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Cover Page Footnote

The Arkansas Game and Fish Commission issued a Scientific Collecting Permit to CTM. We thank Drs. S.L. Gardner and G. Racz (HWML) for expert curatorial assistance, and V.V. Tkach (University of North Dakota), N.H. McAllister, and Z.S. McAllister for help collecting hosts.

Hemoparasites (Apicomplexa: *Hepatozoon*; Kinetoplastida: *Trypanosoma*) of Green Frogs, *Rana clamitans* (Anura: Ranidae) from Arkansas

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Running Title: Hemoparasites of Green Frogs in Arkansas

Abstract

The green frog, *Rana clamitans*, has been reported as a host of several hemoparasites, including trypanosomes and *Hepatozoon* spp. In Arkansas, however, there are no reports of any hemoparasites in *R. clamitans* nor from any other anuran from the state. We collected 9 green frogs from Polk County and blood was taken from their facial musculocutaneous vein in heparinized capillary tubes. Thin blood smears were also made and stained with DipQuick stain. Seven out of 9 (78%) *R. clamitans* were infected with hematozoans. Three (33%) were infected with an unknown species of *Hepatozoon* and 4 (44%) were infected with trypanosomes of 3 distinct morphologies. Mixed infections were found in 5 (56%) of the hosts. Here, we provide the first report of hemoparasites in *R. clamitans* from Arkansas, including morphometric data and photomicrographs of the infections.

Introduction

The study of blood parasites (hematozoans) is still in its early stages in herpetofauna of Arkansas. Several studies have addressed this problem and reported hematozoan parasites from reptiles (turtles and snakes) in the state (McAllister and King 1980; McAllister *et al.* 1995, 2014, 2016; McAllister and Robison 2019). Nevertheless, a complete void remains in the study of amphibian hematozoans in Arkansas.

The green frog, *Rana clamitans* (Latreille, 1801) is a medium-sized ranid that ranges from Nova Scotia and Manitoba, Canada, south to central Florida and west to eastern Texas; it is found generally statewide in Arkansas (Powell *et al.* 2016). This frog has been previously reported as a host of several hemoparasites, including trypanosomes and *Hepatozoon* spp. (Southworth *et al.* 1968; Barta and Dessler 1984;

Desser *et al.* 1995; Smith 1996; Leveille *et al.* 2014). In Arkansas, however, there are no reports of any hemoparasites in *R. clamitans* nor from any other anuran from the state. Here, we provide the first report of blood parasites infecting an anuran in Arkansas.

Materials and Methods

During June 2019, 9 adult *R. clamitans* were collected by hand from a pond across from Blue Haze Vista, 4.8 km N of Mena, Polk County (34°37'40.17" N, -94°14'44.4228" W). Blood was drawn from the facial musculocutaneous vein of individuals anesthetized with tricaine methanesulfonate following the methods of Forzán *et al.* (2012) and collected in heparinized capillary tubes. Samples were centrifuged at 4,000 × g for 1 min to separate red blood cells (rbcs) from plasma and concentrating parasites in the buffy coat layer (Woo *et al.* 1969). Capillary tubes were then scored with a glass scratcher right below the buffy coat and gently snapped. Using a capillary pipette bulb, the buffy coat and plasma were dispensed from the tube onto a slide for microscopical examination for hemoparasites. In addition to buffy coat smears, thin blood smears were prepared and air-dried and stained with DipQuick J-322 stain (Jorgenson Labs, Loveland, CO). Stained slides were scanned at high power (100× objective) with an Olympus BX-51 upright research microscope for approximately 10 min to detect intracellular and extracellular parasites. When *Hepatozoon* spp. gamonts were found infecting rbcs, 10 to 20 specimens of each stage were photographed with an Olympus 5-megapixel digital camera. Gamont length and width in micrometers (µm) were measured using ImageJ (Schneider *et al.* 2012) and calibrated with a stage micrometer. Trypanosomes were also measured to the nearest 1.0 µm following Dessler (2001). Parasites were identified to genus based on

previous reports of hematozoa infecting *R. clamitans* in North America (Southworth *et al.* 1968; Barta and Desser 1984; Desser *et al.* 1995; Kim *et al.* 1998; Leveille *et al.* 2014) and photovouchers were deposited in the Harold W. Manter Laboratory of Parasitology (HWML), University of Nebraska, Lincoln, NE.

Results

Seven of 9 (78%) *R. clamitans* were infected with hematozoans. Three (33%) were infected with an unknown species of *Hepatozoon* (HWML 216338; Figs. 1 A–C; Table 1). Four (44%) were infected with trypanosomes of 3 distinct morphologies (Figs. 1 D–F; Table 2). Mixed infections were found in 5 (56%) of the hosts. Three frogs (33%) were infected by 2 forms of trypanosomes (HWML 216339), 2 with forms “a” and “b,” and one with forms “b” and “c.” One frog (11%) was infected by both *Hepatozoon* sp. and *Trypanosoma* sp. “c,” and one frog (11%) had *Hepatozoon* sp. infecting erythrocytes with both fragmented and non-fragmented nuclei. Measurements for these parasites are presented in Tables 1 and 2.

Discussion

Stebbins (1903, 1905) described *Hepatozoon* (= *Haemogregarina*) *catesbiana*e and *Hepatozoon* (= *Karyolysus*) *clamata*e in the blood of American bullfrogs, *Rana catesbeiana*, and *R. clamitans* in New York, respectively (see Desser *et al.* 1995; Smith 1996).

Table 1. Morphometric data (in μm) of *Hepatozoon* sp. gamonts infecting rbc of *Rana clamitans* in Arkansas. Average $\pm$ SD*, ranges in parentheses.

Frog ID #	13 <i>n</i> = 10	14 <i>n</i> = 11	21 <i>n</i> = 11
Gamont length	26.9 $\pm$ 1.8 (25–30)	26.0 $\pm$ 1.7 (22–28)	25.2 $\pm$ 2.5 (22–31)
Gamont width	4.0 $\pm$ 0.5 (3–5)	4.5 $\pm$ 0.5 (4–5)	4.4 $\pm$ 0.5 (4–5)
Effect on host rbc nucleus	Fragmented	Fragmented	Non-Fragmented
Host rbc length	28.9 $\pm$ 2.5 (25–34)	28.5 $\pm$ 2.2 (25–33)	25.8 $\pm$ 1.8 (23–29)
Host rbc width	14.0 $\pm$ 0.8 (13–15)	14.6 $\pm$ 1.5 (11–16)	13.2 $\pm$ 0.9 (11–14)

*One standard deviation.

In addition, *H. catesbiana*e and *H. clamata*e have both been reported from *R. clamitans* from Canada (Kim *et al.* 1998; Boulianne *et al.* 2007; Shutler *et al.* 2009; Trites *et al.* 2013). Traits typically used to differentiate *Hepatozoon* species, such as oocyst morphology in the mosquito vector, were indistinguishable between these 2 species (Boulianne *et al.* 2007). However, *H. clamata*e fragments the nucleus of the rbc's it infects, while *H. catesbiana*e does not (Desser *et al.* 1995; Kim *et al.* 1998; Boulianne *et al.* 2007). Recent research has called this distinction into question, as analyses of genetic data do not consistently separate these species

Table 2. Morphometric data (in μm) of *Trypanosoma* spp. infecting *Rana clamitans* in Arkansas. Average $\pm$ SD*, ranges in parentheses.

	<i>Trypanosoma</i> sp. “a” <i>n</i> = 15	<i>Trypanosoma</i> sp. “b” <i>n</i> = 3	<i>Trypanosoma</i> sp. “c” <i>n</i> = 19
PA	55.4 $\pm$ 6.7 (40–68)	42.0 $\pm$ 5.6 (37–48)	37.5 $\pm$ 6.0 (26–54)
BW	11.8 $\pm$ 2.5 (8–17)	16.7 $\pm$ 1.5 (15–18)	31.7 $\pm$ 3.8 (23–38)
LN	4.1 $\pm$ 0.4 (4–5)	4.0 $\pm$ 0.1 (4–4)	5.0 $\pm$ 0.9 (4–7)
WN	3.4 $\pm$ 0.7 (2–4)	3.7 $\pm$ 0.6 (3–4)	4.4 $\pm$ 0.7 (3–5)
PK	7.2 $\pm$ 2.8 (3–11)	5.7 $\pm$ 4.7 (2–11)	n/a
KN	11.8 $\pm$ 2.9 (5–16)	7.7 $\pm$ 1.2 (7–9)	n/a
NA	36.9 $\pm$ 3.7 (30–45)	28.0 $\pm$ 2.6 (26–31)	n/a
PN	18.9 $\pm$ 4.2 (10–26)	13.3 $\pm$ 4.0 (11–18)	n/a
PK/PN	0.19 $\pm$ 0.07	0.20 $\pm$ 0.18	n/a
PK/PA	0.13 $\pm$ 0.04	0.13 $\pm$ 0.09	n/a
PN/PA	0.67 $\pm$ 0.06	0.67 $\pm$ 0.10	n/a
BW/PA	0.22 $\pm$ 0.05	0.40 $\pm$ 0.07	0.86 $\pm$ 0.12

Abbreviations: PA = body length, BW = maximum body width excluding undulating membrane, LN = nucleus length, WN = nucleus width, PK = distance from posterior end to kinetoplast, KN = distance from kinetoplast to center of nucleus, NA = distance from center of nucleus to anterior end, PN = distance from posterior end to center of nucleus. *One standard deviation.

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Figure 1. Photomicrographs of hematozoans infecting *Rana clamitans*. (A) *Hepatozoon* sp. infecting frog 13. Note fragmented host rbc nucleus (white arrows). (B) *Hepatozoon* sp. infecting frog 14. Note fragmented host rbc nucleus (white arrows). (C) *Hepatozoon* sp. infecting frog 21. Note host rbc nucleus displaced but not fragmented (white arrow). (D) *Trypanosoma* sp. ("a") infecting frog 16. (E) *Trypanosoma* sp. ("b") infecting frog 21. (F) *Trypanosoma* sp. ("c") infecting frog 19. Abbreviations: G = gamont, GN = gamont nucleus, K = kinetoplast, N = nucleus, UM = undulating membrane. Scale bars = 20 µm.

of *Hepatozoon* with their effect on the host rbc nucleus (Boulianne *et al.* 2007).

Both fragmented and non-fragmented types were observed in our study (Figs. 1A–C). These forms were morphologically similar to the descriptions of *H. catesbiana* and *H. clamatae* by Dessler *et al.* (1995) and Kim *et al.* (1998) and were from the same host, *R. clamitans*. However, molecular data and further study of the life cycle, including examinations of stages in the definitive hosts, are needed to identify the parasites in this study (O'Donoghue 2017). Additionally, further phylogenetic analyses are warranted to determine the usefulness of the rbc nucleus fragmentation as a character to differentiate *H. catesbiana* and *H. clamatae*.

Trypanosomes have been reported previously from *R. clamitans*. Southworth *et al.* (1968) reported *Trypanosoma rotatorium* from green frogs from Louisiana. Bartlett-Healy *et al.* (2009) sequenced blood meals of *Culex territans* mosquitoes in New

Jersey, and 50% of blood meals from *R. clamitans* were infected with trypanosomes. Additionally, Barta and Dessler (1984) reported *T. rotatorium* and *T. ranarum* from *R. clamitans* from Ontario, Canada. In the current survey, 3 morphologies of trypanosomes were found in green frogs (Figs. 1D–F). Trypanosomes are pleomorphic, meaning they can change morphology throughout their life cycle (Dessler 2001). Additionally, single amphibian hosts are often infected with multiple morphologies and it is unknown whether these forms represent different species or a single pleomorphic species. Therefore, species of trypanosomes cannot be identified based on morphology alone, and careful isolation, culturing, and experimental infections of frogs are required to describe and genuinely identify them (Dessler 2001).

Additional research will be necessary to describe the species found in the present study, including molecular analyses and discovery of hematophagous arthropod definitive hosts and vectors in their life cycles.

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