 4. Center Lake (42.4792°N, 100.4858°W); 5. Clear Lake (42.5577°N, 100.6171°W); 6. Coleman Lake (42.4611°N, 100.6443°W); 7. Cow Lake (42.4431°N, 100.5350°W); 8. Dad's Lake (42.4983°N, 100.6624°W); 9. Devil's Punch Bowl (42.4900°N, 100.7051°W); 10. Dew Lake (42.4488°N, 100.3506°W); 11. Dewey Lake (42.5450°N, 100.6288°W); 12. Duck Lake (42.5432°N, 100.7262°W); 13. East Sweetwater Lake (42.4492°N, 100.5163°W); 14. East Twin Lake (42.5048°N, 100.5311°W); 15. Hackberry Lake (42.5627°N, 100.6813°W); 16. Homestead Lake (42.4646°N, 100.5655°W); 17. Lee Lake (42.4625°N, 100.4987°W); 18. Little Hay Lake (42.5383°N, 100.5562°W); 19. Lost Lake (42.4891°N, 100.5936°W); 20. Marsh Lakes (42.5182°N, 100.5020°W); 21. McKeel Lake (42.5327°N, 100.5917°W); 22. Mule Lake (42.4862°N, 100.6759°W); 23. Pelican Lake (42.5268°N, 100.6721°W); 24. Pony Lake (42.4874°N, 100.5125°W); 25. Rice Lake (42.5543°N, 100.7227°W); 26. School Lake (42.5270°N, 100.6103°W); 27. Tom's Lake (42.4784°N, 100.5352°W); 28. Twenty-one Lake (42.4712°N, 100.4599°W); 29. Watts Lake (42.5778°N, 100.6887°W); 30. West Long Lake (42.5278°N, 100.7221°W); 31. West Twin Lake (42.5086°N, 100.5623°W); 32. Whitewater Lake (42.5338°N, 100.6484°W); 33. Willow Lake (42.5521°N, 100.5865°W).

LAKES WITHOUT BULLFROGS: 1. Crooked Lake (42.4429°N, 100.3877°W); 2. East Long Lake (42.4244°N, 100.4003°W).