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Hemoparasites (Apicomplexa: *Hepatozoon*; Kinetoplastida: *Trypanosoma*) of Two Anurans (Hylidae; Ranidae), from Polk County, Arkansas

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Running Title: Hemoparasites of Anurans in Arkansas

Abstract

Arkansas supports 26 species/subspecies of anurans and only one (4%), the green frog, *Rana clamitans*, has been previously reported with hemoparasites. Here, we collected blood samples from three species of anurans, five American green treefrogs, *Dryophytes cinereus*, five American bullfrogs, *Rana catesbeianus*, two southern leopard frogs, *Rana sphenocephalus utricularius*, and two Fowler's toads, *Anaxyrus fowleri* from Polk County and examined each for hemoparasites. American green treefrogs and American bullfrogs harbored hemoparasites, including two (40%) *D. cinereus* and four (80%) *R. catesbeianus* with trypanosomes, and one (20%) *R. catesbeiana* with a *Hepatozoon* sp. This is the first time these two anurans have been reported with hemoparasites from Arkansas.

Introduction

Compared to Arkansas' reptiles (turtles and snakes, see McAllister *et al.* [2016]), we know very little about blood parasites (hemoparasites) in the 26 anuran amphibians (frogs and toads) of the state. The only previous report of hemoparasites from Arkansas amphibians was by McAllister *et al.* (2020) who reported a *Hepatozoon* sp. and trypanosomes from green frogs, *Rana clamitans* (Latreille) from Polk County.

The green treefrog, *Dryophytes* (formerly *Hyla cinerea*) *cinereus* (Schneider) is a bright green hylid that ranges from the Delmarva Peninsula and Indiana and Illinois south to the Florida Keys and west to Texas (Powell *et al.* 2016). In Arkansas, this anuran inhabits the Gulf Coastal Plain, extending up the Arkansas River Valley, and into the Ouachita uplift; it is mostly absent from the Ozarks (Trauth *et al.* 2004). This frog has been previously reported as a host of an undescribed trypanosome in Florida (Johnson *et al.* 1993). We are unaware of any additional reports of hemoparasites in green treefrogs.

The American bullfrog, *Rana catesbeianus* (Shaw) is the largest North American anuran. This frog ranges from the Atlantic Provinces and southern Québec, Canada, southward to southern Florida and west to the Rocky Mountains and south into northeastern México; it has been introduced widely elsewhere, including Hawaii (Powell *et al.* 2016). In suitable habitat, *R. catesbeianus* is found statewide in Arkansas (Trauth *et al.* 2004).

The American bullfrog has been the subject of several studies on its hemoparasites, including those reporting trypanosomes and/or *Hepatozoon catesbianae* (= *Haemogregarina catesbiana*, [Stebbins, 1903]) in specimens from Louisiana, Long Island, New York, and Ontario, Canada (Stebbins 1903; Barta and Desser 1984; Desser *et al.* 1995; Smith 1996; Kim *et al.* 1998; Boulianne *et al.* 2007). A similar hemoparasite of *R. catesbeianus* and *R. clamitans*, was described by Stebbins (1905) with him noting morphological differences that the gamonts produced hypertrophy and fragmentation of host red blood cell (rbc) nuclei and hence, named it *Karyolysus clamatae*. Later, Smith (1996) reclassified this hemoparasite as *Hepatozoon clamatae.*

Here, for the first time, we document hemoparasites in *D. cinereus* and *R. catesbeianus* in individuals collected from Arkansas, including photomicrographs of the infection.

Materials and Methods

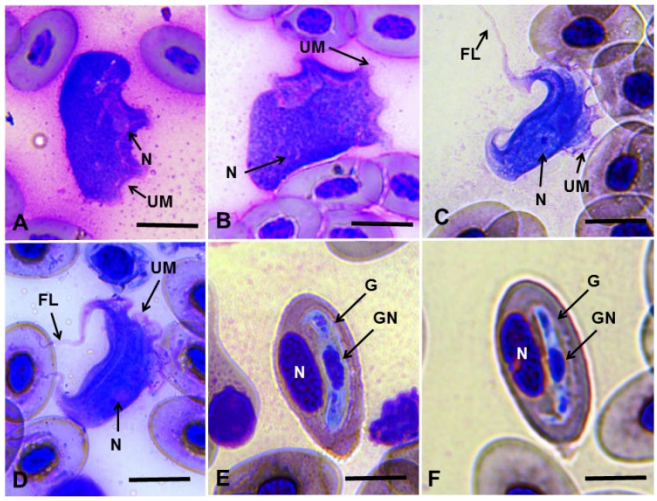
Between May and July 2019, five adult (4 male, 1 female) *D. cinereus* (mean ± SD snout-vent length

[SVL] = 55.8 ± 3.2, range 51–61 mm), five juvenile and adult (3 male, 2 female) *R. catesbeianus* (142.5 ± 61.5, 75–220 mm SVL), two adult (male and female) *Rana sphenocephalus utricularius* (Harlan) (68 and 89 mm SVL) and two adult (females, 84–90 mm SVL) Fowler's toad *Anaxyrus fowleri* (Hinckley) were collected by hand from two sites in Polk County: (1) a pond across from Blue Haze Vista, 4.8 km N of Mena off St. Hwy. 88 (34°37'40.17"N, -94°14'44.4228"W) and (2) a pond feeding Butcherknife Creek at the Ouachita Mountains Biological Station (OMBS), Polk County (34°27'43.4484"N, -93°59'54.3264"W). Following the methods of Forzán *et al.* (2012), blood was obtained from the facial musculocutaneous vein of anesthesized individuals with a concentrated solution of tricaine methanesulfonate (TMS-222) and collected in heparinized capillary tubes. Thin blood smears were air-dried, fixed in absolute methanol, and stained with Wright's-Giemsa stain for 30 min. Stained slides were scanned at high power (100× oil immersion objective) for approximately 10 min. to detect intracellular and extracellular parasites. When *Hepatozoon* sp. gamonts were found infecting rbcs, individuals were photographed with an Swift model M10 light microscope (Microscope Central, Feasterville, PA) fitted with a digital camera. Gamont length and width in micrometers (µm) were measured using a calibrated stage micrometer. Trypanosomes were also measured to the nearest 1.0 µm following Desser (2001) and photographed. Parasites were identified to genus based on previous reports of hematozoa infecting *D. cinereus* and *R. catesbeianus* in North America (Johnson *et al*. 1993; Desser *et al.* 1995) and photovouchers were deposited in the Harold W. Manter Laboratory of Parasitology (HWML), University of Nebraska, Lincoln, NE. Host vouchers were deposited in the Eastern Oklahoma State College Vertebrate Collection (EOSC), Idabel, OK. We follow Yuan *et al*. (2016) in the adoption of the genus *Rana* instead of *Lithobates* for North American ranid frogs.

Results

Two of five (40%; males, 56, 61 mm SVL) *D. cinereus* were infected with trypanosomes (HWML 216944, Figs. 1A–B), four of five (80%, 3 males, 1 female; 142.5 ± 62.5, 75–220 SVL) *R. catesbeianus* had trypanosomes (HWML 216945, Figs. 1C–D), and one (20%; male, 220 mm SVL) *R. catesbeianus* possessed gamonts of an unknown species of *Hepatozoon* sp. with typical non-fragmented nuclei (HWML 216946, Figs. 1E–F) in erythrocytes. Mixed infections were found of trypanosomes and *Hepatozoon* sp. in a single (20%) *R. catesbeianus*. The two *R. s. utricularius* and *A. fowleri* were not infected with hematozoans.

Measurements (n = 10) of the body (excluding the undulating membrane) of the *Trypanosoma* sp. from *R. catesbeianus* (L × W ± SD) was 56.6 ± 7.0 (41–69) × 12.4 ± 2.5 (9–18) µm. Measurements of the length and width (n = 10) of *Hepatozoon* sp. gamonts from *R. catesbeianus* were 27.2 ± 2.0 (25–30) × 4.0 ± 0.5 (3–5) µm. Unfortunately, not enough trypanosomes were observed from *D. cinereus* for measurements.



Figures 1A-F. Photomicrographs of hemoparasites infecting *Dryophytes cinereus* and *Rana catesbeianus*, (A–B) *Trypanosoma* sp. from *D. cinereus*. (C–D) *Trypanosoma* sp. from *R. catesbeianus*; note long flagellum (FL). (E–F) *Hepatozoon* sp. from *R. catesbeianus*: note host eccentric erythrocytic nucleus (N) displaced but not fragmented. Abbreviations: gamont (G); gamont nucleus (GN); undulating membrane (UM). Scale bars = 20 µm.

Discussion

Johnson *et al.* (1993) 159 of 215 (72%) male green treefrogs (but none of the 31 female frogs) collected in Alachua and Levy counties, Florida, were infected with an undescribed *Trypanosoma* sp. Here, trypanosomes from two male green treefrogs (Figs. 1A–B) from Arkansas were morphologically similar to those from same host species from Florida (Johnson *et al*. 1993). Unfortunately, species of trypanosomes cannot be identified based on morphology alone, and vigilant isolation, culturing, and experimental infections of frogs are required to describe and genuinely identify them (Desser 2001).

The adult females of at least some species of frog-biting midges of the genus *Corethrella* transmit *Trypanosoma* between calling (male) frogs. Johnson *et al.* (1993) noted that conspecific trypanosomes also

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were found in the mid- and hind guts of female *Corethrella wirthi* Stone (Diptera; Corethrellidae Edwards) collected at or near the study sites and experimental transmission of the hemoparasite to uninfected frogs was demonstrated. Because female adults of these hematophagous midges are attracted to the call of male frogs and their mouthparts are modified to obtain a blood meal, species are restricted to areas where there are frogs. It appears that other hosts reported for *C. wirthi* are also hylids, including bird-voiced treefrog, *Dryophytes avivoca* Viosca and Cope's gray treefrog, *Dryophytes chrysoscelis* Cope from the Tuskegee National Forest, Alabama (Camp and Irby 2017). To date, all localities reported for this fly are east of the Mississippi River and, as such, does not apparently occur in Arkansas but a congener, *Corethrella brakeleyi* Coquillett reported to infect Southern cricket frogs, *Acris gryllus* (LeConte), *R. clamitans*, and *R. s. ultricularius* from Statesboro, Georgia (Camp and Irby 2017), has also been reported to occur in Marion, Lee County, Arkansas (Stone 1968). Interestingly, Camp and Irby (2017) found that *R. s. utricularius* and *R. clamitans* were the principal hosts selected by *C. brakeleyi*. Unfortunately, we did not attempt to collect midges from our study site.

Stebbins (1903) was the first to report *H.* (=*Ha. catesbiana*) *catesbianae* in the blood of American bullfrogs from New York (see Desser *et al.* 1995; Smith 1996). Other reports of *H. catesbeiana* from *R. catesbeianus* have been from surveys conducted in Ontario, Canada (Desser *et al.* 1995; Smith 1996; Smith and Desser 1997; Kim *et al.* 1998; Smith *et al.* 2000; Boulianne *et al.* 2007). Characters typically used to differentiate *Hepatozoon* spp. such as oocyst morphology in the mosquito vector is usually indistinguishable between species (Boulianne *et al*. 2007) although *H. catesbeianae* does not cause fragmentation of the nuclei of erythrocytes it infects (see also McAllister *et al.* 2020). Recent investigation has noted this distinction into vagueness, as analyses of molecular data does not consistently separate these species of *Hepatozoon* with their effect on the host erythrocytic nucleus (Boulianne *et al.* 2007).

The non-fragmented forms of *Hepatozoon* sp. observed in our study (Figs. 1E–F) were morphologically similar to the descriptions of *H. catesbianae* by Desser *et al.* (1995) from *R. catesbeianus* and a *Hepatozoon* sp. reported from *R. clamitans* from Arkansas by McAllister et al. (2020, their Fig. 1C). Examinations of life cycle stages in the definitive host and further study of the life cycle as well as inclusion of molecular data are vital to identify the parasites in this study (see O'Donoghue 2017). Additionally, as noted previously by McAllister *et al.* (2020), further phylogenetic analyses are warranted to determine the usefulness of the fragmentation of the erythrocytic nucleus as a character to differentiate *H. catesbianae* from *H. clamatae*.

In the current survey, a single morphology of a trypanosome was found in *R. catesbeianus* (Figs. 1C–D). Trypanosomes are pleomorphic, meaning they can change morphology throughout their life cycle (Desser 2001). Additionally, individual anuran hosts are often infected with multiple morphologies as was recently shown by McAllister *et al.* (2020) with three forms (designated "A", "B", and "C", see their Table 2) of trypanosomes from *R. clamitans* from Arkansas. In addition, it is unknown whether those forms represent different species or a single pleomorphic species.

The data presented here illustrate that additional surveys should be performed on anurans to understand the relationship between the hematophagous arthropod definitive hosts and discovery of and vectors in their life cycles, attraction of hosts, and selection of hosts in this group of midges. Future research will be necessary to describe the unknown species found in the present study, including inclusion of molecular analyses and experimental infections of suitable vectors.

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