# AMPHIBIAN BLOOD PARASITES AND THEIR POTENTIAL VECTORS IN THE GREAT PLAINS OF THE UNITED STATES

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Bachelor of Science in Microbiology

Oklahoma State University

Stillwater, Oklahoma

2013

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2016

AMPHIBIAN BLOOD PARASITES AND THEIR POTENTIAL VECTORS IN THE GREAT PLAINS OF THE UNITED STATES

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# ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Matthew Bolek for his guidance, patience, and support throughout the course of this project. His enthusiasm and support have made working in his lab both enjoyable and rewarding. I am also grateful for Dr. Bolek's expertise in numerous parasite systems and his willingness to allow me to investigate understudied organisms like the amphibian blood protozoa.

I would also like to thank my committee members, Drs. Monica Papeş and Bruce Noden, for their guidance and the advice that they provided during this project and on this manuscript. Additionally, I would like to thank the previous and current members of the Bolek lab for their assistance with collecting specimens, increasing my knowledge and appreciation of our field through our discussions of parasitology, and finally for their friendship. These include Cleo Harkins, Dr. Heather Stigge, Dr. Kyle Gustofson, Chelcie Pierce, Christina Anaya, and Ryan Koch.

I would like to acknowledge my parents Chris and Terry Shannon, and brothers Eric Shannon and Kevin Shannon for their continued support and contributions to my education. Lastly, I'd like to thank my girlfriend Ashley Love for her assistance in collecting amphibians and setting up mosquito traps, feedback on manuscripts, and overall support and companionship throughout this project.

Name: RYAN PATRICK SHANNON

Date of Degree: JULY, 2016

Title of Study: AMPHIBIAN BLOOD PARASITES AND THEIR POTENTIAL VECTORS IN THE GREAT PLAINS OF THE UNITED STATES

Major Field: Zoology

Abstract: Between May 2014 and August 2015, 200 larval, newly metamorphosed and adult amphibians from 5 families and 9 species were examined for blood protozoa, leeches and leech hematomas from five locations in north central Oklahoma. Of those, only adult bullfrogs (*Lithobates catesbeianus*) and newly metamorphosed and adult southern leopard frogs (*Lithobates sphenocephalus*) were infected with *Hepatozoon* and/or *Trypanosoma* spp. Six species/morphotypes of blood protozoans infected southern leopard frogs and bullfrogs and conformed to previous descriptions of (1) *Hepatozoon catesbianae*, (2) *Trypanosoma ranarum*, (3) *T. schmidti*, (4) *T. loricatum*, (5) *T. rotatorium*, and (6) *T. chattoni*. Because leeches or leech hematomas were never observed on any amphibians collected, I sampled for potential leech and dipteran vectors and examined tadpoles, newly metamorphosed and adult southern leopard frogs for blood infections from a single location. One species of turtle leech (*Placobdella rugosa*), and 2 species of mosquitoes that are known to feed on amphibians (*Culex erraticus* and *Uranotaenia sapphirina*), were collected as potential vectors. Blood protozoan prevalence and species richness increased as southern leopard frogs aged and became more terrestrial in their habitats, being 0 in tadpoles, 0.09 ± 0.3 (9%) in newly metamorphosed frogs and 1.10 ± 1.34 (52%) in adult frogs, suggesting that frogs acquired infections with blood protozoans in a terrestrial environment and potentially via dipteran vectors after metamorphosis. Sequencing of the 18s rRNA gene and phylogenetic analyses of the blood parasites in this study revealed that the trypanosome morphotypes are genetically distinct and the Oklahoma strain of *H. catesbianae* formed a polytomy with previous sequences of *H. catesbianae* and *H. clamatae* from Canada. Broader sampling of blood parasite species/morphotypes with additional genetic markers is necessary to resolve the phylogenetic relationships within these blood protozoans and to better support trypanosome species identifications.

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# OVERVIEW

Parasitism is a form of symbiotic association between two organisms. The most widely used definition states that it is a symbiotic and intimate relationship between two organisms where the parasite lives on, or in a host, and at the expense of the host (Roberts et al. 2013). Some parasites can be highly pathogenic to their hosts, causing mechanical tissue damage or stimulating inflammatory and/or immune responses (Roberts et al. 2013). Other parasites can be more subtle, simply diverting resources away from the host to various degrees, where in some cases the harm caused from the parasites presence is debatable (Combes, 2001). Importantly, parasites are metabolically dependent on their hosts, and possess remarkable adaptations to uptake resources from the host and survive host immunological defenses (Roberts et al. 2013).

Price (1980) estimated that more than 50% of all plant and animal species are parasitic at some point in their life cycle. Just as importantly, it has been estimated that almost all animals and plants serve as hosts for parasites (Esch and Fernandez, 1993). Therefore, parasitism is a very common ecological relationship which is represented in most species. To the parasite, the host represents a resource and a habitat where the parasite can grow and reproduce (Combes, 2001). Once produced, parasite reproductive stages are released from the host into the external environment or taken up by a vector

host where they undergo development, and must find their way back in to another host (Roberts et al. 2013). Therefore, unlike most free living organisms, one of the major problems faced by parasites is for individuals of a particular species to find the correct host to propagate the next generation and complete the life cycle. This is a statistical problem of colonization, where parasites face spatial and temporal difficulties of transfer from one host to another, which must be overcome by enormous reproductive outputs and/or by exploiting complex ecological associations between successive hosts (Tinsley, 1990).

Combes (2001) identified the processes which limits a given parasites' range of suitable hosts into two general categories defined as filters. The encounter filter includes ecological barriers to parasite transmission. Ecological barriers can result through a biodiversity parameter, where the geographic ranges of the parasite and host must overlap for successful transmission, and a behavioral parameter, where the spatial and temporal activities of the parasite and host must coincide for parasite transmission to occur. Once a parasite is in contact with a potential host, the compatibility filter further excludes some hosts through physiological or immunological barriers. Physiological barriers can result through a resource parameter, where the host must be able to spatially or metabolically support the requirements of a parasite's infection, and a defense parameter, where the parasite must be able to evade host defenses such as immune responses or other mechanisms (Combes, 2001).

Despite these barriers, parasites are immensely successful organisms, and as mentioned above, are estimated to have colonized almost all animal and plant species (Esch and Fernandez, 1993). Parasites overcome the ecological barriers in the "encounter

filter" and the physiological barriers of the "compatibility filter" with complex adaptations including enormous reproductive outputs, exploiting ecological associations between successive hosts, and remarkable mechanisms to evade the host defensive responses. In narrowing the range of suitable hosts for a given parasite, these filters reflect the adaptations possessed by the parasite, allowing for studies that provide insight into parasite biology through inferences made from the ecologies of infected hosts.

Amphibians reside in a multitude of habitats and have varied life history patterns, reproductive modes, body sizes, foraging modes, and trophic levels (Petranka, 2010; Dodd, 2013). Therefore, amphibian communities are excellent models for studies on host parasite interactions because multiple amphibian species with diverse life histories can be sampled for parasites at a single location, and prevalence and species richness data can give insight into the ecological relationships determining parasite species distribution and their transmission within amphibian hosts.

Numerous studies on amphibian parasites have concentrated on the life histories, distribution, and community structure of helminth parasites (Brooks, 1976; Aho, 1990; Bolek and Coggins, 2000, 2001, 2003; Bolek and Janovy, 2007a, 2007b, 2008; Yoder and Coggins, 2007; Bolek et al. 2009, 2010; Langford and Janovy, 2009, 2013). These studies have focused on a wide array of amphibian hosts and provided life history information, host specificity, prevalence and intensity data for nematodes, trematodes and cestodes of true frogs (Ranidae), treefrogs (Hylidae) and true toads (Bufonidae) (see Muzzal and Peebles, 1991; Bolek and Coggins, 1998a; Bolek and Coggins, 2000, 2003; McAllister, 2013). Similar helminthological data has been collected for caudatan amphibians in three families including mole salamanders (Ambystomatidae), lungless

salamanders (Plethodontidae), and newts (Salamandridae) (see Muzzal, 1990, 1991; Muzzal and Schinderle, 1992; Bolek, 1997; Bolek and Coggins, 1998b; Muzzal et al. 2003; Rhoden and Bolek, 2012). Although less common, amphibian intestinal protozoa surveys are also abundant in the literature. These include work on amphibian coccidia (McAllister, 1987, 1989; Bolek et al. 2003; Duzynski et al. 2007), myxozoans (Jirků et al. 2006; Hartigan, 2013; McAllister et al. 2013), opalinid flagellates (Delvinquier and Desser, 1996; Bolek and Coggins, 1998a), and nyctotheran ciliates (Bolek and Coggins, 1998a; McAllister et al. 2013).

All of these studies clearly suggest that life cycle strategies of amphibian parasites are adapted to the environment and ecology of their hosts; however, few comprehensive amphibian surveys have concentrated on amphibian blood protozoa. Currently only a single North American study has examined multiple species of anurans and caudatans from a single location for all groups of amphibian blood protozoans (Barta and Desser, 1984). Additionally, Werner and Walewski (1976) examined multiple species of caudatans and anurans for trypanosome infections from a single location in Michigan. As a result, we know very little about the distribution, host specificity and community structure of these amphibian protozoa and how they are transmitted among and within amphibian hosts.

One of the reasons amphibian blood protozoa are largely understudied is the simple fact that there are few useful characters to differentiate between species. Many amphibian trypanosome species are polymorphic, where the same species gives rise to multiple cell morphologies. Often multiple trypanosome morphotypes can be observed infecting a single amphibian host and it is unclear whether these morphotypes represent

different species or a single polymorphic species (Diamond, 1965). Additionally, gamonts of some species of *Hepatozoon* infecting red blood cells of true frogs are morphologically indistinguishable but have different effects on the host red blood cell nuclei. For example, gamonts of *H. clamatae* distort and fragment the erythrocyte nucleus; whereas *H. catesbianae* gamonts do not (Kim et al. 1998). However, mixed infections are possible where within the same host individual, some red blood cells possess fragmented nuclei and some do not, and it is not clear if these represent an immature *H. clamatae* infection, or a mixed infection with both *H. clamatae* or *H. catesbianae* (personal observation). The difficulties in identification of amphibian blood protozoa have undoubtedly led to these parasites being largely ignored by the scientific community. However, recent technological advances have made obtaining genetic information easier and have therefore created the potential to use gene sequence data as additional markers for amphibian blood parasite identification.

This thesis investigates the distribution, host specificity, and community structure of amphibian blood protozoa as well as provides insight into how they are transmitted among and within amphibian hosts. This work had three objectives: 1) to determine the blood parasite prevalence and species richness infecting adult, larvae and newly metamorphosed stages of amphibians of multiple species residing in different ecological habitats and compare infection parameters among amphibian species and different amphibian life stages, 2) to identify potential leech and dipteran vectors at locations containing amphibians infected with blood protozoa, and 3) to morphologically and molecularly characterize the species of blood protozoa found infecting amphibians in north central Oklahoma.

Oklahoma has a rich amphibian diversity represented by 11 families and 58 species with varied habitat preferences and foraging strategies (Sievert and Sievert, 2011). This diversity makes Oklahoma an excellent location for comparative studies on amphibian blood protozoan communities. By examining numerous species of amphibians at different life stages for their blood protozoan species and communities, this study provides valuable information on the distribution of these protozoans within amphibian communities and across aquatic and terrestrial habitats. Additionally, this study gives evidence regarding the ecology of the vector responsible for blood parasite transmission by collecting potential vectors and comparing the ecologies of infected hosts. Finally, this study provides insight into the identification of blood parasite species by supplementing morphological data with molecular data.

My thesis consists of three chapters: Chapter I summarizes the sampling of amphibian hosts for blood parasites, the surveys conducted for potential leech and dipteran vectors present at a site containing infected amphibians, and the analyses of prevalence and species richness data across amphibian hosts and life stages to make inferences about the ecologies of potential vectors. In Chapter II, the five morphotypes of *Trypanosoma* found infecting bullfrogs and southern leopard frogs in north central Oklahoma are characterized morphologically and molecularly. In Chapter III, the apicomplexan species *Hepatozoon catesbianae* found infecting bullfrogs and southern leopard frogs in north central Oklahoma is characterized morphologically and molecularly.

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# CHAPTER I

## HOST ASSOCIATIONS OF AMPHIBIAN BLOOD PROTOZOA AND THEIR POTENTIAL VECTORS IN NORTH CENTRAL OKLAHOMA

ABSTRACT: Between May 2014 and August 2015, 200 larval, newly metamorphosed and adult amphibians from 5 families and 9 species were examined for blood protozoa, leeches and leech hematomas from five locations in north central Oklahoma. Of the amphibians surveyed, only adult bullfrogs (*Lithobates catesbeianus*) and newly metamorphosed and adult southern leopard frogs (*Lithobates sphenocephalus*) were infected with *Hepatozoon* and/or *Trypanosoma* spp. Six species/morphotypes of blood protozoans infected southern leopard frogs and bullfrogs and conformed to previous descriptions of (1) *Hepatozoon catesbianae*, (2) *Trypanosoma ranarum*, (3) *T. schmidti*, (4) *T. loricatum*, (5) *T. rotatorium*, and (6) *T. chattoni*. Because leeches or leech hematomas were never observed on any amphibians collected I sampled for potential leech and dipteran vectors and examined tadpoles, newly metamorphosed and adult southern leopard frogs for blood infections from a single location. One species of turtle leech (*Placobdella rugosa*), and 2 species of mosquitoes which are known to feed on amphibians (*Culex erraticus* and *Uranotaenia sapphirina*), were collected as potential vectors. Blood protozoan prevalence and species richness increased as southern leopard

frogs aged and became more terrestrial in their habitats, being 0 in tadpoles, 0.09 ± 0.3 (9%) in newly metamorphosed frogs and 1.10 ± 1.34 (52%) in adult frogs, suggesting that frogs acquired blood protozoan infections in a terrestrial environment and potentially via dipteran vectors after metamorphosis.

## INTRODUCTION

Compared to other amphibian parasites, few comprehensive surveys have concentrated on amphibian blood protozoa. Currently only a single North American study has examined multiple species of anurans and caudatans from a single location in Ontario for all groups of amphibian blood protozoans (Barta and Desser, 1984). Additionally, Werner and Walewski (1976) examined multiple species of caudatans and anurans for trypanosome infections from a single location in Michigan. These studies suggest that host specificity and life cycle strategies of amphibian blood protozoans are associated with the ecology of their amphibian hosts and specific vectors found in aquatic or terrestrial environments. However, in general we know very little about the distribution, host specificity and community structure of these amphibian protozoa and how they are transmitted among and within amphibian individuals and species.

Two major groups of blood protozoa have been reported in anurans and caudatans from North America (Diamond, 1965; Bardsley and Harmsen, 1973; Barta and Desser, 1984; Martin and Desser, 1990; Barta, 1991; Desser, 1993; Desser et al., 1995; Smith, 1996; Kim et al., 1998; Barta et al., 2001; Desser, 2001; Paperna et al., 2009). These include the apicomplexans (*Hepatozoon* spp., *Lankesterella* spp., and *Babesiasoma* spp.)

and the kinetoplastids (*Trypanosoma* spp.). Although little information is available on the life cycles of most species of blood protozoans of amphibians, all species for which life cycles are known use leeches or blood feeding dipterans such as frog biting midges (Corethrellidae), sandflies (Psychodidae) and potentially mosquitoes (Culicidae) as vectors (Anderson and Ayala, 1968; Ramos and Urdaneta-Morales, 1977; Tse et al., 1986; Barta 1991; Johnson et al., 1993; Desser, 1995; Kim et al., 1998; Bartlett-Healy et al., 2009).

One reason so little information is available on the life cycles and transmission of amphibian blood protozoans is that until recently there was little known about the potential invertebrate vector hosts for these parasites. However, over the last three decades we have gained a better understanding of leech and dipteran interactions with their amphibian hosts.

In North America, amphibian leech interactions have been reported for a wide variety of leech and amphibian species and these interactions appear to be relatively common (Barta and Sawyer, 1990; Bolek and Janovy, 2005; Moser et al., 2005; Siddall and Bowerman, 2006; McCallum et al., 2011; Rhoden and Bolek, 2012). With one exception (*Placobdella picta*), most of these studies indicate that amphibian leeches are restricted to a single amphibian host species or those leech species have a restricted geographical distributions (Moser et al., 2005; Siddall and Bowerman, 2006).

As with leeches, all information regarding dipterans feeding on amphibian hosts has been primarily limited to visual observation (Shannon, 1915; Crans, 1970; Blosser and Lounibos, 2012). However, two recent advances including (1) the ability to identify

blood meals from recently fed dipterans by molecular techniques and (2) new methods of trapping dipterans that preferentially feed on amphibians have allowed scientists to identify blood feeding dipterans which specialize on amphibians (McKeever and Hartberg, 1980; McKeever and French, 1991; Cupp et al., 2004; Toma et al., 2005; Borkent and Belton, 2006; Camp, 2006; Bartlett-Healy et al., 2008a; Tamashiro et al., 2011). These studies indicate that in North America, frog biting midges in the genus *Corethrella* and mosquitoes including *Culex territans*, *Culex peccator*, *Uranotaenia* spp. and *Deinocerites* spp. among others specialize on amphibian blood (Cupp et al., 2004; Burkett-Cadena et al., 2008a, Tamashiro et al., 2011). Additionally, life history studies on some of these dipterans indicate that they are highly associated with the amphibian habitat (Burkett-Cadena et al., 2008b; Bartlett-Healy et al., 2008b). Finally, discoveries by McKeever and colleagues indicate that frog biting midges are attracted to amphibians by their mating calls (McKeever, 1977; McKeever and Hartberg, 1980; McKeever and French, 1991). By developing modified CDC light traps and playing frog calls, McKeever and Hartberg (1980) were able to collect frog biting midges exclusively. More recently, researchers conducting studies in Japan, Costa Rica and the United States were able to collect mosquitoes that specialize on amphibians using these modified frog call traps (Toma et al., 2005; Borkent and Belton, 2006; Bartlett-Healy et al., 2008a; Tamashiro et al., 2011). This new information suggests that mosquitoes and other dipterans that specialize on amphibians can be collected relatively easily and this technique can provide new information for potential vectors of amphibian blood protozoans.

In this study, I first surveyed for blood parasites in adult, larvae and newly metamorphosed stages of amphibians of multiple species residing in different ecological habitats and then compared infection rates among amphibian species and different amphibian life stages. Second, at one of the sites containing infected amphibians, I surveyed for potential leech and dipteran vectors using newly developed collecting techniques. This is the first study of potential vectors of amphibian blood parasites at a location with confirmed infected amphibians, and provides valuable information on the distribution of these protozoans within amphibian communities and across aquatic and terrestrial habitats.

Oklahoma has a rich amphibian diversity represented by 11 families and 58 species with varied habitat preferences and foraging strategies (Sievert and Sievert, 2011). This diversity makes Oklahoma an excellent location for comparative studies on amphibian blood protozoan communities. By examining numerous species of amphibians at different life stages for their blood protozoan species and communities and collecting potential vectors, this comparative study addressed the following questions: 1) What species of blood protozoans infect a community of amphibians? 2) What life stages of amphibians are infected with blood protozoans? and 3) What potential vectors are present in aquatic and terrestrial habitats at these locations?

## MATERIALS AND METHODS

### Description of field site and anuran collections

Between April and September 2014 and April and July 2015 eight anuran and one caudatan species representing four families were collected from a single location in Guthrie, Logan County, and four locations in Stillwater, Payne County, Oklahoma U.S.A. and examined for the presence of blood protozoa. The sites included an ephemeral stream located in Guthrie, Logan County, and its surrounding area, referred to as Guthrie Stream (N 35° 24.666', W 97° 24.160'), four sites in Stillwater, Payne County which included: Sanborn Lake and surrounding area, referred to as Sanborn Lake (N 36° 09.31', W 97° 04.68'), Chapel Ridge Apartment complex and its surrounding area, referred to as Apartment Pond (N 36° 8.337', W 97° 2.922'), OSU Microscopy Facility, referred to as OSU Microscopy Pond (N 36° 6.352', W 97° 6.384'), and Teal Ridge (N 36° 6.050', W 97° 4.790'), a non-irrigated restored semi-permanent wetland (Fig. 1). Of the five sampled locations, only Teal Ridge Wetland contained both a diverse amphibian community and was identified to be positive for amphibian blood protozoans. Therefore, Teal Ridge was an ideal location for studying the blood parasites and their potential vectors of a single amphibian community. As a result, all amphibian and potential vector collections were conducted exclusively at Teal Ridge between April and July 2015.

The ephemeral stream in Guthrie is located in a Postoak-Blackjack Oak Forest ecosystem and meanders for approximately 1 km through Triple Crown Meadow subdivision. The stream is approximately 1 m in width and contains slow moving water during heavy rains. Small pools of water remain in the stream throughout the year.

Sanborn Lake contains two permanent man-made connected lakes that measures 495 m long by 135 m wide and 158 m long by 90 m wide and are both over 2 m deep. The lakes are located in a mixed grass prairie ecosystem and surrounded by various trees and emergent vegetation such as (*Carex* spp.) and cattails (*Typha* spp.). Apartment Pond is a permanent man-made pond that measures 47 m long by 30 m wide and approximately 1 m deep with no emergent vegetation surrounding the pond. The OSU Microscopy Pond is also a permanent man-made pond that measures 90 m long by 70 m wide and is approximately 1 m deep with cattails (*Typha* sp.) surrounding the pond. Finally, Teal Ridge is situated in a mixed grass prairie ecosystem and is 9.31 hectares of restored non-irrigated semi-permanent wetland containing four distinct ponds surrounded by sedges (*Carex* spp.) and cattails (*Typha* spp.). The four ponds range in size from 183 m by 150 m to 100 m by 40 m and are each less than 1 m deep. The largest pond is surrounded by the three smaller ponds, which are located 33 m to the north and east side of the largest pond.

Amphibians from all locations sampled were collected by hand or by dip-net and examined for leeches and/or leech hematomas according to Rhoden and Bolek (2012). Adult and newly metamorphosed amphibians were placed in moist cotton bags; whereas all larval amphibians were placed in 18.9 L buckets filled with pond water and transported to the laboratory. In the laboratory, amphibians were double pithed and examined for blood protozoans within 12 hours of capture. Snout-vent length (SVL) to the nearest 1.0 mm was recorded for each metamorphosed anurans. Snout-vent length and total length (TL) was recorded for salamanders and anuran tadpoles according to Petranka (1998), and McDiarmid and Altig (1999), respectively. When possible, sex was determined for all individuals by gonadal inspection during necropsy.

**Blood and processing and examination**

Blood was collected from adult and newly metamorphosed amphibians by making a small incision behind the foramen magnum and drawing the blood from the area with heparinized capillary tubes. For tadpoles, the tail vein was punctured with a 30-gauge needle and blood was drawn in heparinized capillary tubes, as previously described by Diamond (1965). For each amphibian individual, blood from a single capillary tube was deposited on a glass slide with a pipette bulb and a thin smear prepared. To increase the probability of detecting *Trypanosoma* spp., a modified micro-hematocrit technique was employed (Woo, 1969). Briefly, a second capillary tube full of blood was plugged at one end with clay Cha-Seal tube sealing compound (Kimble Chase, NJ) and centrifuged for 2 minutes at 3,600 RPM. The junction between the red blood cells and plasma, referred to as the buffy layer, was examined under a dissecting microscope for movement indicative of a trypanosome infection. If positive, the plugged capillary tube was scored at the blood-plasma junction and the buffy layer deposited on a glass slide for further examination with a compound microscope. Additionally, 200 μL of blood from each infected amphibian was fixed in 100% ethanol in 1.5 ml Eppendorf tubes and stored at -80 °C for molecular analyses.

Thin blood smears were air-dried, fixed in methanol for 1 minute and stained with Giemsa (1:10 in phosphate-buffered water) for 10 minutes and then air-dried according to Barta and Desser (1984). Slides were examined with a 10x objective for approximately 5 minutes for trypanosomes and for an additional 5 minutes with a 100x objective for intracellular parasites using an Olympus BX-51 upright research microscope configured for bright-field and differential interference contrast microscopy with plain fluorite

objectives. Digital photographs of all protozoans were taken with an Olympus 5 megapixel digital camera.

All blood protozoa were identified based on morphological descriptions in Bardsley and Harmsen (1973), Barta and Desser (1984), Desser et al. (1995), Kim et al. (1998), Smith et al. (1999; 2000), and Desser (2001). Additionally, morphological characteristics were recorded for all *Trypanosoma* species/morphotypes and *Hepatozoon* species and either 18s rRNA or 18s rRNA and ITS 1 sequences were obtained for all species/morphotypes of blood protozoans (see Chapters III and IV). Voucher stained blood smears containing protozoa will be deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska.

**Potential dipteran vector trapping, processing, and identification**

Adult mosquitoes and other potential dipteran vectors were collected using Center for Disease Control and Prevention (CDC) miniature light traps (Model No. 1012, John W. Hock Company, Gainesville, Florida, USA) modified according to McKeever and Hartberg (1980) and Toma et al. (2005). These modifications included, first, the addition of nylon mesh stretched over the polypropylene collection cups to prevent smaller insects from escaping. Second, the bulbs were removed from traps and recorded frog calls were broadcasted over the intake of each trap using small, 8 GB MP3 player (SanDisk SansaClip+) with a portable rechargeable speaker (Goal Zero Rock Out; Fig. 2). The remaining three miniature CDC light traps were used as controls. The control traps were run with the bulb on and no sound for three of the five sampling dates and with no light

and no sound for two of the five dates sampled. The three Miniature CDC control traps and four frog call traps were hung 0.5 m above the ground on camera tripods (Vivitar 50" Tripod), and were positioned 1-0.5 m from the shore of the ponds. Traps were alternated approximately 50 m apart along a line of shore adjacent to a wooded habitat. The speaker playing frog calls was pointed toward the nearest body of water according to McKeever and Hartberg (1980). One night in July, 2014 and once a month between May and August 2015, miniature CDC control and frog call traps were set before sunset, turned on approximately 30 minutes after sunset and turned off at sunrise. The call of a non-native barking treefrog, *Hyla gratiosa*, obtained from a commercially available recording (Elliot et al., 2009) was used to attract potential vector hosts. The recordings consisted of 30 seconds of an individual *H. gratiosa* frog advertisement call followed by 10 seconds of silence; this sequence was repeated for the duration of time traps were running from sunset to sunrise. The *H. gratiosa* call was used as the dipteran attractant based on previous work by Borkent and Belton (2006) and Camp (2006). Briefly, in previous studies Borkent and Belton (2006) and Camp (2006) found that the calls of *H. gratiosa* were the best attractant to frog-feeding dipterans, even though *H. gratiosa* was not native to their sampling locations in Costa Rica and Georgia, U.S.A., respectively.

All captured mosquitoes and other dipterans from each trap were placed on ice in coolers and transported to the laboratory. Dipterans were then placed in a refrigerator and individuals from each trap were killed with ethyl acetate, and stored in a refrigerator at 4° C until identified within 24 hr of being collected. Mosquitoes were identified using keys in Carpenter and LaCasse (1955), Darsie and Ward (2005) and Burkett-Cadena (2013); whereas all other dipterans were identified using keys in McAlpine et al. (1981).

Representative dipterans were pinned and voucher specimens will be deposited in the K.C. Emerson Entomology Museum, Oklahoma State University-Stillwater.

**Potential leech vector trapping, processing, and identification**

Leeches were collected from submerged aquatic plants with a dip-net or by hand from submerged debris located a few feet from the shore of ponds from which amphibians were collected at Teal Ridge. Some of the debris (a sign and two beer bottles) were checked for leeches and replaced on seven different dates from May through July, 2015. Leeches were removed from debris, placed in plastic 75 ml capped jars filled with pond water and brought to the laboratory for identification. All leeches were identified alive using a stereomicroscope and keys by Klemm (1985, 1991), Moser et al. (2012a, b; 2013a, b) and Moser et al. (2016). Once identified, representative leeches were relaxed in tap water narcotized with 10% ethanol, and preserved in 70% ethanol according to Rhoden and Bolek (2012). Voucher specimens of leeches will be deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska.

**Statistical analyses**

Prevalence for blood protozoan morphotypes/species was calculated according to Bush et al. (1997) and 95% confidence intervals were calculated for appropriate sample sizes (Rohlf and Sokal, 1995). Mean protozoan species richness was calculated as the sum of protozoan morphotypes/species per individual amphibian, including uninfected

individuals, divided by the total sample size for each amphibian species examined according to Bolek and Coggins (2003). The Chi square test for independence was calculated, for infected amphibian species with appropriate sample sizes, to compare differences in prevalence among amphibian life stages, sexes, and/or species. Student's *t*-test was used to compare differences in mean morphotype/species richness between amphibian life stages, sexes, and amphibian species. Approximate *t*-tests were calculated when variances were heteroscedastic. Pearson's correlation was used to determine the relationships among amphibian SVL and TL and protozoan morphotype/species richness (Sokal and Rohlf, 1981).

## RESULTS

### Amphibian collections and blood protozoan diversity

In total, 10 adults of a single species of salamander and 190 adults, recently metamorphosed individuals, and/or tadpoles of eight anuran species were collected from five sites in north central Oklahoma (Table I). The mean SVL ± 1 SD and/or range for all metamorphosed anurans collected were as follows: bullfrogs 75.8 ± 35.8 mm, 29 – 170 mm; Woodhouse's toads 75.4 ± 18.5 mm, 51 – 98 mm; southern leopard frogs 62.7 ± 7.6 mm, 48 – 80 mm; American toads 58 – 65 mm; gray treefrogs 42.9 ± 3.78 mm, 37 – 48 mm; narrow-mouthed toad 24.5 ± 3.2 mm, 17 – 31 mm; spotted chorus frogs 23 ± 3.74 mm, 20 – 29 mm; and Blanchard's cricket frogs 22.4 ± 3.1 mm, 14 – 28 mm. The mean SVL ± 1 SD and range, and mean TL ± 1 SD and range for tadpoles and adult salamanders collected were as follows: southern leopard frog tadpoles SVL 24.3 ± 2.96

mm, 19 – 32 mm; TL 57.5 ± 5.53 mm, 47 – 73 mm; gray treefrog tadpoles SVL 14.9 ± 1.44 mm, 12 – 17 mm; TL 34.2 ± 3.5 mm, 28 – 39 mm; small mouth salamanders were SVL 50.9 ± 4.28 mm, 45 – 57 mm; TL 91.3 ± 10.6 mm, 74 – 112 mm.

Of the nine amphibian species examined, only two anuran species were positive for blood protozoa. Twenty-seven percent (19/70) of southern leopard frogs (*Lithobates sphenocephalus*) and 18% (5/28) of bullfrogs were infected with blood protozoa. Of those, 7 % (2/29) of adult southern leopard frogs and 7% (2/28) of adult bullfrogs were infected with a *Hepatozoon* sp. (Table II). Based on morphological description by Desser et al. (1995) and Kim et al. (1998), this species conformed to *H. catesbianae* (Fig. 3). In addition, 26% (18/70) of southern leopard frogs and 14% (4/28) of bullfrogs (*Lithobates catesbeianus*) were infected with five morphologically distinct *Trypanosoma* morphotypes/species. The five trypanosome morphotypes conformed to descriptions of *T. ranarum*, *T. schmidti*, *T. loricatum*, *T. chattoni*, and *T. rotatorium* (Fig. 3) (Diamond, 1965; Bardsley and Harmsen, 1973; Desser, 2001). Prevalence for the five *Trypanosoma* morphotypes/species ranged from as high as 45% (13/29) for *T. loricatum* and *T. rotatorium* in adult southern leopard frogs to as low as 7% (1/28) for *T. schmidti* in adult bullfrogs (Table II).

The majority of the infected frogs with trypanosomes (86%) contained multiple trypanosome morphotypes. Three frogs infected with *T. schmidti* were also infected with at least *T. ranarum*. Additionally, 80% (12/15) of frogs that were infected with *T. loricatum* were also infected with *T. rotatorium*, and 90% (9/10) of frogs that were infected with *T. chattoni* were also infected with either *T. loricatum* or *T. rotatorium*.

**Infection parameters in adult southern leopard frogs and adult bullfrogs**

Overall prevalence of all blood protozoans was significantly higher in adult southern leopard frogs (62%, 18/29) than adult bullfrogs (18%, 5/28; $\chi^2 = 11.569$, $P < 0.001$). Additionally, mean species/morphotype richness of blood protozoans was significantly higher in southern leopard frogs (1.52 ± 1.43) than in adult bullfrogs (0.39 ± 1.07; $t' = 3.37$, $P < 0.001$; Fig. 4). There was no significant difference in the overall prevalence of blood protozoans among male (60%, 9/15), female (100%, 6/6), and unsexed (38%, 3/8; $\chi^2 = 5.75$, $P = 0.0566$) southern leopard frogs or male (40%, 4/10), female (10%, 1/10), and unsexed (0%, 0/8; $\chi^2 = 5.50$, $P = 0.0638$) bullfrogs.

Trypanosome prevalence was also significantly higher in adult southern leopard frogs (59%, 17/29) than adult bullfrogs (14%, 4/28; $\chi^2 = 12.034$, $P < 0.001$). Additionally, mean trypanosome species/morphotype richness was significantly higher in southern leopard frogs (1.45 ± 1.40) than in adult bullfrogs (0.32 ± 0.904; $t' = 3.615$, $P < 0.001$). However, trypanosome prevalence was not significantly different between male (60%, 9/15), female (83%, 5/6), and unsexed (38%, 3/8; $\chi^2 = 2.99$, $P = 0.2238$) southern leopard frogs or male (30%, 3/10), female (10%, 1/10), and unsexed (0%, 0/8; $\chi^2 = 3.5$, $P = 0.1738$) bullfrogs.

**Prevalence and species richness of blood protozoa in different life stages of southern leopard frogs from Teal Ridge**

Within different life stages of southern leopard frogs from Teal Ridge, blood protozoan prevalence was significantly higher in adult frogs (52%, 11/21) than newly

metamorphosed frogs (9%, 1/11), or in tadpoles (0%, 0/30; $\chi^2 = 22.617$, $P < 0.0001$). Furthermore, mean species richness was significantly higher in adults (1.10 ± 1.34) than in metamorphs (0.09 ± 0.30; $t' = 3.2841$, $P < 0.01$; Fig. 5). Additionally, there was a significant positive correlation between southern leopard frog SVL and blood protozoan species/morphotype richness for all metamorphosed leopard frogs collected at Teal Ridge ($r = 0.495$, $P < 0.05$).

**Dipteran collections from Teal Ridge**

In total, 13 families of dipteran flies were captured on five different trapping nights at Teal Ridge. These families included: Aulacigastridae n = 1, Cecidomyiidae n = 195, Chaoborinae n = 21, Chironomidae n = 118, Dixidae n = 98, Phoridae n = 1, Psychodidae n = 274, Sciaridae n = 11, Sphaeroceridae n = 1, Syrphidae n = 1, Tipulidae n = 11, Ceratopogonidae n = 5, and Culicidae n = 33. Of these, only two families are known to feed on the blood of vertebrates and include the Culicidae and the Ceratopogonidae (Table III). Seven different species of mosquitoes (Culicidae) were collected. However of these, only two species (*Culex erraticus* and *Uranotaenia sapphirina*) have been reported to feed on amphibians (Burkett-Cadena, 2013). *Culex erraticus* was only collected from modified CDC frog call traps (n = 4; Fig. 6). Additionally, the only *U. sapphirina* (n = 1) specimen collected was a male captured in a control trap without a light (Fig. 6). Of the Ceratopogonids collected, three individuals of a *Forcipomyia* sp. were collected, each from a call trap, a control light trap, and a control

trap with no light (Fig. 6). However, all three specimens could only be identified to genus level.

**Leech collections from Teal Ridge**

Twenty-one leeches representing two families, Glossiphoniidae and Erpobdellidae were collected from Teal Ridge. Five individuals of the glossiphonid leech, *Placobdella rugosa*, were collected from both the plastic sign (n = 4) and a glass bottles (n = 1) and, 15 individuals of the glossiphonid leech, *Helobdella* sp. were collected from the plastic sign (n = 9), a glass bottle (n = 5), and by dip-net (n = 1). Additionally, a single erpobdellid leech was collected from the plastic sign (n = 1). Of those, only *P. rugosa* has been reported to feed on the blood of vertebrate hosts, predominantly turtles (Sawyer, 1972; Moser et al., 2012b; Fig. 6). Finally, no leeches were found on any of the amphibians collected and no leech hematomas were ever observed on any amphibians collected at Teal Ridge or any other amphibian collected from the other four locations.

**DISCUSSION**

Of the nine species of amphibians sampled for blood protozoa, only bullfrogs and southern leopard frogs were infected with at least one species/morphotype of blood protozoan. These blood protozoa include five kinetoplastid species/morphotypes, *T. ranarum*, *T. schmidti*, *T. loricatum*, *T. chattoni*, and *T. rotatorium*, and one apicomplexan, *H. catesbianae*. Prevalence of the five trypanosome species ranged from a

low of 4% (*T. schmidti*, *T. loricatum*, *T. rotatorium*) to a high of 14% (*T. ranarum*) in bullfrogs, and a low of 7% (*T. schmidti*) to a high of 45% (*T. rotatorium*, *T. loricatum*) in southern leopard frogs. In contrast, the prevalence of *H. catesbianae* was similar in bullfrogs and in southern leopard frogs, being 7% in both anuran species.

**Species designation of blood protozoans infecting Oklahoma amphibians**

*Trypanosoma ranarum* infected 14% of bullfrogs and 17% of southern leopard frogs. This species has been reported infecting 5 species of true frogs (*Lithobates catesbeianus*, *L. clamitans*, *L. pipiens*, *L. septentrionalis*, *L. sylvaticus*) and one hylid (*Hyla versicolor*) in the Great Lakes area of the United States and Canada (Diamond, 1965; Woo, 1969, 1983; Werner and Walewski, 1976; Barta and Desser, 1984; Woo and Bogart, 1984; Jones and Woo, 1986; 1989). Southern leopard frogs are a new host record for *T. ranarum* and Oklahoma is a new locality record for the trypanosome. *Trypanosoma schmidti* was found in 4% bullfrogs and 7% of southern leopard frogs. This species has only been reported from southern leopard frogs in Florida by Diamond (1965), making bullfrogs a new host record for this species. Interestingly, *T. schmidti* was only found in frogs co-infected with *T. ranarum*. Oklahoma is a new locality record for *T. schmidti*.

*Trypanosoma chattoni* occurred in 7% of bullfrogs and 28% of southern leopard frogs. *Trypanosoma chattoni* has been reported from the northern leopard frog (*L. pipiens*) in Minnesota (Diamond, 1965) and Ontario (Jones and Woo, 1986, 1989). Additionally, Werner and Walewski (1976) found *T. chattoni* in true frogs in Michigan, but considered it a morphotype of *T. rotatorium* and did not specify which frogs were

infected with this morphotype. One reason Werner and Walewski (1976) considered the round trypanosome they observed to be a morphotype of *T. rotatorium* was that it was always found co-infecting the host with at least one other trypanosome morphotype. In the current study, 90% of frogs infected with *T. chattoni* were also infected with either *T. loricatum* or *T. rotatorium*. One frog infected with *T. chattoni* was not infected with *T. loricatum* or *T. rotatorium*, but was also infected with *T. ranarum*. Jones and Woo (1986) also found *T. chattoni* only in frogs that were also co-infected with other trypanosome species. These observations suggest that *T. chattoni* is a morphotype of a polymorphic *Trypanosoma* species. However, data from laboratory culture and genetic studies suggest that *T. chattoni* is a distinct species (Diamond, 1965; Martin et al., 2002). Diamond (1965) isolated *T. chattoni* from norther leopard frogs in Minnesota and maintained it in culture for 7 years, and never observed any changes in its morphology. Additionally, when culture forms were inoculated into frogs by Diamond (1965), only the round forms of *T. chattoni* were observed. Martin et al. (2002) performed phylogenetic analyses using the 18s rRNA gene and found *T. chattoni* to be distinct and basal to the other anuran trypanosomes, some of which have been reported to co-occur with *T. chattoni* in natural anuran infections (Jones and Woo, 1986). In this study, I report the round trypanosome as *T. chattoni* based on morphological similarities to *T. chattoni* as reported by Diamond (1965), Jones and Woo (1986), and Desser (2001). Bullfrogs and southern leopard frogs are new host records for *T. chattoni*, and Oklahoma is new locality record for this species.

*Trypanosoma loricatum* was found in 4% of bullfrogs and 45% of southern leopard frogs. *Trypanosoma loricatum* has been reported infecting ranid frogs worldwide (Bardsley and Harmsen, 1973; Desser, 2001; McKenzie and Starks, 2008).

Morphologically, this trypanosome species conforms to reports of *T. rotatorium* as reported from the Great Lakes area of North America (Woo, 1969; Barta and Desser, 1984). However, the descriptions of *T. rotatorium* and *T. loricatum* indicate that *T. rotatorium* has a free flagellum; whereas *T. loricatum* does not as they were originally described by Mayer (1843) from anurans in France. In contrast, the *T. rotatorium* as reported by Woo (1969) and Barta and Desser (1984) did not have a free flagellum, suggesting these reports were dealing with two distinct species of trypanosomes or two different morphotypes of *T. rotatorium*. Diamond (1965) indicated that *T. rotatorium* does not exist in North America because the *T. rotatorium* reported in North America does not have a free flagellum like that of its European counterpart. Additionally, the form observed in this study did not have a free flagellum, and therefore I follow the designation of Desser (2001) for New World anuran trypanosomes and refer to this form as *T. loricatum* instead of *T. rotatorium*. However, it should be noted that *T. loricatum* as re-described from edible frogs (*Pelophylax* spp.) from France by França and Athias (1906) was reported to have a free flagellum, making the identification of *T. loricatum* and *T. rotatorium* even more problematic. Clearly it is unlikely that the morphotypes of *T. loricatum* and *T. rotatorium* infecting North America anurans represent the same species than these morphotypes reported from anurans in Europe. In order to resolve this taxonomical confusion it will be necessary to compare the North American and European *T. loricatum* and *T. rotatorium* species/morphotypes using morphological and molecular markers. However, currently no sequences are available for any trypanosome infecting European anurans.

The morphotype referred to as *Trypanosoma rotatorium* was found in 4% of bullfrogs and 45% of adult southern leopard frogs. This species is the most commonly reported trypanosome of anurans and has been reported in at least 21 different anuran host species from three families and one salamander family worldwide (Bardsley and Harmsen, 1973). Additionally, many morphologically distinct trypanosomes have been reported as *T. rotatorium*. Because of the multiple species of amphibians reported as hosts for *T. rotatorium* and the extreme morphological variation of *T. rotatorium* reported in these studies, it is likely that the literature on *T. rotatorium* actually represents multiple trypanosome species (Bardsley and Harmsen, 1973). Of the many forms reported for *T. rotatorium*, one has been described as a cornucopia with a curved anterior end (Laveran and Mesnil, 1907). However, to my knowledge an anuran trypanosome morphotype like the one found in this study, which has a relatively small size (~30 μm) and a curved anterior end without a free flagellum has not been described previously. Interestingly, of the 15 frogs infected with *T. rotatorium*, 12 were also co-infected with *T. loricatum*. Whether this is due to trypanosome polymorphism or similar transmission strategies using the same vector host remains unclear. However, no other reports of *T. loricatum* and *T. rotatorium* morphotypes have been reported to co-infect anurans in North America (Bardsley and Harmsen, 1973; Barta and Desser, 1984; Desser, 2001). Details of morphological and genetic characterization of all five trypanosomes reported in this study are detailed in Chapter II.

Finally, the apicomplexan *H. catesbianae* was found infecting the red blood cells of 7% of adult bullfrogs and 7% of adult southern leopard frogs. This species has been reported from bullfrogs and green frogs in eastern North America, and is transmitted

between frogs by the mosquito *Culex territans* (Stebbins, 1904; Desser et al. 1995; Kim et al. 1998). A similar species, *Hepatozoon clamatae* infects bullfrogs, green frogs, and northern leopard frogs, and differs from *H. catesbianae* in that it distorts and fragments the nuclei of the host red blood cells (Stebbins, 1905; Kim et al. 1998). The *Hepatozoon* gamonts found in this study were never observed to distort or fragment the red blood cell nuclei. Oklahoma is a new locality record and southern leopard frogs are a new host record for *H. catesbianae*. Details of morphological and genetic characterization of *H. catesbianae* reported in this study are presented in Chapter III.

**Blood protozoan host specificity in Oklahoma amphibians**

Of the two species of amphibians infected with blood protozoans, bullfrogs are primarily aquatic frogs and are restricted to permanent lakes, rivers, streams and ponds, and they spend most of their time in the water but occasionally feed on the shore of aquatic habitats (Conant and Collins, 1998). In contrast, southern leopard frogs are considered semi-terrestrial anurans and reside along the shores of marshes, swamps, lakes ponds, rivers and creeks, and during the summer, wander great distances from water (Collins, 1993). The overall prevalence of blood parasites was significantly higher in southern leopard frogs than bullfrogs. However, none of the other arboreal, semi-arboreal and semi aquatic treefrog species, fossorial narrow-mouth toads and salamanders, or terrestrial toads was infected with any blood protozoans.

Although some of the amphibian sample sizes in this study were small, the absence of infected treefrogs, toads, and salamanders could result from blood protozoan

host specificity. In North America, trypanosomes have been reported from numerous species of true frogs, true toads, and treefrogs as well as mole salamanders and newts (Lehmann, 1952, 1954, 1959; Woo, 1969; Woo et al. 1980; Reilly and Woo, 1982; Woo and Bogart, 1984, 1986; Martin and Desser, 1990; Schotthoefer et al., 2009). Unfortunately, these accounts provide little information on field host specificity of amphibian trypanosomes because these studies only examined individual amphibian species for trypanosomes. However, host specificity of a few species has been investigated with cross-inoculation experiments in the laboratory and these species appear to be host specific at the family level (Reilly and Woo, 1982; Werner et al., 1988; Martin and Desser, 1991a). For example, Reilly and Woo (1982) demonstrated that intraperitoneal inoculation of culture forms of *Trypanosoma andersoni* and *T. grylli* were infective to the eastern gray treefrog (*Hyla versicolor*) and the spring peeper (*Pseudacris crucifer*), but were not infective to 10 other species of amphibians exposed with these trypanosomes. Additionally, Werner et al. (1988) demonstrated that *T. fallisi* was infective to toads but not true frogs, and *T. ranarum* was infective to true frogs but not toads. Finally, Martin and Desser (1991a) demonstrated that neither *T. ranarum* nor *T. fallisi* could persist longer than one day in the eastern gray treefrog.

In addition to trypanosomes, five species of *Hepatozoon* have been described infecting North American true frogs (Lehman, 1959a, 1959b, 1960; Desser et al. 1995; Kim et al., 1998). *Hepatozoon* species have not been reported in North American toads, treefrogs, or salamanders. However *Hepatozoon* species have been reported infecting toads and treefrogs in Asia and Africa (Smith, 1996). Laboratory investigations of amphibian host specificity to *Hepatozoon* species have focused on *H. catesbianae* and *H.*

*clamatae*, which infect bullfrogs, green frogs (*Lithobates clamitans*) and northern leopard frogs in nature (Kim et al., 1998). However Kim et al. (1998) and Harkness et al. (2010) attempted transmission of *H. catesbianae* and *H. clamatae* into American toads and blue spotted salamanders (*Ambystoma laterale*) and *H. clamatae* into wood frogs (*Lithobates sylvaticus*), respectively. Interestingly, only wood frogs, which are never infected with *Hepatozoon* spp. in nature, became infected. These experimental host specificity studies suggest that at least some amphibian trypanosomes and *Hepatozoon* species are physiologically adapted to their hosts and highlight the possibility that the blood parasites found in this study could be host specific to true frogs. Clearly, controlled laboratory host specificity studies are needed to be able to evaluate the amphibian host specificity of the *Trypanosoma* and *Hepatozoon* species found in this study.

Host specificity can also arise ecologically, where the habitats or activity levels of the host and vector differ. Additionally, even if hosts and vectors overlap spatially and temporally, vector-feeding preferences can preclude some species of parasites transmission to the amphibian host. The leech, *Placobdella picta*, and diperans, *Lutzomyia vexator*, *Corethrella wirthi*, and *Culex territans*, have been reported as vectors for amphibian blood protozoans in North America (Diamond 1965; Anderson and Ayala, 1968; Woo, 1969; Werner and Walewski 1976; Woo and Bogart 1986, Martin and Desser, 1991b; Johnson et al., 1993). In North America, leeches which feed on amphibians are restricted to the aquatic habitat and feed on amphibian larval and metamorphosed stages when amphibians are at least partially submerged in aquatic habitats; whereas dipteran vectors would come into contact with metamorphosed amphibians above the water and also feed on amphibians in a terrestrial environment. The

vectors for the blood parasites found in this study are unknown, but for an amphibian to become infected, they will have to come into contact with their vector host in a specific habitat.

**Potential vectors and transmission of blood protozoans in southern leopard frogs**

In this study, blood protozoan prevalence was highest in adult southern leopard frogs, significantly lower in newly metamorphosed frogs, and no tadpoles were ever infected with blood protozoans. Additionally, blood protozoans species/morphotype richness increased significantly with frog age and metamorphosed frog size, being 0 in tadpoles, 0.09 ± 0.30 in newly metamorphosed frogs and 1.10 ± 1.34 in adult frogs. Because, larger southern leopard frogs are typically older and spend more time out of the water (Dodd, 2013), the correlation between frog size and species richness suggests that as frogs spend more time in a terrestrial environment they are more likely to be infected with blood protozoans. In a previous study by Diamond (1965), 35% of northern leopard frog tadpoles collected in Minnesota, USA, were naturally infected with *Trypanosoma pipientis*, a trypanosome transmitted by the leech *Placobdella picta*. However, the absence of infected tadpoles and species of leeches that feed on amphibian hosts during this study suggests that leeches may not be the vectors for the trypanosomes recorded in this study, and dipteran vectors or other arthropods may play a role in transmission.

The only blood feeding leech collected during this study was *Placobdella rugosa*, which has been reported to feed on turtles (Moser et al. 2012a). Until recently and throughout most of its range, *P. rugosa* was known as *Placobdella ornate*. However,

Moser et al. (2012a; 2012b) synonymized most reports of *P. ornata* from the continental United States as *P. rugosa*. Sawyer (1986) reported that *P. ornata* feeds on amphibians, but it is unclear if he was referring to what is now *P. rugosa*, the only blood feeding leech collected in this study, or if he was dealing with *P. ornata* from its restricted locality in Connecticut. However, no leeches or leech hematomas were ever observed on any amphibian collected in this study suggesting that *P. rugosa* does not feed commonly on amphibians at my study site. The reported North American leech vector for amphibian trypanosomes, *P. picta*, was not found at Teal Ridge, and has not been reported from Oklahoma (Bolek and Janovy, 2005). However, *P. picta* has been reported from Arkansas, Kansas and Texas and most likely occurs in Oklahoma (Bolek and Janovy, 2005).

During the current study, three dipteran species known to feed on amphibians were collected during this study. These included two mosquito species (*Culex erraticus*, *Uranotaenia sapphirina*) and a species of biting midge (*Forcipomyia* sp.). *Culex erraticus* shows little discrimination in its animal targets for blood meals and reportedly feeds on birds, mammals, reptiles, and amphibians (Cupp et al., 2004; Burkett-Cadena et al., 2008b; Burkett-Cadena, 2013). Importantly, *C. erraticus* was the only mosquito collected exclusively from frog call traps. Additionally, a single male *Uranotaenia sapphirina* was recovered in a control trap without a light. Females of this mosquito species specialize in feeding on amphibians (Burkett-Cadena, 2013). It is interesting that no females of the genus *Uranotaenia* were recovered from frog call traps, unlike previous studies by Toma et al. (2005), Borkent and Belton (2006), and Tamashiro et al. (2011). However, recovering a male *U. sapphirina* indicates that females of this species are also

present at this location and are most likely feeding on frogs. *Uranoteania sapphirina* is one of the two known *Uranoteania* species that have been identified in past surveys of mosquito species in Oklahoma (Noden et al. 2015).

At least one member of the genus *Forcipomyia* is reported to feed on frogs, *Forcipomyia* (*Lasiohelea*) *fairfaxensis* (Desser, 1992). However, the three individuals found in this study could only be identified to genus. Therefore, because not all members of the genus feed on anurans, it is unclear if the species of *Forcipomyia* collected in this study feeds on amphibian blood. However, in a previous study on *F. fairfaxensis*, flies were commonly observed hovering around and landing on frogs to take a blood meal (Desser, 1992). In this study, no flies were observed on or near any frogs collected (personal observation), which suggests that the *Forcipomyia* sp. recovered in this study may not feed on amphibian blood, at least during the times in which frogs were collected and observed.

The reported vector for *H. catesbianae* and *H. clamatae* is the mosquito *Culex territans*, which was not collected during this study. *Culex territans* is a frog specialist and has been reported from Oklahoma and from Stillwater (Bohart, 1948; Carpenter and LaCasse, 1955; Noden et al. 2015). One reason *C. territans* was not collected during this study may be that this mosquito species has not been collected from frog call traps used in surveys by previous workers. Bartlett-Healy et al. (2008b) was unsuccessful in trapping *C. territans* using frog call traps but collected *C. territans* using resting traps positioned near ponds inhabited by frogs. It is worth mentioning that other *Hepatozoon* species have been described infecting frogs in areas outside of *C. territans* range, and it is unlikely that *C. territans* is the only vector for anuran *Hepatozoon* species. Additionally,

Harkness et al. (2010) was able to infect *Culex pipiens*, a bird/mammal feeding mosquito, in the laboratory with *H. clamatae*, further suggesting that amphibian *Hepatozoon* species may have multiple species of mosquito vector hosts.

In addition to the possible evidence that multiple mosquito species transmit amphibian *Hepatozoon*, there is evidence indicating that mosquitoes may be vectors for amphibian trypanosomes. Desser et al. (1973) and Ramos and Urdaneta-Morales (1977) attempted laboratory infections of mosquito species with amphibian trypanosomes and their findings highlight the possibility of mosquitoes serving as vectors for amphibian trypanosomes in nature. Desser et al. (1973) was able to infect *C. territans* with *T. rotatorium* and observed trypanosome development and multiplication in the mosquito hindgut. However, attempts at transmit trypanosomes back to amphibian hosts by allowing infected mosquitos to feed on frogs and feeding infected mosquitoes to frogs were unsuccessful. In contrast, Ramos and Urdaneta-Morales (1977) were able to infect laboratory strains of *Aedes aegypti* and *C. pipiens* with *T. rotatorium* from naturally infected South American frogs and asexual trypanosome reproduction was observed in the mid and hindgut of both mosquito species. More importantly, they were able to infect anuran hosts by feeding infected *A. aegypti* to frogs and injecting intestinal trypanosomes recovered from mosquito hindguts intraperitoneally. *Aedes aegypti* predominantly feeds on humans and other mammals (Burkett-Cadena, 2013), and it is doubtful that this species serves as an ecologically relevant vector for amphibian trypanosomes in nature. However, because *A. aegypti* can transmit trypanosomes to frogs in the laboratory, and *T. rotatorium* infects and develops in at least two other mosquito species, a natural mosquito vector for amphibian trypanosomes seems plausible. Specifically, two mosquito species

found in this study, *C.erraticus* and *U. sapphirina*, are excellent potential vector candidates because they feed on amphibians and are highly associated with amphibian habitats and/or are attracted to frog calls for feeding. However, their potential for transmit trypanosomes has not been evaluated. Additional vector sampling and laboratory investigations of trypanosome life cycles are necessary to definitively determine the vectors of the trypanosomes found in this study.

**Conclusions**

This is one of the first studies to examine both multiple species of amphibian hosts for blood protozoans and sample for possible vectors at the same location. The life cycles of the blood protozoa found in this study have yet to be elucidated. However, the results of my vector surveys and the blood protozoan prevalence and species richness data across multiple amphibian life stages provide the necessary data for future studies of potential vector candidates for transmission of amphibian trypanosomes. The possibility that frogs are most likely becoming infected with trypanosomes in the terrestrial environment is supported by four facts: 1) the absence of *P. picta*, the reported leech vector for North American amphibian trypanosomes at Teal Ridge, 2) the absence of leech hematomas on collected amphibians, 3) the absence of infected tadpoles at Teal Ridge, and 4) the higher prevalence and species richness of trypanosomes in older and larger southern leopard frogs. Other than leeches, the only reported vectors for amphibian blood protozoans are blood-feeding dipterans. Two mosquito species were collected during this study that feed on amphibians, and are probable vector candidates. Clearly,

additional vector sampling and laboratory investigations into amphibian blood protozoan life cycles are needed to evaluate the possibility of mosquito transmission of trypanosomes found in this study. I hope my study provides insight for other parasitologists to examine the potential vectors and life cycles of these neglected amphibian parasites.

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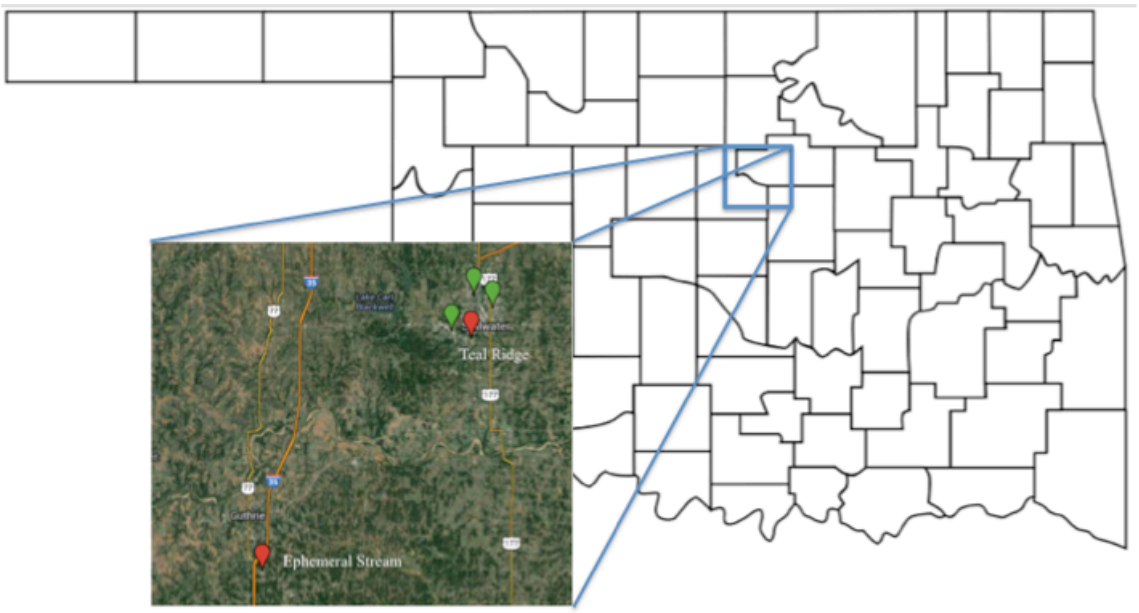


**Figure 1**. Map of Oklahoma and the five localities where amphibians were collected and examined for blood parasites in Payne and Logan counties, Oklahoma, USA (Google Maps, 2016). Red pins indicate locations where amphibians were positive for blood protozoans and green pins indicate locations where none of the amphibians sampled were infected with blood protozoans.

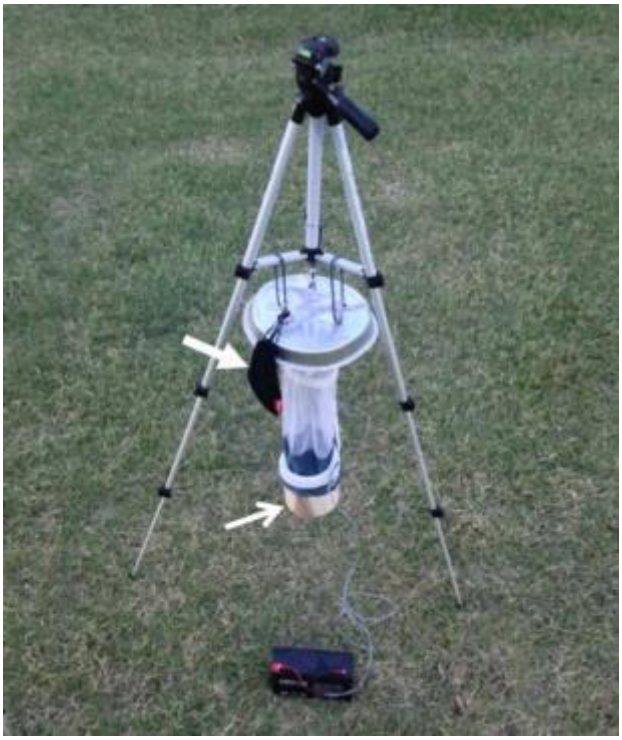

**Figure 2**. Miniature CDC light trap (Model No. 1012, John W. Hock) modified as a frog call trap and hung on a tripod. Note the nylon hose covering the collecting cup (small white arrow) and MP3 player (SanDisk SansaClip+) equipped with a portable speaker (Goal Zero Rock Out) hung in a small black cotton bag next to the fan intake of the trap (large white arrow).

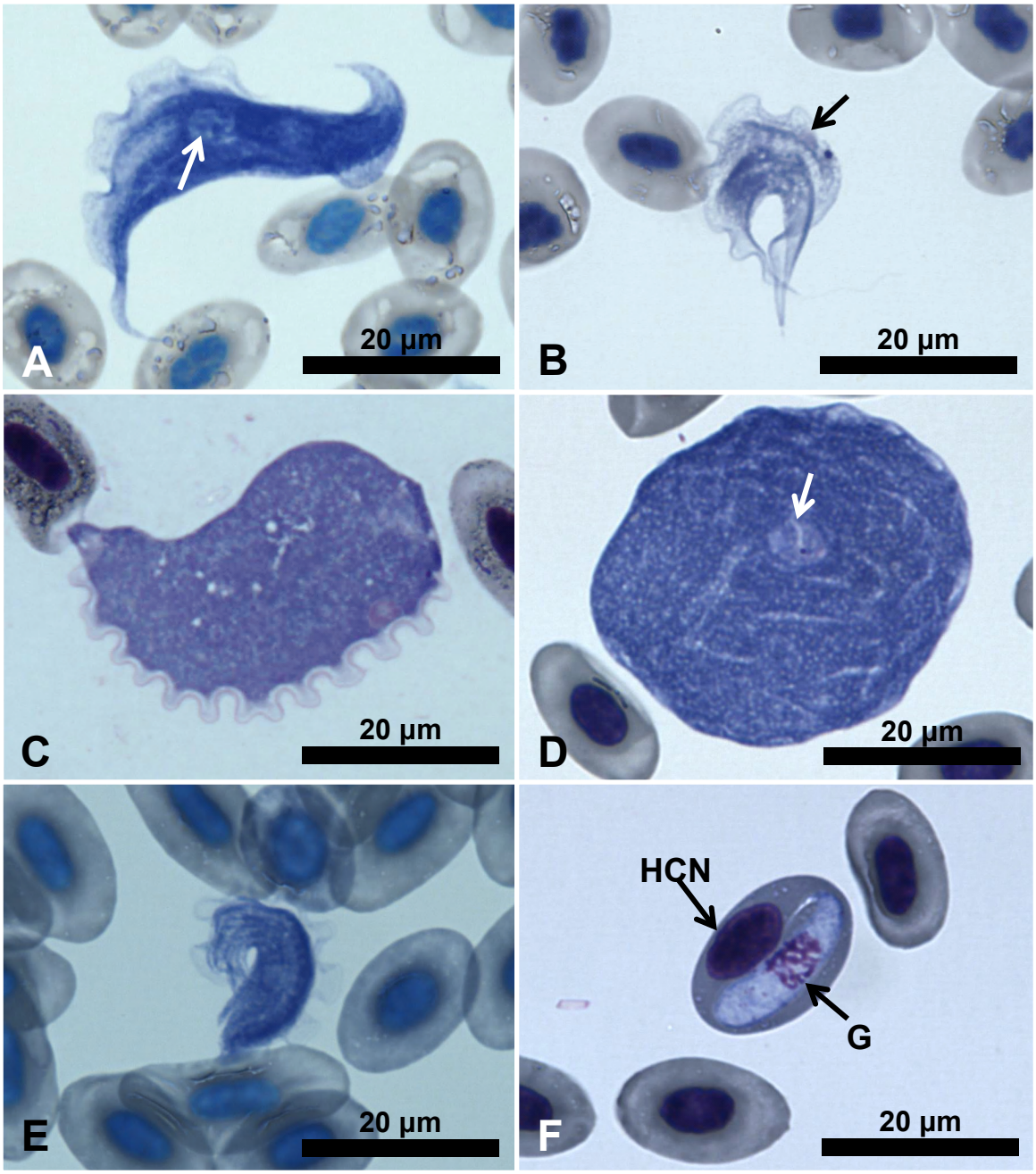


**Figure 3**. Photomicrographs of *Trypanosoma* spp. and *Hepatozoon catesbianae* infecting southern leopard frogs (*L. sphenocephalus*) and bullfrogs (*L. catesbeianus*) from north central Oklahoma, USA. **A**. *Trypanosoma ranarum*, note undulating membrane beginning approximately halfway along the cell body and relatively large nucleus (white arrow). **B**. *Trypanosoma schmidti*, note undulating membrane beginning approximately halfway along the cell body and relatively small nucleus (black arrow). **C**. *Trypanosoma loricatum*, note undulating membrane spanning almost the entire length of the cell body. **D**. *Trypanosoma chattoni*, note round cell shape and internal flagella (white arrow). **E**. *Trypanosoma rotatorium* note curved anterior end and undulating membrane spanning the length of the cell body. **F**. *Hepatozoon catesbianae*, note host red blood cell nucleus (HCN) and intracellular gamont (G).

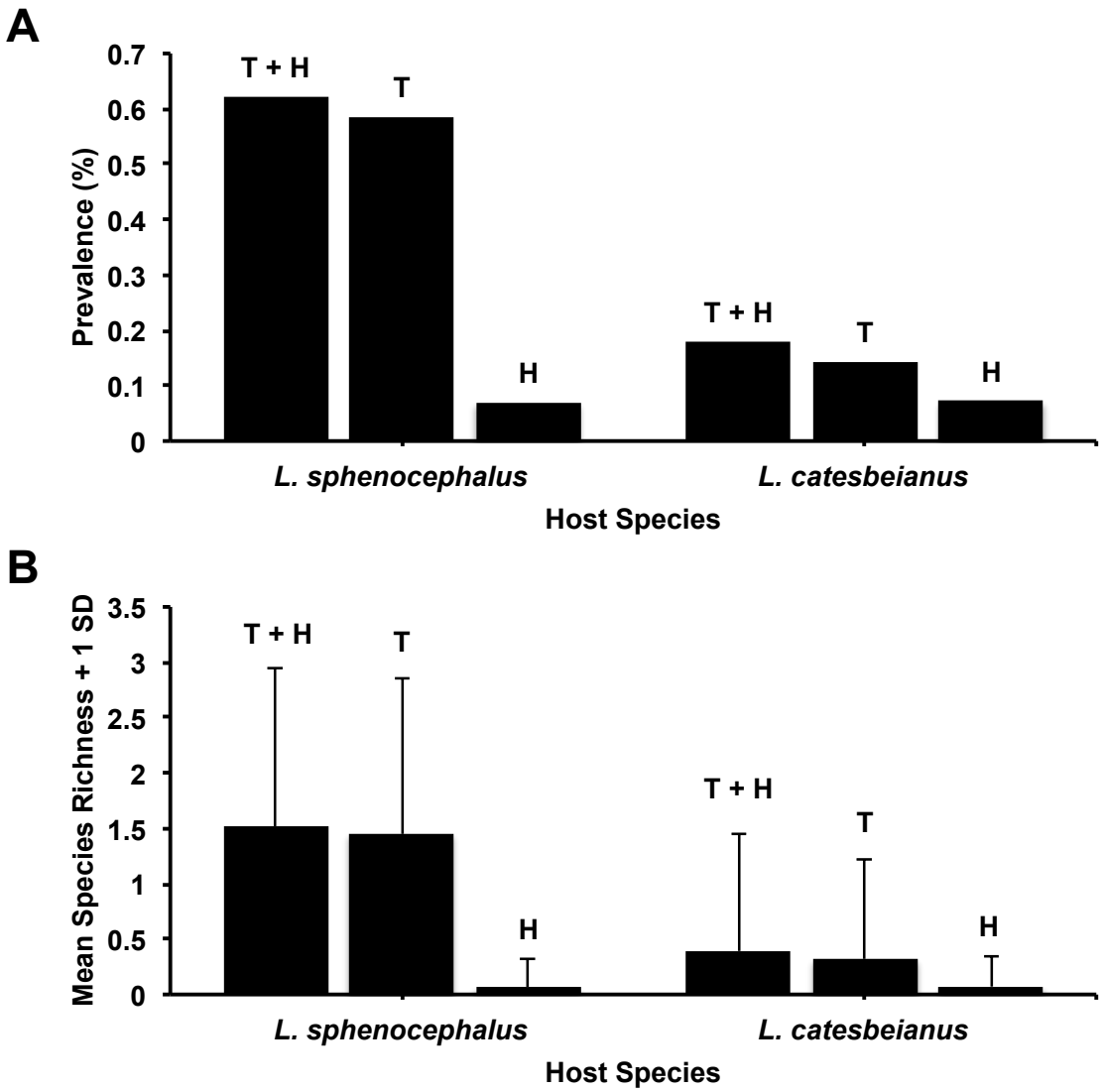


**Figure 4**. Prevalence and mean species richness of *Trypanosoma* and *Hepatozoon* species infecting adult frogs from north central Oklahoma. **A**. Prevalence of *Trypanosoma* and *Hepatozoon* species infecting southern leopard frogs and bullfrogs. **B**. Mean species richness of *Trypanosoma* and *Hepatozoon* species infecting southern leopard frogs and bullfrogs. Letters above columns represent parasite species: T + H, *Trypanosoma* and *Hepatozoon* species; T, *Trypanosoma* species only; H, *Hepatozoon* species only.

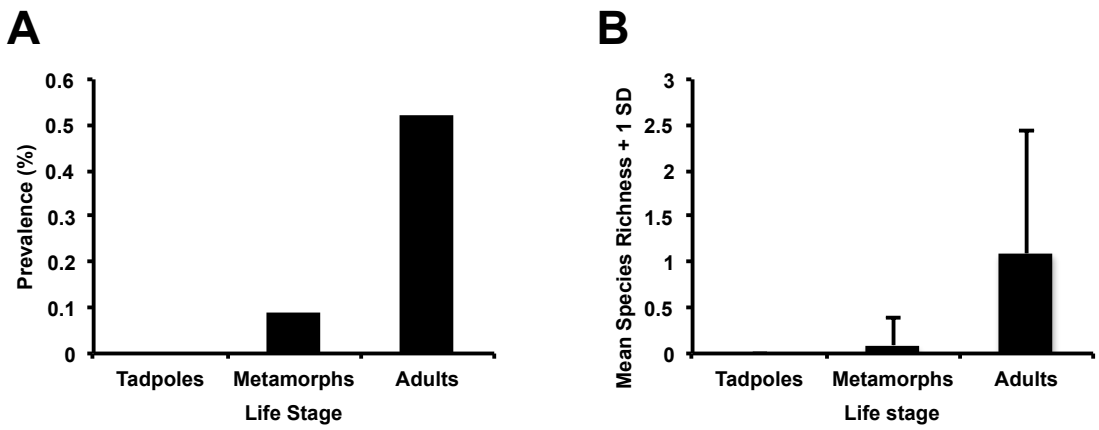


**Figure 5**. Prevalence and mean species richness of blood protozoan species infecting different life stages of southern leopard frogs collected from Teal Ridge, Payne Co., Oklahoma. **A**. Prevalence of blood protozoan species infecting tadpoles, metamorphs, and adults. **B**. Mean species richness of blood protozoan species infecting tadpoles, metamorphs, and adults. For both figures, N = 30 for tadpoles, N= 11 for metamorphs, and N = 21 for adults.

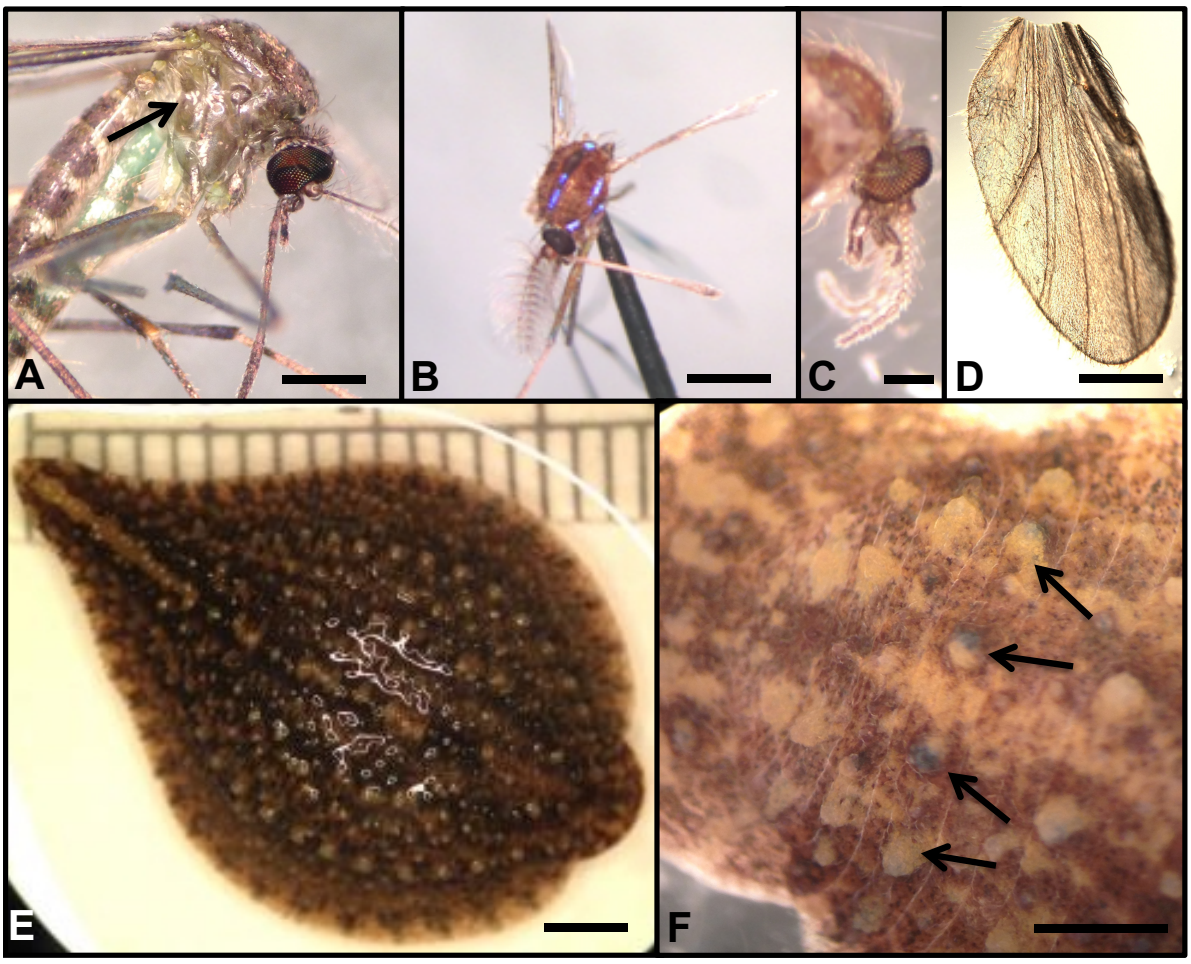


**Figure 6**. Potential vectors for amphibian blood parasites collected at Teal Ridge, Payne Co., Oklahoma. **A**. *Culex erraticus* female, note the median patch of white scales on the lateral thoracic plate below wing base (arrow). **B**. *Uranotaenia sapphirina* male, note the longitudinal stripe of iridescent blue scales on the scutum. **C** and **D**. Head and wing of *Forcipomyia* sp., respectively. **E** and **F**. *Placobdella rugosa*, note the 2 pairs of paramedial papillae (arrows) characteristic of the species.

Table 1. Locations, species, and numbers of amphibians (separated by year and life stage) collected in north central Oklahoma and examined for blood protozoa.

| Location | Amphibian Species and Life Stage | No. Examined in 2014 | No. Examined in 2015 | Total |
|---|---|---|---|---|
| Teal Ridge Payne Co. | *Acris blanchardi* | | | |
| | Adult | 15 | 3 | 18 |
| | *Anaxyrus americanus* | | | |
| | Adult | 1 | 1 | 2 |
| | *Anaxyrus woodhousii* | | | |
| | Adult | 2 | 3 | 5 |
| | *Gastrophryne olivacea* | | | |
| | Adult | 17 | 11 | 28 |
| | *Hyla versicolor* | | | |
| | Adult | 10 | 1 | 11 |
| | Tadpole | 13 | 0 | 13 |
| | *Lithobates catesbeianus* | | | |
| | Adult | 2 | 8 | 10 |
| | *Lithobates sphenocephalus* | | | |
| | Adult | 5 | 16 | 21 |
| | Metamorph | 11 | 0 | 11 |
| | Tadpole | 30 | 0 | 30 |
| | *Pseudacris clarkii* | | | |
| | Adult | 1 | 4 | 5 |
| | *Ambystoma texanum* | | | |
| | Adult | 10 | 0 | 10 |
| Sanborn Lake Payne Co. | *Acris blanchardi* | | | |
| | Adult | 10 | 0 | 10 |
| | *Lithobates catesbeianus* | | | |
| | Adult | 16 | 0 | 16 |
| Apartment Pond Payne Co. | *Lithobates catesbeianus* | | | |
| | Adult | 2 | 0 | 2 |
| OSU Microscopy Pond Payne Co. | *Lithobates sphenocephalus* | | | |
| | Adult | 1 | 0 | 1 |
| Guthrie Stream Logan Co. | *Lithobates sphenocephalus* | | | |
| | Adult | 7 | 0 | 7 |
| | Total | 153 | 47 | 200 |

Table II. Prevalence (number infected /number examined) and 95% confidence intervals for six species of blood protozoans infecting nine species of amphibians collected from north central Oklahoma during April-September of 2014 and April-July 2015.

| | *Trypanosoma ranarum* | *Trypanosoma schmidti* | *Trypanosoma loricatum* | *Trypanosoma chattoni* | *Trypanosoma rotatorium* | *Hepatozoon catesbianae* | Total Blood Protozoa |
|---|---|---|---|---|---|---|---|
| **Anura** | | | | | | | |
| **Bufonidae** | | | | | | | |
| *Anaxyrus americanus* | | | | | | | |
| Adult | 0% (0/2), 0 to 84 | 0% (0/2), 0 to 84 | 0% (0/2), 0 to 84 | 0% (0/2), 0 to 84 | 0% (0/2), 0 to 84 | 0% (0/2), 0 to 84 | 0% (0/2), 0 to 84 |
| *Anaxyrus woodhousii* | | | | | | | |
| Adult | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 |
| **Hylidae** | | | | | | | |
| *Acris blanchardi* | | | | | | | |
| Adult | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 |
| *Hyla versicolor* | | | | | | | |
| Adult | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 |
| Tadpole | 0% (0/13), 0 to 25 | 0% (0/13), 0 to 25 | 0% (0/13), 0 to 25 | 0% (0/13), 0 to 25 | 0% (0/13), 0 to 25 | 0% (0/13), 0 to 25 | 0% (0/13), 0 to 25 |
| *Pseudacris clarkii* | | | | | | | |
| Adult | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 | 0% (0/5), 0 to 52 |
| **Microhylidae** | | | | | | | |
| *Gastrophryne olivacea* | | | | | | | |
| Adult | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 | 0% (0/28), 0 to 12 |
| **Ranidae** | | | | | | | |
| *Lithobates catesbeianus* | | | | | | | |
| Adult | 14% (4/28), 4 to 33 | 4% (1/28), 0.1 to 18 | 4% (1/28), 0.1 to 18 | 7% (2/28), 0.9 to 24 | 4% (1/28), 0.1 to 18 | 7% (2/28), 0.9 to 24 | 18% (5/28), 6 to 37 |
| *Lithobates sphenocephalus* | | | | | | | |
| Adult | 21% (6/29), 8 to 40 | 7% (2/29), 0.9 to 23 | 45% (13/29), 26 to 64 | 28% (8/29), 13 to 47 | 45% (13/29), 29 to 67 | 7% (2/29), 0.9 to 23 | 62% (18/29), 42 to 79 |
| Metamorph | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 0% (0/11), 0 to 29 | 9% (1/11), 0.2 to 41 | 0% (0/11), 0 to 29 | 9% (1/11), 0.2 to 41 |
| Tadpole | 0% (0/30), 0 to 12 | 0% (0/30), 0 to 12 | 0% (0/30), 0 to 12 | 0% (0/30), 0 to 12 | 0% (0/30), 0 to 12 | 0% (0/30), 0 to 12 | 0% (0/30), 0 to 12 |
| **Caudata** | | | | | | | |
| **Ambystomatidae** | | | | | | | |
| *Ambystoma texanum* | | | | | | | |
| Adult | 0% (0/10), 0 to 31 | 0% (0/10), 0 to 31 | 0% (0/10), 0 to 31 | 0% (0/10), 0 to 31 | 0% (0/10), 0 to 31 | 0% (0/10), 0 to 31 | 0% (0/10), 0 to 31 |
| **Total Amphibians** | 5% (10/200), 2 to 9 | 2% (3/200), 0.3 to 4 | 7% (14/200), 4 to 11 | 5% (10/200), 2 to 9 | 8% (15/200), 4 to 12 | 2% (4/200), 0.6 to 5 | 12% (24/200), 8 to 17 |

Data in cells represent No. Infected/No. Examined (%), 95% Confidence Interval. Confidence intervals were calculated using the binomial distribution for the appropriate sample size.

Table III. Dipteran species known to be blood feeders, collected in modified frog call and control CDC traps from Teal Ridge, Payne Co., Oklahoma during July, 2014 and May-August 2015.

| Family | Species | Call traps | Light traps | No light traps | Total |
|---|---|---|---|---|---|
| Ceratopogonidae | | | | | |
| | *Forcipomyia* sp.* | 1 | 1 | 1 | 3 |
| Culicidae | | | | | |
| | *Aedes cinereus* | 0 | 2 | 0 | 2 |
| | *Aedes sollicitans* | 2 | 1 | 0 | 3 |
| | *Aedes vexans* | 2 | 18 | 0 | 20 |
| | *Culex erraticus** | 4 | 0 | 0 | 4 |
| | *Culex restruans* | 0 | 1 | 0 | 1 |
| | *Culex salinarius* | 1 | 1 | 0 | 2 |
| | *Uranotaenia sapphirina** | 0 | 0 | 1 | 1 |

*possible vector for amphibian blood protozoa

# CHAPTER II

## CHARACTERIZATION OF THE MORPHOLOGY, MOTILITY, AND PHYLOGENETIC RELATIONSHIPS OF TRYPANOSOME MORPHOTYPES INFECTING RANID FROGS IN NORTH CENTRAL OKLAHOMA

ABSTRACT**:** In a survey of blood protozoans of 9 species of amphibians from Oklahoma, 5 trypanosome morphotypes were found infecting bullfrogs (14%) and southern leopard frogs (59%). Trypanosome morphotypes separated into distinct groups based on morphological measurements and video-microscopy revealed distinct differences in trypanosome motility. Based on cell morphology, the five trypanosome morphotypes conform to previous descriptions of (1) *Trypanosoma ranarum*, (2) *T. schmidti,* (3) *T. loricatum*, (4) *T. rotatorium*, and (5) *T. chattoni*. However, trypanosome species can be polymorphic, where two or three trypanosome morphotypes can be observed infecting a single amphibian host and it is unclear whether these morphotypes represent different species or a single polymorphic species. To investigate if the 5 different morphotypes observed in this study are the result of polymorphism and also further support species identifications, a 561 bp fragment of the 18s rRNA gene was sequenced. Phylogenetic analyses of the 18s rRNA gene fragment revealed genetic differences between morphotypes. However obtaining the entire 18s rRNA gene

sequence, broader sampling of amphibian trypanosomes, and additional genetic markers such as the gGAPDH gene may be necessary to better resolve the relationships among these morphotypes.

## INTRODUCTION

The genus *Trypanosoma* (Trypanosomatida, Kinetoplasta) infects the circulatory system of all classes of vertebrates. *Trypanosoma* species are known to cause various diseases in humans, domestic animals and wildlife, including sleeping sickness and Chagas disease (Roberts et al. 2013). The majority of *Trypanosoma* species are transmitted by hematophagous vectors such as insects or annelids. Within the vector host, trypanosomes reproduce asexually by binary and/or multiple fission, and are found in the gut and/or salivary glands of the vector. In addition to rare cases of direct transmission between vertebrate hosts through cannibalism or coprophagy for *T. cruzi* and direct transmission for *Trypanosoma equiperdum*, two types of transmission mechanisms have been reported among different species of *Trypanosoma* (Lee et al. 2000). In stercorarian or posterior station transmission, the vector deposits infective trypanosome stages in its feces on the skin of the vertebrate host while taking a blood meal and the vertebrate host inoculates itself while scratching the bite. In contrast, in salivarian or anterior station transmission, the vector inoculates infective trypanosome stages from its salivary glands into the vertebrate host while taking a blood meal. Within the vertebrate host, trypanosomes reproduce asexually and are found in the bloodstream or intracellular in various tissues (Roberts et al. 2013).

Estimates suggest that there are over 70 species of *Trypanosoma* infecting the class amphibia worldwide (Bardsley and Harmsen, 1973; Desser, 2001). At least twenty-six species of trypanosomes have been reported from 36 amphibian species in North America (Diamond, 1965; Bardsley and Harmsen, 1973; Martin and Desser, 1990; Bernal and Pinto, 2016). However, the validity of several of those species is questionable because many *Trypanosoma* spp. can be polymorphic. Often two or three trypanosome morphotypes can be observed infecting a single amphibian host and it is unclear whether these morphotypes represent different species or a single polymorphic species (Diamond, 1965). Additionally, amphibian host specificity of *Trypanosoma* spp. varies tremendously among trypanosome species. For example, *Trypanosoma rotatorium* has been reported from 21 different amphibian species; whereas *Trypanosoma fallisi* has been reported from a single host species, the American toad, *Bufo americanus* (Martin and Desser, 1990).

The life cycles of North American amphibian trypanosomes are also variable among species. Two distinct groups of vectors have been reported for amphibian trypanosomes and include (1) aquatic leech vectors or (2) terrestrial dipteran vectors. Because leeches are usually restricted to aquatic habitats, they transmit trypanosomes to amphibian species that spend a significant portion of their time in water when adults, such as true frogs and/or aquatic larval stages of amphibians (Diamond, 1965; Martin and Desser, 1991b). In contrast, hematophageous dipterans such as sand flies, frog biting midges and mosquitoes have been reported to transmit trypanosomes to primarily terrestrial amphibians, such as the true toads and treefrogs (Anderson and Ayala, 1968; Ramos and Urdaneta-Morales, 1977; Johnson et al., 1993).

In this study, a total of 200 individuals representing nine species in four families of amphibians were collected from a single location in Guthrie, Logan County, and four locations in Stillwater, Payne County, and Oklahoma U.S.A. during 2014 and 2015 and examined for the presence of blood protozoa. A total of five *Trypanosoma* species/morphotypes were found infecting bullfrogs and southern leopard frogs in the survey. This chapter details the motility and morphological and molecular characterization of the five trypanosome species/morphotypes found in bullfrogs and southern leopard frogs.

## MATERIALS AND METHODS

### Morphological characterization of trypanosomes

Amphibians were screened for trypanosome infections by both capillary tube centrifugation and observation of Giemsa stained slides using methods described previously in Chapter I. Giemsa stained slides of amphibian blood infected with trypanosomes were examined with an Olympus BX-51 upright research microscope using 100x magnification. When found, trypanosomes were photographed at 1000x magnification with an Olympus 5 megapixel digital camera.

Photomicrographs of trypanosomes were measured using ImageJ (Schneider et al. 2012) calibrated with a stage micrometer (Reichert, NY). When present, at least ten different measurements were taken on each trypanosome species/morphotype, including: PF = total length including free flagellum, PA = body length excluding free flagellum, FF = length of free flagellum, BW = maximum body width excluding undulating membrane,

LN = length of nucleus, WN = width of nucleus, 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 (Fig. 1). At least six trypanosomes were photographed and measured from each infected frog, however not all measurements could be taken for all specimens because often organelles or flagella were not visible for each individual trypanosome. For the round form resembling *T. chattoni*, the maximum cell diameter was recorded for body length and the diameter perpendicular to the maximum diameter measurement was recorded for the body width.

For Principle Components Analysis (PCA), measurements of each morphotype body width (BW), body length (PA), length of nucleus (LN), width of nucleus (WN), and ratios of the kinetoplast to the posterior end of the cell body length over the body length (PK/PA), and nucleus to the posterior end of the cell body length over the body length (PN/PA) were analyzed using Canoco 5 with default settings. The assumption of PCA is that the total variance of a variable reflects the sum of explained and error variance (Grimm and Yarnold, 1995). Voucher specimens of trypanosome morphotypes from the same individual amphibians for which sequences were obtained will be deposited in the H. W. Manter Parasitology Collection, University of Nebraska, Lincoln, Nebraska.

**Characterization of trypanosome motility**

To characterize the motility of trypanosome morphotypes, trypanosomes were observed swimming in blood plasma isolated by the modified micro-hematocrit technique

previously described in Chapter I (Woo, 1969). Briefly, capillary tubes of amphibian blood containing trypanosomes were scored at the blood-plasma junction and the buffy layer deposited on a glass slide. Slides were then observed with a 40x objective on an Olympus BX-51 upright research microscope. Within 30 seconds of placing the trypanosomes on a slide, videos of the motility of each trypanosome morphotype were recorded from the droplet of blood plasma using the camera of an iPhone 5C cell phone (Apple, CA) mounted on the ocular lens with a Skylight adaptor. Slide cover slips were not used as flattening the drop of plasma interfered with the trypanosomes' natural motility patterns. Individuals of each morphotype were observed for at least 1 minute and the mechanisms (using a flagellum, undulating membrane, or no motility) and directions (anterior, lateral, spiral, etc) of movement were observed and documented.

**DNA extraction**

For extraction of trypanosome DNA from frog blood, 20 µL of infected frogs' blood preserved in 100% ethanol and stored at -80℃ was thawed and dried using a Thermolyne dri-bath heated to 56 °C for approximately 30 minutes. DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, CA) following the recommended protocol for samples of nucleated blood (Qiagen, 2006).

**PCR amplification and sequencing**

The 18s rRNA gene was amplified from extracted DNA samples by nested PCR

using previously described primers (Noyes et al. 1999, 2000). The external forward and reverse primers were TRY927F 5'-GAA ACA AGA AAC ACG GGA G-3' and TRY927R 5'-CTA CTG GGC AGC TTG GA-3' and internal forward and reverse primers were SSU561F 5'-TGG GAT AAC AAA GGA GCA-3' and SSU561R 5'-CTG AGA CTG TAA CCT CAA AGC-3'. Amplification of the 18s rRNA gene sequence was performed by an initial PCR using primers TRY927F/R and the following materials: 10 μL master mix (HotStarTaq Master Mix Kit, Qiagen, CA), 0.5 μL forward primer, 0.5 μL reverse primer, 1 μL template DNA, and 13 μL sterile water, giving a total reaction volume of 25 μL per reaction. A Hot Start touchdown PCR profile was implemented on a AB 2720 thermocycler (Applied Biosystems) using the following touchdown PCR program: initial activation for 600s at 95°C, annealing for 30s at 65°C decreasing by 2°C -55°C over 10 cycles, and extension for 60s at 72°C, repeated for 30 total cycles before a final extension period of 400s at 72°C. The PCR product of this reaction was diluted 1:10 with sterile water, and 1 μL was used as template for the second PCR using the internal primers SSU561F/R. PCR success was determined by running 4 μL of PCR product on a 1% agarose gel using TBE buffer on a horizontal electrophoresis system (VWR, PA) at constant voltage (100V) for 20 min. Bands were visualized using a UV light and single, solid bands were prepped for sequencing using the QIAquick PCR Purification Kit (Qiagen, CA). For sequencing PCR amplicons, SSU561 forward and reverse internal primers (Noyes et al. 1999; 2000) were used with the Sanger sequencing chemistry on an Applied Biosystems 3730 capillary sequencer at the Oklahoma State University core facility.

**Cloning**

Due to the inability of sequencing two DNA sequences at once, blood samples containing two or more trypanosome morphotype sequences were first separated using a cloning technique. To separate the 18s rRNA gene of each morphotype from a single blood sample, the mixed PCR amplicon was ligated into the pDrive plasmid vector and transformed into competent cells using the PCR CloningPlus Kit (Qiagen, Valencia, CA) and plated on AMP, IPTG, X-GAL plates (Teknova, Inc) and incubated at 37°C overnight. Single, white colonies were picked using sterile technique (Slonczewski and Foster, 2013), re-amplified with PCR, and sequenced as described above.

**Phylogenetic analyses**

The five 18s rRNA gene sequences from five amphibian trypanosome morphotypes obtained in this study were combined with 17 sequences retrieved from GenBank and aligned using the MUSCLE (Edgar, 2004) feature of MEGA 6.06 (Tamura et al. 2013). The alignment was inspected by eye and ambiguous regions were removed from further analyses. The final alignment consisted of 469 base pairs and 24 taxa. Trypansomes infecting terrestrial vertebrates (birds: *T. avium*, *T. grayi*, mammals: *T. lewisi*, *T. theileri*, *T. melophagium*), and aquatic vertebrates (fish: *T. boissoni*, *T. triglae*, *T. cobitus*, reptiles: *T. chelodinae*, mammals: *T. binneyi*) were used as outgroups to the amphibian clade (see Table I).

Phylogenies were estimated using both Maximum Likelihood (ML) and Bayesian inference frameworks. The model testing feature of MEGA 6.06 (Tamura et al. 2013)

yielded the K2P + G model (Kimura, 1980) as the best evolutionary model for the ML analysis. ML trees were created in MEGA 6.06 (Tamura et al. 2013) using the K2P + G evolutionary model with 1000 bootstrap replications. Bayesian phylogenies were created using MrBayes 3.2.4 (Ronquist and Huelsenbeck, 2003), using 1,000,000 Monte Carlo Markov generations using 1 hot chain and 3 cold chains. Nucleotide substitutions were allowed to be different for transitions and transversions and the rates were gamma distributed. Additionally, pairwise genetic distances and their standard errors were calculated among trypanosome 18s rRNA gene sequences with MEGA 6.06 (Tamura et al. 2013), using the K2P + G (Kimura, 1980) model.

## RESULTS

### Morphology, motility, diagnosis and comments of trypanosome morphotypes

The five trypanosome species/morphotypes infecting southern leopard frogs and bullfrogs from north central Oklahoma were morphologically distinct (Fig. 1). The morphology of each morphotype is provided below, along with comparisons to descriptions of taxa named by previous authors. Additionally, all morphotype measurements and ratios of organelle positions are reported in Table II.

The first morphotype, *Trypanosoma schmidti* as described by Diamond (1965) and reported in Werner and Walewski (1976) infected 7% (2 of 29) of southern leopard frogs and 4% (1 of 28) of bullfrogs. The cell body of *T. schmidti* measured 49.0 ± 9.8 μm in length by 6.8 ± 2.3 μm in width (Table II). The nucleus was oval and positioned halfway along the cell body (Fig. 1). The kinetoplast is circular and just posterior to the

nucleus. The undulating membrane begins approximately halfway along the cell body, and ends on the anterior region of the cell body. A free flagellum of 11.8 ± 7.9 μm in length is present on the anterior region of the cell body. In blood plasma, *T. schmidti* moves forward by whipping its flagellum, while its body spirals forward in a corkscrew like motion about central cell axis in the direction of movement.

The second morphotype conforms with measurements and descriptions of *Trypanosoma ranarum* by Diamond (1965), Woo (1969), and Barta and Desser (1984) and was found in 17% (5 of 29) of southern leopard frogs and 14% (4 of 28) of bullfrogs. This is a wide trypanosome, with an average body length of 54.7 ± 8.9 μm and an average body width of 12.8 ± 4.0 μm (Table II, Fig. 1). The nucleus is circular and positioned approximately half way from the posterior to the anterior end of the cell body. The kinetoplast is circular and located posterior to the nucleus. The undulating membrane arises posteriorly just to the center of the cell body and continues anteriorly until it becomes a free flagellum about 10 μm in length. Like *T. schmidti*, *T. ranarum* whips its flagellum forward to pull itself through the blood plasma, however the cell body of *T. ranarum* rotates in a wider spiral than *T. schmidti*.

The third morphotype conformes to descriptions of both *T. rotatorium* as reported by Woo (1969), Barta and Desser (1984) and *T. loricatum* by Desser (2001). *Trypanosoma loricatum* was observed in 45% (13 of 29) of southern leopard frogs and 4% (1 of 28) of bullfrogs. Both *T. rotatorium* and *T. loricatum* as reported by Woo (1969), Barta and Desser (1984) and Desser (2001) do not have a free flagellum. *Trypanosoma rotatorium* and *T. loricatum* were originally described by Mayer (1843) from the blood of anurans in France, and *T. rotatorium* was described having a free

flagellum and *T. loricatum* was described as not having a free flagellum (Laveran and Mesnil, 1907). The form observed in this study did not have a free flagellum, and therefore I follow Desser (2001) and designate this form as *T. loricatum*. *Trypanosoma loricatum* has an average cell body length of 56.6 ± 8.4 μm and an average cell body width of 14.8 ± 4.5 μm (Fig. 1). The nucleus is circular and located in the posterior portion of the cell body. The kinetoplast is circular and located approximately half way from the nucleus to the posterior end. The undulating membrane starts about one fifth of the way from the posterior portion of the cell body and continues along the cell body ending at the anterior end and no free flagellum is present. *Trypanosoma loricatum* moves sideways, using its undulating membrane to pull itself in the direction perpendicular to the anterior-posterior axis of the cell body.

The fourth morphotype, *T. chattoni* as reported by Diamond (1965), Jones and Woo (1986), and Desser (2001) was found in 28% (8 of 29) of southern leopard frogs and 7% (2 of 28) of bullfrogs. This is an unusual trypanosome, with a round or oval cell shape, no undulating membrane, and an internal flagellum. *Trypanosoma chattoni* has an average maximum cell diameter (PA) of 35.0 ± 6.8 μm and an average perpendicular diameter (BW) of 30.3 ± 5.1 μm (Table II). The nucleus is round and positioned in the center of the cell body. The kinetoplast is located near the nucleus or sometimes superimposed on top of the nucleus, where the internal flagellum begins (Fig. 1). In contrast to the other morphotypes, *T. chattoni* does not exhibit any motility.

The fifth morphotype conforms to previous reports of the cornucopia form of the polymorphic trypanosome species *T. rotatorium* in Laveran and Mesnil (1907). However, the cornucopia morphotype of *T. rotatorium* has never been reported from North

America. This was the smallest and most common morphotype observed in this study, and occurred in 48% of adult southern leopard frogs and 4% of bullfrogs. Additionally, one (9%) metamorph southern leopard frog was infected with this morphotype. The most distinctive characteristic of this morphotype is its curved anterior end, giving the cell the shape of a cornucopia (Fig. 1). The average cell body length is 35.8 ± 3.8 μm and the average cell body width is 12.4 ± 5.4 μm (Table II). The nucleus is approximately one third of the way from the posterior end of the cell body, and the kinetoplast is located at the posterior end of the cell. The undulating membrane spans almost the entire length of the cell body, ending at the anterior end with no free flagellum being present. In blood plasma, *T. rotatorium* moves towards its curved anterior end, always being pulled forward by its undulating membrane.

The majority of the infected frogs with trypanosomes (86%) contained multiple trypanosome morphotypes. Three frogs infected with *T. schmidti* were also infected with at least *T. ranarum*. Additionally, 80% (12/15) of frogs that were infected with *T. loricatum* were also infected with *T. rotatorium*, and 90% (9/10) of frogs that were infected with *T. chattoni* were also infected with either *T. loricatum* or *T. rotatorium*.

PCA produced distinct clusters of morphotypes based on cellular measurements (Fig. 2). PCA axis 1, 2, and 3 explained 34.22%, 29.03%, and 21.43% of the variation, respectively. Plotting PCA axes 1 and 2 resulted in *T. loricatum* and *T. rotatorium* clustering together (Fig. 2A), where they formed distinct clusters when PCA axes 1 and 3 were plotted (Fig. 2B).

**Phylogenetic analysis of trypanosome morphotypes**

Genetic divergence between the ingroup (aquatic hosts) and outgroup (terrestrial hosts) ranged from 5.23% to 13.76%. Within the new sequences generated for the five Oklahoma anuran trypanosome morphotypes, genetic divergence ranged from 0.7% to 9.6%. Additionally, the genetic divergence between these morphotypes and previously reported amphibian trypanosomes was found to range from 1.3% to 14.2% (Table III).

The ML and Bayesian analyses produced trees with topologies consistent with previous analyses of trypanosome 18s rRNA sequences (Fig. 3, Martin et al. 2002; Hamilton et al. 2007; Bernal and Pinto, 2016). The tree was rooted with trypanosomes infecting terrestrial hosts, and trypanosomes of anura came out within the aquatic clade, however they did not form a monophyletic clade. Eight anuran trypanosome species were monophyletic, while six anuran trypanosomes were more closely related to the non-anuran aquatic trypanosomes in hosts including fish, turtles, and platypus, than to the eight monophyletic anuran trypanosomes.

The five morphotypes added in this study were located within the aquatic trypanosome clade (Fig. 3). Three of the five Oklahoma trypanosome morphotypes were more closely related to anuran trypanosomes or other aquatic clade trypanosomes collected from other locations than to the distinct morphotypes from the same individual frog infected with multiple trypanosome morphotypes from Oklahoma. Although with low support values, the *T. ranarum* morphotype from Oklahoma was sister to *T. ranarum* from Ontario, CA; *T. schmidti* morphotype from Oklahoma was sister to *T. fallisi* from Ontario, CA, and the Oklahoma moprhotype of *T. chattoni* was basal to a clade of aquatic

trypanosomes including a previous sequence of *T. chattoni* from Minnesota, USA, the Oklahoma morphotypes of *T. loricatum* and *T. rotatorium*, and other non-amphibian aquatic trypanosomes. However, sequences from the morphotypes conforming to *T. loricatum* and *T. rotatorium* were sister to each other. The genetic distance between the sister taxa *T. loricatum* and *T. rotatorium* (1.8%) was greater than between that of *T. ranarum* and *T. schmidti* (0.7%), which were more closely related to other trypanosome species (Table III, Fig. 3).

## DISCUSSION

At least twenty-six species of trypanosomes have been reported from 36 amphibian species in North America (Diamond, 1965; Bardsley and Harmsen, 1973; Martin and Desser, 1990; Bernal and Pinto, 2016). However, *Trypanosoma* spp. can be polymorphic, and therefore the validity of many of these species is questionable. Desser (2001) argued that for a species to be considered valid, morphological and morphometric data, host associations and geographic locality, as well as culture forms and vector stages must be described for the species. Additionally, developmental changes of trypanosomes in the blood of experimentally infected frogs must be tracked and documented over time. This implies that considerable work must be done to not only describe new amphibian trypanosome species, but also to confidently identify the species found in a given survey. Of the amphibian trypanosomes in North America, only *T. pipientis* and *T. fallisi* have been characterized with all of these criteria (Desser, 2001). Most amphibian trypanosomes have not been fully characterized and therefore the comparison data

needed to identify a given trypanosome infection are not available. For these reasons, the closest conforming species based on morphology are documented here. These species designations should be considered tentative identifications and until additional life cycle or genetic data is collected should be interpreted cautiously.

**Comparisons between trypanosome morphotype morphologies and motilities**

PCA analysis of four trypanosome morphotypes excluding *T. chattoni* yielded distinct clusters for each morphotype indicating statistically significant morphological differences between the amphibian trypanosome morphotypes observed in this study. In addition to differences in morphology, differences in motility were observed between the five trypanosome morphotypes. This is to be expected because trypanosome motility is directly related to their morphological characteristics. For example, the presence of a free flagellum results in the cell moving in a spiral and being pulled through the blood plasma with the flagellum as observed in the morphotypes identified as *T. schmidti* and *T. ranarum* in this study. In contrast, trypanosome morphotypes identified as *T. loricatum* and *T. rotatorium* did not possess a free flagellum, and used their undulating membranes resulting in the cell bodies of these morphotypes moving toward the fluttering of their undulating membranes. Additionally, having a reduced, internal flagellum and no undulating membrane along with a spherical to round shape resulted in *T. chattoni* not exhibiting any motility.

**Molecular characterization of trypanosome morphotypes**

Since Desser (2001) published the criteria for identifying and describing amphibian trypanosome species based on morphological and life cycle data, technological advances have made obtaining genetic information much easier and adding genetic markers to help identify resolve the taxonomic conundrum of amphibian trypanosomes. However, the power of genetic methods is limited to the availability of sequences of identified trypanosomes for comparative purposes, and currently sequences for the 18s rRNA gene are only available for seven amphibian trypanosome species/morphotypes on GenBank. Additionally, the 18s rRNA gene may not be useful for resolving species delineations and is typically used for resolving deep relationships between broad ranges of taxa (Meyer et al. 2010). Clearly, more research is needed on the phylogenetic relationships of trypanosomes, including sequencing other faster evolving genes such as gGAPDH which are available for some trypanosome species.

The phylogeny generated in this study recovered topologies consistent with previous analyses of trypanosomes using 18s rRNA gene sequences (Martin et al. 2002; Hamilton et al. 2007; Bernal and Pinto, 2016). However, the tree created in this study had lower support values than previous analyses. This is most likely because only a 561 base pair fragment of the 18s rRNA gene was used to estimate the phylogenetic relationships among aquatic and terrestrial trypanosomes. However, my phylogenetic analysis indicates that morphotypes of the Oklahoma amphibian trypanosomes with similar morphologies and motilities were found to be more closely related to each other than to other Oklahoma morphotypes with more distinct morphologies and motilities. For example, the Oklahoma morphotypes of *T. schmidti* and *T. ranarum* both move through

the blood plasma in a spiral motion by whipping their free flagellum, and the genetic divergence of the 18s rRNA gene sequences was 0.66% between these morphotypes (Table III). Additionally, *T, loricatum* and *T. rotatorium* both lack a free flagellum and move in the direction of the fluttering undulating membranes, and their 18s rRNA gene divergence was 1.83%. In addition, in the phylogenetic analyses, the Oklahoma *T. schmidti* isolate was sister to *T. fallisi* a species of amphibian trypanosome with a free flagellum specific to toads (Martin and Desser, 1991). Finally, the distinctive large and round shaped non-motile morphotype designated as *T. chattoni* Mathis and Leger, 1911 had similar cell body width and length measurements to reports of *T. chattoni* by Diamond (1965), Jones and Woo (1986), and Desser (2001), and was basal to a clade containing a previously reported isolate of *T. chattoni* from Martin et al. (2002). These data suggest that at least in some amphibian trypanosomes, their morphology is congruent with their phylogenetic placement.

**Similarities between trypanosome morphotypes**

The morphotypes designated as *T. loricatum* and *T. rotatorium* were found infecting the same frog host 90% (9 of 10) of the time, and were the second least divergent of the five trypanosome morphotypes recovered in this study based on a partial 18s rRNA gene (1.83%). In addition, they had similar organelle positions (see Figs. 1 and 2), and were observed to swim through the blood plasma in a similar motion, using their undulating membranes. The main differences between these two morphotypes was (1) their cell body length with *T. loricatum* being almost twice as long as *T. rotatorium*, and

(2) the shapes of their anterior ends with *T. rotatorium* having a curved anterior end while *T. loricatum* did not have a curved anterior end.

The morphotypes designated as *T. ranarum* and *T. schmidti* shared similar characteristics as well. All three of the frogs infected with *T. schmidti* were also infected with *T. ranarum*, and these two morphotypes were the least divergent of the five morphotypes recovered in this study based on a partial 18s rRNA gene (0.66%). Additionally, they both used a free flagellum to swim through the blood plasma. However, these two morphotypes formed distinct clusters in the PCA analysis of their organelle positions and cell body lengths and widths.

Clearly, comparisons of additional 18s rRNA gene sequences of these morphotypes obtained from different frog hosts will be necessary to confirm the low divergence between *T. loricatum* and *T. rotatorium*, as well as between *T. ranarum* and *T. schmidti*. Additionally, tracking trypanosome development throughout laboratory infections of uninfected frogs is needed to determine if pleomorphic changes in morphology are occurring in what I refer to as four distinct morphotypes.

**Conclusion**

My study is the first to examine an amphibian community for *Trypanosoma* species in the Great Plains region of North America and Oklahoma is a new locality record for amphibian trypanosomes. Additionally, this study adds new molecular and morphological data for five amphibian trypanosome morphotypes and demonstrates that the morphotypes found co-infecting a host are genetically distinct. However, my study

also indicates that broader sampling of amphibian trypanosome species/morphotypes with additional genetic markers is necessary to resolve the phylogenetic relationships within this group of blood protozoans and to better support trypanosome species identifications and phylogenetic relationships.

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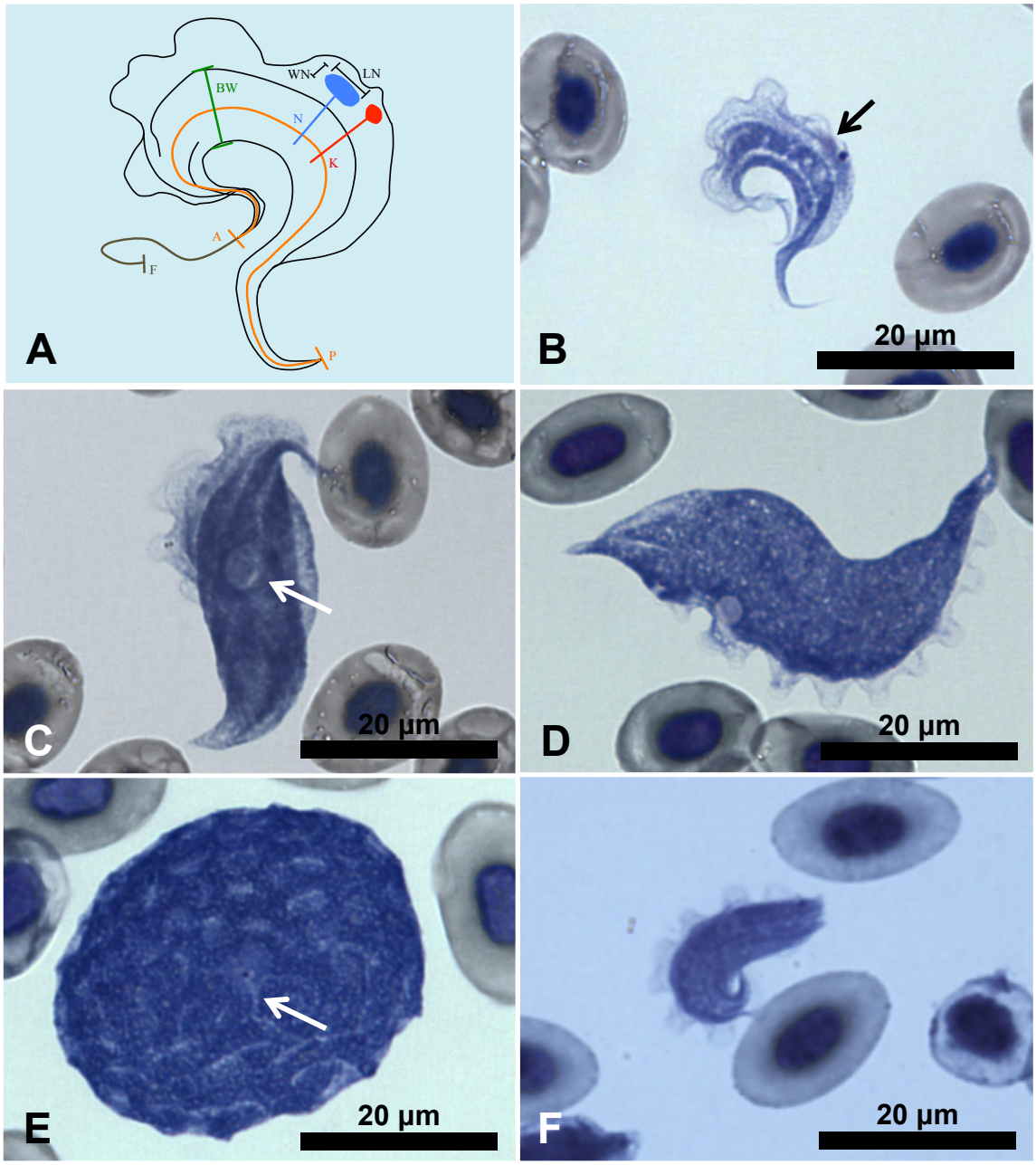


**Figure 1**. Photomicrographs of trypanosome morphotypes found in southern leopard frogs and bullfrogs from north central Oklahoma. **A**. Measurements taken on trypanosomes to characterize their morphology. **B**. *Trypanosoma schmidti*, note undulating membrane beginning approximately halfway along the cell body and relatively small nucleus (black arrow). **C**. *Trypanosoma ranarum*, note undulating membrane beginning approximately halfway along the cell body and relatively large nucleus (white arrow). **D**. *Trypanosoma loricatum*, note undulating membrane spanning almost the entire cell body. **E**. *Trypanosoma chattoni*, note round cell shape and internal flagella (white arrow). **F**. *Trypanosoma rotatorium*, note curved anterior end and undulating membrane spanning the length of the cell body.

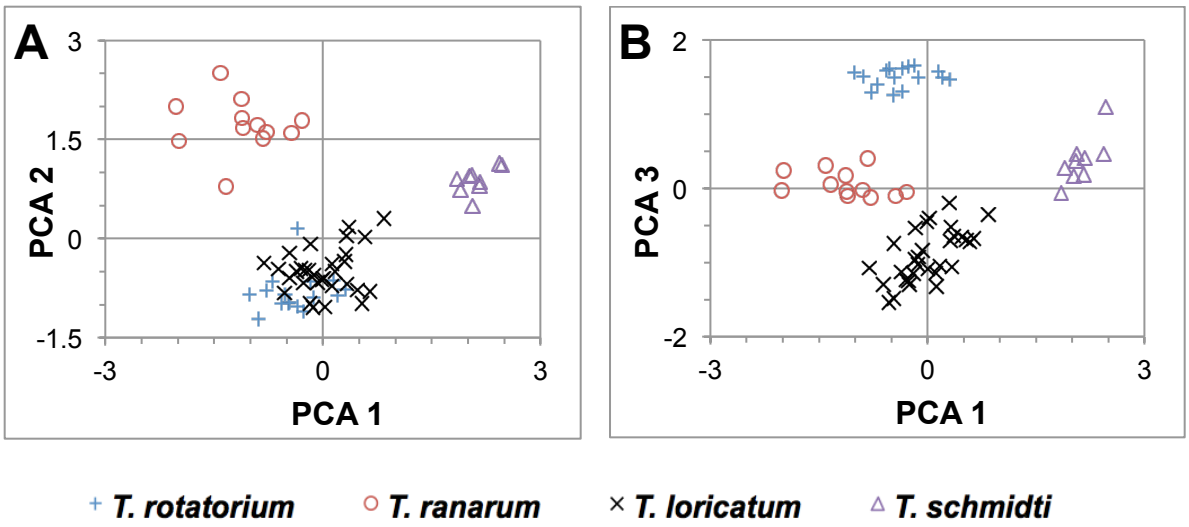


**Figure 2**. Principal Components Analysis of 6 metrics measured from trypanosome morphotypes: PA, BW, LN, WN, PK/PA, PN/PA. PCA axis 1, 2, and 3 explained 34.22%, 29.03%, and 21.43% of the variation, respectively. *Trypanosoma chattoni* was not included in the analysis because this morphotype's round shape made it unable to calculate applicable ratios used to characterize organelle locations in the cell.

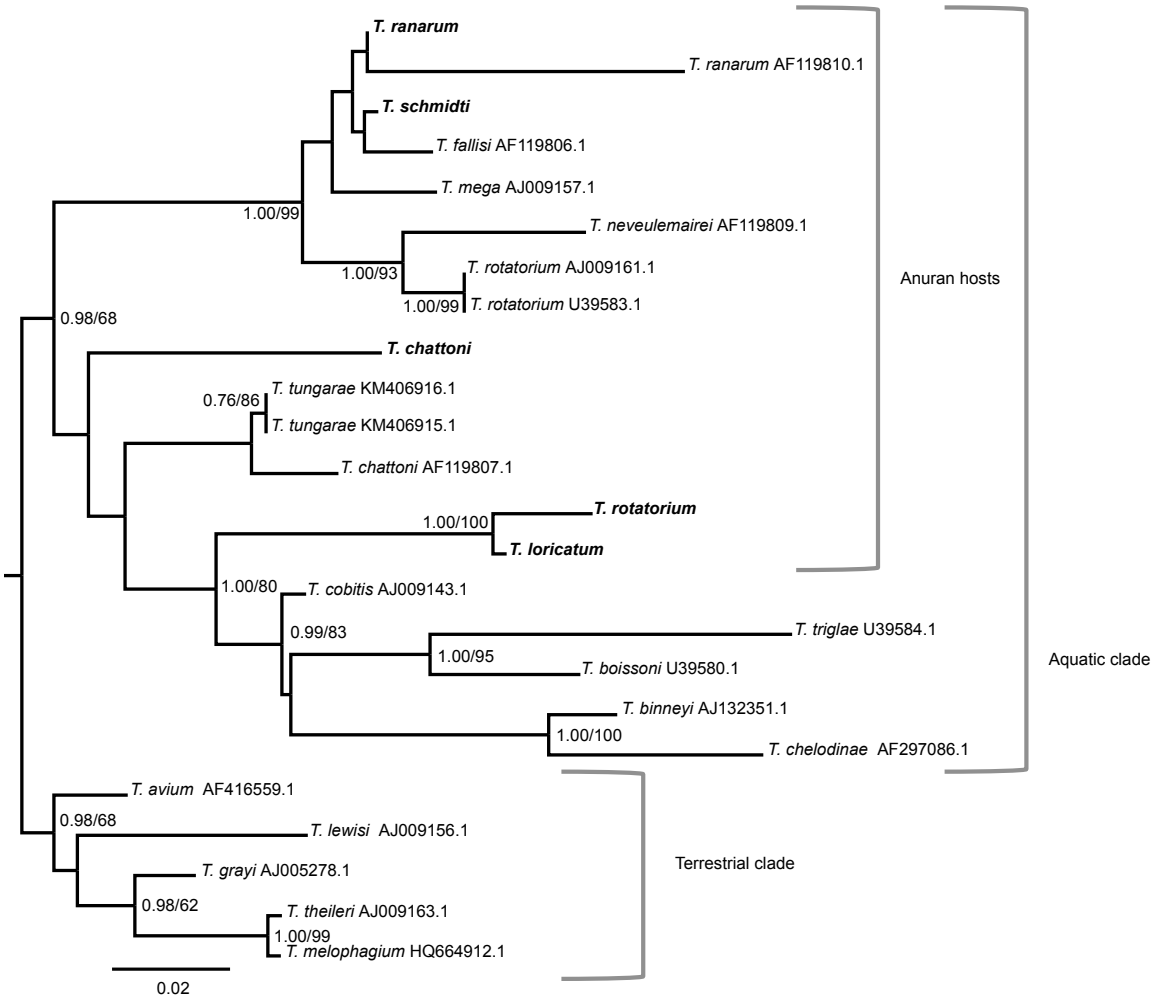


**Figure 3**. Phylogeny of the 18s rRNA gene based on an alignment of 522 base pairs and rooted with trypanosomes that infect terrestrial hosts. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values, respectively. Posterior probabilities <70 and bootstrap values <50 were omitted. Taxa added in this study are in bold.

Table I. Trypanosome 18s rRNA gene sequences sampled for phylogenetic analysis, their hosts, geographic origins, GenBank identification numbers, and sequence lengths.

| Trypanosome taxa | Host Species* | Geographic origin | GenBank ID | Sequence Length (bp) |
|---|---|---|---|---|
| *T. avium* | Lesser-spotted eagle *Aquila pomarina* | Slovakia | AF416559.1 | 2098 |
| *T. binneyi* | Platypus *Ornithorhynchus anatinus* | Tasmania, Australia | AJ132351.1 | 2136 |
| *T. boissoni* | Striped panray *Zanobatus schoenleinii* | Green Cape, Dakar, Senegal | U39580.1 | 2195 |
| *T. chattoni* | Northern leopard frog *Lithobates pipiens* | Anaoka County, Minnesota, USA | AF119807.1 | 2180 |
| *T. chattoni* | True frogs *Lithobates* spp. | Payne and Logan Cos. Oklahoma | TBA | 561 |
| *T. chelodinae* | Brisbane River tortoise *Emydura signata,* | Brisbane, Queensland, Austrailia | AF297086.1 | 2044 |
| *T. cobitis* | Stone Loach *Noemacheilus barbatulus* | River Lee, England | AJ009143.1 | 2134 |
| *T. fallisi* | American Toad *Anaxyrus americanus* | Algonquin Park, Ontario, Canada | AF119806.1 | 2239 |
| *T. grayi* | Tsetse fly *Glossina palpalis gambiensis* | Abuko and Bansang, The Gambia | AJ005278.1 | 2140 |
| *T. lewisi* | Rat *Rattus sp.* | England | AJ009156.1 | 2155 |
| *T. loricatum* | True frogs *Lithobates* spp. | Payne and Logan Cos. Oklahoma | TBA | 555 |
| *T. mega* | African Toad *Amietophrynus regularis* | Africa | AJ009157.1 | 2211 |
| *T. melophagium* | Sheep Ked *Melophagus ovinus* | Goljak, Croatia | HQ664912.1 | 2142 |
| *T. neveulemairei* | Edible frog *Pelophylax* kl. e*sculentus* | Yugoslavia | AF119809.1 | 2,197 |
| *T. ranarum* | Bullfrog *Lithobates catesbeianus* | Algonquin Park, Ontario, Canada | AF119810.1 | 2,203 |
| *T. ranarum* | True frogs *Lithobates* spp. | Teal Ridge, Payne Co., Oklahoma | TBA | 611 |
| *T. rotatorium* B2-II | Bullfrog *Lithobates catesbeianus* | Algonquin Park, Ontario, Canada | AJ009161.1 | 2176 |
| *T. rotatorium* B2-I | Bullfrog *Lithobates catesbeianus* | Algonquin Park, Ontario, Canada | U39583.1 | 2172 |
| *T. rotatorium* | True frogs *Lithobates* spp. | Payne and Logan Cos. Oklahoma | TBA | 556 |
| *T. schmidti* | True frogs *Lithobates* spp. | Teal Ridge, Payne Co., Oklahoma | TBA | 603 |
| *T. theileri* | Cattle *Bos taurus* | Scotland | AJ009163.1 | 2141 |
| *T. triglae* | Sea robin *Trigloporus lastoviza* | Eastern Atlantic Ocean | U39584.1 | 2242 |
| *T. tungarae* | Túngara frog *Engystomops pustulosus* | Gamboa, Panama | KM406915.1 | 1688 |
| *T. tungarae* | Túngara frog *Engystomops pustulosus* | Gamboa, Panama | KM406916.1 | 1689 |

*Current host taxonomy is given

Table II. Measurements for the 5 species/morphotypes of trypanosomes observed in frogs from Payne and Logan Cos., Oklahoma.

| Metric | *Trypanosoma schmidti* | *Trypanosoma ranarum* | *Trypanosoma loricatum* | *Trypanosoma chattoni* | *Trypanosoma rotatorium* |
|---|---|---|---|---|---|
| PF | 56.5 ± 7.9<br>47.3 - 66.7 (N = 5) | 70.8 ± 6.3<br>61.0 - 77.1 (N = 5) | n/a | n/a | n/a |
| PA | 49.0 ± 9.8<br>26.8 - 67.8 (N = 13) | 54.7 ± 8.9<br>33.5 - 71.1 (N = 66) | 56.5 ± 8.4<br>32.8 - 76.6 (N = 74) | 35.0 ± 6.8*<br>24.6 - 49.7 (N = 30) | 35.8 ± 3.8<br>27.1 - 46.5 (N = 53) |
| FF | 11.8 ± 7.9<br>2.5 - 23.0 (N = 5) | 10.2 ± 6.7<br>4.4 - 21.7 (N = 5) | n/a | n/a | n/a |
| BW | 6.8 ± 2.3<br>2.4 - 10.4 (N = 13) | 12.8 ± 4.0<br>8.0 - 35.1 (N = 67) | 14.8 ± 4.5<br>6.4 - 26.8 (N = 74) | 30.3 ± 5.1*<br>22.0 - 43.5 (N = 30) | 12.4 ± 5.4<br>5.7 - 25.4 (N = 53) |
| LN | 2.3 ± 0.4<br>1.8 - 2.9 (N = 9) | 5.1 ± 0.7<br>3.5 - 6.7 (N = 44) | 3.4 ± 0.5<br>2.5 - 4.5 (N = 59) | 6.1 ± 1.2<br>4.3 - 7.4 (N = 10) | 3.7 ± 0.5<br>2.8 - 4.8 (N = 47) |
| WN | 1.7 ± 0.4<br>1.1 - 2.5 (N = 9) | 4.0 ± 0.9<br>2.7 - 6.7 (N = 44) | 2.9 ± 0.5<br>1.8 - 3.9 (N = 59) | 5.1 ± 0.8<br>3.8 - 6.4 (N = 10) | 3.3 ± 0.6<br>2.0 - 4.7 (N = 47) |
| PK | 22.2 ± 4.7<br>12.9 - 28.6 (N = 11) | 23.0 ± 4.7<br>12.9 - 29.0 (N = 12) | 11.2 ± 3.5<br>4.6 - 18.0 (N = 31) | n/a | 4.6 ± 2.8<br>1.3 - 12.6 (N = 16) |
| KN | 3.3 ± 0.8<br>2.4 - 5.1 (N = 9) | 6.9 ± 1.2<br>5.2 - 9.3 (N = 12) | 8.2 ± 1.9<br>3.7 - 12.9 (N = 31) | n/a | 8.1 ± 1.8<br>4.4 - 11.0 (N = 16) |
| NA | 26.4 ± 6.6<br>10.6 - 34.4 (N = 9) | 29.3 ± 7.7<br>12.3 - 46.9 (N = 45) | 39.3 ± 8.7<br>12.9 - 55.9 (N = 58) | n/a | 24.6 ± 4.0<br>17 - 33 (N = 47) |
| PN | 23.0 ± 3.9<br>16.1 - 28.4 (N = 9) | 25.9 ± 5.8<br>15.9 - 38.0 (N = 45) | 18.9 ± 4.0<br>11.7 - 33.0 (N = 58) | n/a | 12.3 ± 1.6<br>8.8 - 16.9 (N = 47) |
| PK/PN | 0.91 ± 0.06 (N = 9) | 0.82 ± 0.10 (N = 12) | 0.56 ± 0.12 (N = 31) | n/a | 0.36 ± 0.17 (N = 16) |
| PK/PA | 0.47 ± 0.07 (N = 11) | 0.38 ± 0.07 (N = 12) | 0.20 ± 0.05 (N = 31) | n/a | 0.13 ± 0.08 (N = 16) |
| PN/PA | 0.50 ± 0.06 (N = 9) | 0.48 ± 0.08 (N = 45) | 0.34 ± 0.08 (N = 58) | n/a | 0.34 ± 0.05 (N = 47) |
| BW/PA | 0.14 ± 0.05 (N = 13) | 0.24 ± 0.08 (N = 66) | 0.26 ± 0.08 (N = 74) | 0.88 ± 0.10 (N = 30) | 0.34 ± 0.14 (N = 53) |
| FF/PA | 0.33 ± 0.32 (N = 5) | 0.18 ± 0.14 (N = 5) | n/a | n/a | n/a |

Table cells include measurement mean ± 1 SD, range, and number of trypanosomes measured. All measurements are in micrometers (µm PF = total length including free flagellum, PA = body length excluding free flagellum, FF = length of free flagellum (n/a = no free flagellum), BW = maximum body width excluding undulating membrane, LN = length of nucleus, WN = width of nucleus, 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.

*PA for *T. chattoni* was measured as the maximum cell diameter, and BW was the diameter perpindicular to PA.

Table III. Pairwise genetic distances between trypanosome 18s rRNA sequences using the K2P + G model, expressed as percentages and given below the diagonal. Standard error estimates are based on bootstrap analysis with 1000 pseudoreplicates and are given above the diagonal.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *T. avium* AF416559.1 | | 0.017 | 0.015 | 0.013 | 0.013 | 0.018 | 0.012 | 0.014 | 0.009 | 0.011 | 0.015 | 0.013 | 0.010 | 0.015 | 0.019 | 0.012 | 0.015 | 0.015 | 0.017 | 0.013 | 0.010 | 0.019 | 0.011 | 0.011 |
| 2 | *T. binneyi* AJ132351.1 | 10.73 | | 0.017 | 0.017 | 0.019 | 0.010 | 0.012 | 0.022 | 0.017 | 0.020 | 0.017 | 0.020 | 0.018 | 0.023 | 0.026 | 0.020 | 0.023 | 0.023 | 0.019 | 0.021 | 0.018 | 0.019 | 0.016 | 0.016 |
| 3 | *T. boissoni* U39580.1 | 8.45 | 9.95 | | 0.016 | 0.017 | 0.019 | 0.010 | 0.019 | 0.015 | 0.017 | 0.015 | 0.018 | 0.015 | 0.021 | 0.023 | 0.018 | 0.020 | 0.020 | 0.016 | 0.018 | 0.015 | 0.014 | 0.014 | 0.014 |
| 4 | *T. chattoni* AF119807.1 | 6.06 | 10.50 | 8.79 | | 0.014 | 0.019 | 0.012 | 0.016 | 0.013 | 0.015 | 0.015 | 0.016 | 0.015 | 0.018 | 0.018 | 0.015 | 0.019 | 0.019 | 0.017 | 0.016 | 0.015 | 0.018 | 0.006 | 0.006 |
| 5 | *T. chattoni* | 6.82 | 12.24 | 9.55 | 7.43 | | 0.021 | 0.014 | 0.018 | 0.014 | 0.016 | 0.016 | 0.015 | 0.016 | 0.020 | 0.022 | 0.016 | 0.019 | 0.019 | 0.016 | 0.017 | 0.016 | 0.020 | 0.014 | 0.014 |
| 6 | *T. chelodinae* AF297086.1 | 12.20 | 4.49 | 12.00 | 12.29 | 14.08 | | 0.015 | 0.023 | 0.019 | 0.021 | 0.020 | 0.022 | 0.019 | 0.025 | 0.027 | 0.021 | 0.025 | 0.025 | 0.021 | 0.022 | 0.019 | 0.022 | 0.018 | 0.018 |
| 7 | *T. cobitis* AJ009143.1 | 5.75 | 5.76 | 4.77 | 5.78 | 7.59 | 7.61 | | 0.017 | 0.011 | 0.014 | 0.012 | 0.016 | 0.011 | 0.018 | 0.020 | 0.015 | 0.018 | 0.018 | 0.014 | 0.016 | 0.011 | 0.015 | 0.011 | 0.011 |
| 8 | *T. fallisi* AF119806.1 | 7.10 | 13.91 | 11.37 | 8.87 | 9.62 | 15.46 | 9.03 | | 0.016 | 0.018 | 0.018 | 0.008 | 0.014 | 0.012 | 0.012 | 0.006 | 0.009 | 0.009 | 0.019 | 0.005 | 0.014 | 0.020 | 0.016 | 0.016 |
| 9 | *T. grayi* AJ005278.1 | 3.47 | 10.69 | 8.45 | 6.56 | 7.32 | 12.17 | 4.71 | 8.68 | | 0.012 | 0.015 | 0.015 | 0.009 | 0.016 | 0.019 | 0.014 | 0.016 | 0.016 | 0.017 | 0.015 | 0.009 | 0.018 | 0.012 | 0.012 |
| 10 | *T. lewisi* AJ009156.1 | 4.97 | 12.88 | 10.51 | 8.78 | 8.69 | 13.75 | 7.38 | 10.20 | 5.48 | | 0.017 | 0.016 | 0.014 | 0.019 | 0.021 | 0.015 | 0.019 | 0.019 | 0.019 | 0.016 | 0.014 | 0.020 | 0.014 | 0.014 |
| 11 | *T. loricatum* | 8.47 | 9.88 | 7.93 | 8.01 | 8.77 | 12.57 | 5.77 | 10.22 | 8.73 | 9.93 | | 0.015 | 0.017 | 0.019 | 0.021 | 0.016 | 0.018 | 0.018 | 0.006 | 0.016 | 0.017 | 0.018 | 0.014 | 0.014 |
| 12 | *T. mega* AJ009157.1 | 6.57 | 12.95 | 9.87 | 8.60 | 8.19 | 15.14 | 8.76 | 3.00 | 8.12 | 8.70 | 8.18 | | 0.013 | 0.013 | 0.014 | 0.007 | 0.011 | 0.011 | 0.016 | 0.007 | 0.013 | 0.018 | 0.015 | 0.015 |
| 13 | *T. melophagium* HQ664912.1 | 4.25 | 11.07 | 8.75 | 7.95 | 8.74 | 12.53 | 5.49 | 7.59 | 3.24 | 6.84 | 10.21 | 6.51 | | 0.016 | 0.019 | 0.013 | 0.015 | 0.015 | 0.018 | 0.013 | 0.003 | 0.018 | 0.013 | 0.013 |
| 14 | *T. neveulemairei* AF119809.1 | 8.46 | 15.80 | 13.82 | 10.59 | 12.01 | 18.17 | 10.73 | 5.44 | 10.09 | 11.89 | 11.67 | 6.76 | 9.84 | | 0.018 | 0.012 | 0.010 | 0.010 | 0.020 | 0.011 | 0.017 | 0.020 | 0.018 | 0.018 |
| 15 | *T. ranarum* AF119810.1 | 11.26 | 18.05 | 15.25 | 10.87 | 14.19 | 19.79 | 12.30 | 6.33 | 11.52 | 13.76 | 13.29 | 7.72 | 12.14 | 11.11 | | 0.011 | 0.016 | 0.016 | 0.022 | 0.012 | 0.020 | 0.024 | 0.019 | 0.019 |
| 16 | *T. ranarum* | 5.76 | 12.62 | 10.46 | 7.73 | 8.76 | 14.13 | 7.90 | 1.57 | 7.29 | 8.41 | 8.75 | 2.28 | 6.24 | 5.69 | 5.02 | | 0.009 | 0.009 | 0.016 | 0.004 | 0.013 | 0.018 | 0.014 | 0.014 |
| 17 | *T. rotatorium* B2-II AJ009161.1 | 7.40 | 15.25 | 12.92 | 11.02 | 10.88 | 17.55 | 10.20 | 3.70 | 9.55 | 11.35 | 10.80 | 4.97 | 8.74 | 3.95 | 9.09 | 3.45 | | 0.000 | 0.018 | 0.008 | 0.016 | 0.020 | 0.018 | 0.018 |
| 18 | *T. rotatorium* B2-I U39583.1 | 7.40 | 15.25 | 12.92 | 11.02 | 10.88 | 17.55 | 10.20 | 3.70 | 9.55 | 11.35 | 10.80 | 4.97 | 8.74 | 3.95 | 9.09 | 3.45 | 0.00 | | 0.018 | 0.008 | 0.016 | 0.020 | 0.018 | 0.018 |
| 19 | *T. rotatorium* | 9.70 | 11.40 | 9.36 | 9.78 | 8.78 | 14.23 | 7.40 | 10.83 | 9.95 | 11.24 | 1.83 | 9.34 | 11.53 | 12.94 | 13.97 | 9.34 | 10.80 | 10.80 | | 0.017 | 0.019 | 0.018 | 0.016 | 0.016 |
| 20 | *T. schmidti* | 6.55 | 13.24 | 10.75 | 8.58 | 9.03 | 14.77 | 8.45 | 1.34 | 8.10 | 9.26 | 9.03 | 2.51 | 7.04 | 4.93 | 5.79 | 0.66 | 2.73 | 2.73 | 9.62 | | 0.014 | 0.019 | 0.015 | 0.015 |
| 21 | *T. theileri* AJ009163.1 | 4.24 | 11.04 | 8.73 | 7.93 | 8.72 | 12.51 | 5.48 | 7.61 | 3.23 | 6.59 | 10.19 | 7.04 | 0.44 | 10.42 | 12.76 | 6.77 | 9.30 | 9.30 | 11.50 | 7.57 | | 0.018 | 0.013 | 0.013 |
| 22 | *T. triglae* U39584.1 | 11.56 | 12.25 | 7.08 | 11.66 | 12.11 | 15.09 | 7.88 | 12.15 | 11.24 | 12.46 | 10.42 | 10.60 | 10.97 | 13.01 | 16.34 | 11.20 | 12.71 | 12.71 | 11.33 | 11.19 | 11.56 | | 0.018 | 0.018 |
| 23 | *T. tungarae* KM406915.1 | 5.23 | 9.27 | 7.62 | 1.60 | 6.84 | 11.29 | 4.97 | 8.81 | 5.48 | 7.88 | 7.66 | 7.96 | 6.82 | 10.53 | 12.01 | 7.12 | 10.04 | 10.04 | 9.40 | 7.94 | 6.81 | 10.97 | | 0.000 |
| 24 | *T. tungarae* KM406916.1 | 5.23 | 9.27 | 7.62 | 1.60 | 6.84 | 11.29 | 4.97 | 8.81 | 5.48 | 7.88 | 7.66 | 7.96 | 6.82 | 10.53 | 12.01 | 7.12 | 10.04 | 10.04 | 9.40 | 7.94 | 6.81 | 10.97 | 0.00 | |

# CHAPTER III

## MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *HEPATOZOON* SP. FROM OKLAHOMA ANURANS

ABSTRACT**:** Of the 5 North American *Hepatozoon* species infecting anurans, *H. catesbianae* and *H. clamatae*, have been reported on multiple occasions infecting bullfrogs (*Lithobates catesbeianus*) and green frogs (*Lithobates clamatans*) in Eastern North America. However, no accounts exist for these *Hepatozoon* species west of the Mississippi River. The two species are morphologically similar, but can be distinguished by the effect of the gametocyte on the host erythrocyte nucleus. Infections with *H. clamatae* distort and fragment the erythrocyte nucleus; whereas *H. catesbianae* infections do not. In a survey of blood protozoans of 9 species of amphibians from Oklahoma, *Hepatozoon* gametocytes and merozoites were found in 7% (2/28) of adult bullfrogs and 7% (2/29) of adult southern leopard frogs (*Lithobates sphenocephalus*). Morphological measurements of the *Hepatozoon* sp. from this study conformed to previous descriptions of *H. catesbianae*. Additionally, the gamonts and merozoites infecting red blood cells of bullfrogs and southern leopard frogs from this study were never observed to distort the cell nucleus in either infected bullfrogs or infected southern leopard frogs. Based on these morphological characteristic and a close phylogenetic association to other ITS-1

sequence isolates for *H. catesbianae* but not *H. clamatae*, I report a new locality for *H. catesbianae* in Oklahoma, extending the known range for this species by at least 1800 km.

## INTRODUCTION

The genus *Hepatozoon* (Adeleorina, Apicomplexa) includes intracellular protozoan parasites that infect erythrocytes, leukocytes, and hepatocytes of mammals, birds, reptiles, amphibians and fish (Kim et al., 1998). Hemophagous invertebrates including arthropods (ticks, mites, lice, fleas, reduviid bugs, sandflies, tsetse flies, and mosquitoes) and annelids (leeches) have been reported as vectors and definitive hosts for *Hepatozoon* spp. Depending on the species, gamogany (sexual reproduction) occurs in the invertebrate definitive host, forming oocysts (Desser, 1995; Smith, 1996). The oocyst then undergoes sporogany (asexual reproduction) producing sporocysts containing sporozoites (Levine, 1988). Transmission to the vertebrate host can occur either by ingestion of an infected arthropod by mammal, bird, reptile and amphibian hosts or through the bite of an annelid of reptile and fish hosts. Within the vertebrate intermediate host, merogony (asexual reproduction) takes place in internal organs such as the liver, leading to the release of merozoites that infect erythrocytes or leukocytes and develop to gamonts (Smith, 1996). The life cycle is completed when the appropriate hematophagous invertebrate host ingests blood with gamonts from an infected vertebrate. Sexual reproduction (gamogony) followed by asexual reproduction (sporogony) of the parasite occurs within the invertebrate definitive host.

Currently, 44 species of *Hepatozoon* have been described from amphibians around the world (Smith, 1996; Harris et al. 2014). In North America, five species of *Hepatozoon* are known to infect amphibians. Stebbins (1904, 1905) described *H. catesbianae* in bullfrogs (*Lithobates catesbeianus*) and *H. clamatae* in green frogs (*L. clamatans*) from New York. Additionally, Lehmann (1959a, b, 1960) described three species of *Hepatozoon* in anurans from California including *H. sonomae* and *H. boyli* infecting the foothill yellow-legged frog (*Rana boylii*) and *H. aurora* infecting the northern red-legged frog (*R. aurora*). The only other reports of *Hepatozoon* spp. infecting amphibians in North America include, *H. catesbianae* and *H. clamatae* in bullfrogs, green frogs and northern leopard frogs (*L. pipiens*) from Ontario, Canada, *H. clamatae* in green frogs and northern leopard frogs from Illinois, and a unidentified *Hepatozoon* sp. from the Oregon spotted frog (*R. pretiosa*) from Oregon (Kudo, 1922; Barta and Desser, 1984; Desser, 1995; Kim et al. 1998; Stenberg and Bowerman, 2008).

Life cycles for two species of amphibian *Hepatozoon* are currently known. Desser et al. (1995) and Kim et al. (1998) elucidated the life cycles of *H. catesbianae* and *H. clamatae*, respectively. Briefly, gametogenesis, fertilization, and sporogonic development of both species occur in the malpighian tubules of the mosquito *Culex territans*. Upon ingestion of an infected mosquito by the frog, sporozoites invade frog liver cells and form meronts. Within the frog host, merozoites are produced in the liver, infect erythrocytes, and develop into gamonts. *Culex territans* then feeds on the frog and ingest circulating gamonts, which migrate to the malpighian tubules and undergo gametogenesis, continuing the life cycle. More recently, Harkness et al. (2010) was able to infect *Culex*

*pipiens*, a bird/mammal feeding mosquito, with *H. clamatae* in the laboratory, suggesting that amphibian *Hepatozoon* species may not be specific to their mosquito vector hosts.

In this study, a total of 200 individuals representing nine species in four families of amphibians were collected from a single location in Guthrie, Logan County, and four locations in Stillwater, Payne County, and Oklahoma U.S.A. during 2014 and 2015 and examined for the presence of blood protozoa. A total of six blood protozoan species/morphotypes were found infecting bullfrogs and southern leopard frogs in the survey. This chapter details the morphological and molecular characterization of the single *Hepatozoon* species found in bullfrogs and southern leopard frogs.

## MATERIALS AND METHODS

### Slide Examination for *Hepatozoon* spp.

Giemsa stained blood smears of amphibian blood were scanned at high power (100x objective) for the presence of intracellular parasites infecting host red blood cells as described previously in Chapter I. When southern leopard frogs (*Lithobates sphenocephalus)* or bullfrogs (*Lithobates catesbeianus*) were found to be infected with either gamonts or merozoites of a species of a *Hepatozoon*, 10 to 20 specimens of each stage were photographed with an Olympus 5 megapixel digital camera from each frog species and gamonts and merozoite length and width were measured using ImageJ (Schneider et al. 2012) calibrated with a stage micrometer (Reichert, NY).

**DNA Extraction**

For extraction of *Hepatozoon* DNA from frog blood, 20 µL of blood samples preserved in 100% ethanol and stored at -80°C freezer were thawed and dried in a Thermolyne dri-bath heated to 56°C for approximately 30 minutes. DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, CA) following the recommended protocol for samples of nucleated blood (Qiagen, 2006).

**PCR Amplification and Sequencing**

The 18s ribosomal RNA gene and internal transcribed spacer region 1 (ITS-1) were amplified from extracted DNA samples by PCR using previously published primers. For 18s rRNA sequences, the forward and reverse primers HEMO1 5'-TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG-3' and HEMO2 5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3' were used from Perkins and Keller (2001), respectively. For ITS-1 sequences, the forward and reverse primers herpfor 5'-GCG GAA GGA TCA TTC ACA TT-3' and herprev 5'-TCC TTC ATC GAT GCA CAA AC-3' were used from Boulianne et al. (2007), respectively. For both 18s rRNA and ITS-1 gene targets, amplification was performed using 10 µL master mix (HotStarTaq Master Mix Kit, Qiagen, CA), 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL template DNA, and 13 µL sterile water, giving a total reaction volume of 25 µL per reaction. A Hot Start touchdown PCR profile was implemented on a AB 2720 thermocycler (Applied Biosystems) using the following touchdown PCR program: initial activation for 600s at 95°C, annealing for 30s at 65°C decreasing by 2°C to 55°C over 10 cycles, and extension

for 60s at 72°C, repeated for 30 total cycles before a final extension period of 400s at 72°C.

PCR success was determined by running 4 μL of PCR product on a 1% agarose gel using TBE buffer on a horizontal electrophoresis system (VWR, PA) at constant voltage (100V) for 20 min. Bands were visualized using a UV light and only single, solid bands were prepped for sequencing using the QIAquick PCR Purification Kit (Qiagen, CA).

For sequencing PCR amplicons, the same forward and reverse primers mentioned above for each gene sequence were used with the Sanger sequencing chemistry on an Applied Biosystems 3730 capillary sequencer at the Oklahoma State University core facility.

**Phylogenetic Analyses**

The *Hepatozoon* 18s rRNA gene sequence obtained in this study was combined with 13 sequences retrieved from GenBank and aligned using the MUSCLE (Edgar, 2004) feature of MEGA 6.06 (Tamura et al., 2013). The alignment was inspected by eye and any ambiguous regions were removed from further analyses. The final alignment consisted of 914 characters and 14 taxa. Sequences of 18s rRNA genes of other *Hepatozoon* species and isolates of *H. clamatae* and *H. catesbianae* were used for the alignment along with distantly related apicomplexans, *Babesiasoma stableri* and *Dactylsoma ranarum* both of which infect anurans, and the coccidian *Adelina dimidiata*

and the gregarine like apicomplexan *Cryptosporidium serpentis* were used as outgroups (Table I).

The *Hepatozoon* ITS-1 gene sequence obtained in this study was added to the alignment of Boulianne et al. (2007), which included 10 sequences available on GenBank and 6 sequences from Kim et al. (1998). The final alignment consisted of 231 characters (Table II) and contained isolates of both *H. catesbianae* and *H. clamatae* from Ontario and Nova Scotia. Additionally, two sequences of *H. sipedon* from snakes were used as outgroups (Table II).

Phylogenies were estimated using both Maximum Likelihood (ML) and Bayesian inference frameworks. Model test feature of MEGA 6.06 (Tamura et al. 2013) yielded the K2P + G model (Kamura, 1980) as the best evolutionary model for the ML analysis of the 18s rRNA gene, and the T92 model (Tamura, 1992) for the ITS-1 gene. ML trees were created in MEGA 6.06 (Tamura et al. 2013) using the appropriate evolutionary model with 1000 bootstrap replications. Bayesian phylogenies were created using MrBayes 3.2.4 (Ronquist and Huelsenbeck, 2003), using 1,000,000 Monte Carlo Markov generations using 1 hot chain and 3 cold chains. Nucleotide substitutions were allowed to be different for transitions and transversions and the rates were gamma distributed.

## RESULTS

### Morphology

*Hepatozoon* gamonts or merozoites were observed infecting red blood cells in 7% (2/29) of adult southern leopard frogs and 7% (2/28) of adult bullfrogs examined from north central Oklahoma. One southern leopard frog was exclusively infected with gamonts, while the other was infected with both gamonts and merozoites. Additionally, one bullfrog was only infected with gamonts, and the other was only infected with merozoites. Gamonts in bullfrogs measured 22.5 μm ± 1.52 (20.5 – 25.2, n = 10) in length and 5.0 μm ± 0.70 (3.7 – 5.8, n = 10) in width. Merozoites in bullfrogs measured 12.3 μm ± 0.8 (11.0 – 13.9, n = 10) in length and 4.4 μm ± 0.7 (3.5 – 5.7, n = 10) in width. Gamonts in southern leopard frogs measured 22.5 μm ± 1.9 (19.2 – 26.3, n = 20) in length and 4.8 μm ± 0.9 (3.8 – 6.9, n = 20) in width. Merozoites in southern leopard frogs measured 11.8 μm ± 1.9 (8.8 – 15.7, n = 10) in length and 5.5 μm ± 1.0 (4.0 – 7.5, n = 10) in width. Morphologically, gamonts were similar to previously reported measurements of *H. catesbianae* and *H. clamatae* by Desser et al. (1995) and Kim et al. (1998, see Table III). However, the *Hepatozoon* gamonts and merozoites found in this study were not observed to distort the cell nucleus in either infected bullfrogs (n = 20 infected RBCs) or infected southern leopard frogs (n = 30 infected RBCs). Based on the parasite cell dimensions and the effect on the host cell nucleus, the apicomplexan infecting red blood cells of bullfrogs and southern leopard frogs was identified as *Hepatozoon catesbianae*.

**Phylogenetic analyses**

Samples of merozoites and gamonts from two different bullfrogs yielded identical sequences for both the 18s rRNA and ITS-1 genes. The 18s phylogenetic tree indicated that sequences of *H. catesbianae* obtained from Oklahoma bullfrogs occurred in the amphibian *Hepatozoon* clade. However, other sequences of *H. clamatae* and *H. catesbianae* could not be resolved from the Oklahoma bullfrog isolate, resulting in a polytomy (Fig. 3). The ITS-1 tree provided two clades of anuran *Hepatozoon* isolates. The first clade contains the Oklahoma isolate of *H. catesbianae* with isolates of both *H. catesbianae* and *H. clamatae* from Nova Scotia and Ontario. The second clade contains isolates of only *H. clamatae* from both Nova Scotia and Ontario. However, within the first clade the Oklahoma *H. catesbianae* isolate formed a distinct clade within the *H. catesbianae* isolates from Nova Scotia and was distinct from all H. clamatae isolates from Nova Scotia and Ontario (Fig. 4).

**DISCUSSION**

In North America, five species of *Hepatozoon* have been described infecting true frogs and no *Hepatozoon* species have been reported in North American toads, treefrogs, or salamanders (Lehman, 1959a, b, 1960; Desser et al. 1995; Kim et al. 1998; Chapter I). However, other *Hepatozoon* species have been reported infecting toads and treefrogs in Asia and Africa (Smith, 1996). Based on length and width measurements of the gamonts, the *Hepatozoon* found infecting bullfrogs and southern leopard frogs from Oklahoma, most closely conformed to length and width measurements of *H. catesbianae* and *H.*

*clamatae* (Table III). The reported distinguishing characteristic between *H. catesbianae* and *H. clamatae* is the effect of these apicomplexans on the host red blood cell nucleus (Kim et al. 1998). Infections with *H. clamatae* distort and fragment the anuran erythrocyte nucleus; whereas *H. catesbianae* infections do not. The nuclei of the red blood cells infected with *Hepatozoon* gamonts in Oklahoma bullfrogs and southern leopard frogs were not observed to be fragmented, which suggests this species is *H. catesbianae*.

Previous anuran surveys indicate that bullfrogs and green frogs are naturally infected with both *H. catesbianae* and *H. clamatae*, and the host specificity of these two *Hepatozoon* species has been in a state of confusion. Prevalence data and experimental infections suggest that these two species have some host preferences, where *H. catesbianae* has been found to primarily infect bullfrogs, and *H. clamatae* primarily infects green frogs (Kim et al. 1998; Boulianne et al. 2007; Dickson et al. 2013). Boulianne et al. (2007) surveyed bullfrogs and green frogs in Nova Scotia, Canada, and found bullfrogs to be more commonly infected with *H. catesbianae* (17.6%) than with *H. clamatae* (5.9%), where green frogs were more commonly infected with *H. clamatae* (75.2%) than with *H. catesbianae* (29.0%). Additionally, Kim et al. (1998) infected a bullfrog and a green frog with sporocysts of both *H. catesbianae* and *H. clamatae* simultaneously, and found higher levels of parasitemia of infected red blood cells of the bullfrog with *H. catesbianae* (2.7%) than *H. clamatae* (0.4%). In contrast, they found a higher parasitemia of infected red blood cells with *H. clamatae* (4.6%) than *H. catesbianae* (0.6%) in the green frog. In these studies, the parasites' effect on the host red blood cell nucleus was used to differentiate species, and assuming this is a useful

character, these studies suggest that *H. catesbianae* and *H. clamatae* are distinct species. However, genetic work thus far suggests they are conspecific. Boulianne et al. (2007) and Barta et al. (2012) performed phylogenetic analyses of *Hepatozoon* ITS-1 and 18s rRNA gene sequences, respectively, and found *H. catesbianae* and *H. clamatae* to be genetically inseparable. Additionally, in the laboratory, Dickson et al. (2013) used a saline solution to isolate gamonts of *H. catesbianae* and *H. clamatae* from red blood cells of naturally infected bullfrogs and green frogs, respectively. Gamonts of each species were then applied to samples of uninfected bullfrog and green frog blood as well as the blood of uninfected northern water snake (*Nerodia sipedon*). Surprisingly, they found no difference in infectivity of *H. catesbianae* or *H. clamatae* gamonts to bullfrog or green frog red blood cells *in vitro*. Additionally, both *H. catesbianae* and *H. clamatae* gamonts were able to infect the red blood cells of a northern water snake *in vitro*, a host which is never infected with these species in nature. These findings, and as argued by Dickson et al. (2013), suggest there may be other differences in development of these stages in the liver or other organs of bullfrogs and green frogs that could explain their host specificities.

My phylogenetic analyses of the 18s rRNA gene is consistent with previous work by Barta et al. (2012) and indicates that *H. catesbianae* and *H. clamatae* form a monophyletic polytomy. The *H. catesbianae* 18s rRNA isolate added in this study was placed in the unresolved anuran *Hepatozoon* clade (Fig. 3). Additionally, my analyses of the ITS-1 gene are consistent with Boulianne et al. (2007) in which they were unable to differentiate some isolates of *H. catesbianae* and *H. clamatae* from Ontario, Canada. However, the new *H. catesbianae* ITS-1 isolate from Oklahoma bullfrogs formed a

distinct clade within the *H. catesbianae* isolates from Nova Scotia, Canada (Fig. 4). These data suggest that the Oklahoma isolate of *H. catesbianae* is more closely related to the Nova Scotia *H. catesbianae* isolates and genetically distinct from all other isolates of *H. clamatae* from Nova Scotia and Ontario, Canada.

Boulianne et al. (2007) speculated that the ITS-1 *H. clamatae* sequences designated as SM4, SM7, and S34 from Ontario, Canada were misidentified by Kim et al. (1998) because bullfrogs and green frogs can be infected with both species of *Hepatozoon* although at different degrees of parasitemia in each frog species. As a result, Boulianne et al. (2007) suggested that the ITS-1 isolates of *H. clamatae* from Ontario, Canada are in fact isolates of *H. catesbianae*. Along with my data these findings suggest that the ITS-1 gene might be able to differentiate these species into two distinct clades. However, additional samples of *H. clamatae* isolates and *H. catesbianae* isolates along with other anuran *Hepatozoon* species are needed to evaluate this possibility. Taken together, more research is clearly needed, including sequencing other phylogenetically informative genes such as the *CO1* gene, to evaluate if *H. catesbianae* and *H. clamatae* are distinct species.

In conclusion, my study is the first to examine an amphibian community for *Hepatozoon* species in the Great Plains region of North America. Oklahoma is a new locality record and southern leopard frogs are a new host record for what we currently consider *H. catesbianae*. More importantly, my study clearly indicates that other more phylogenetically informative molecular markers are needed to differentiate between what we currently consider *H. catesbianae* and *H. clamatae* and other species of anuran *Hepatozoon*.

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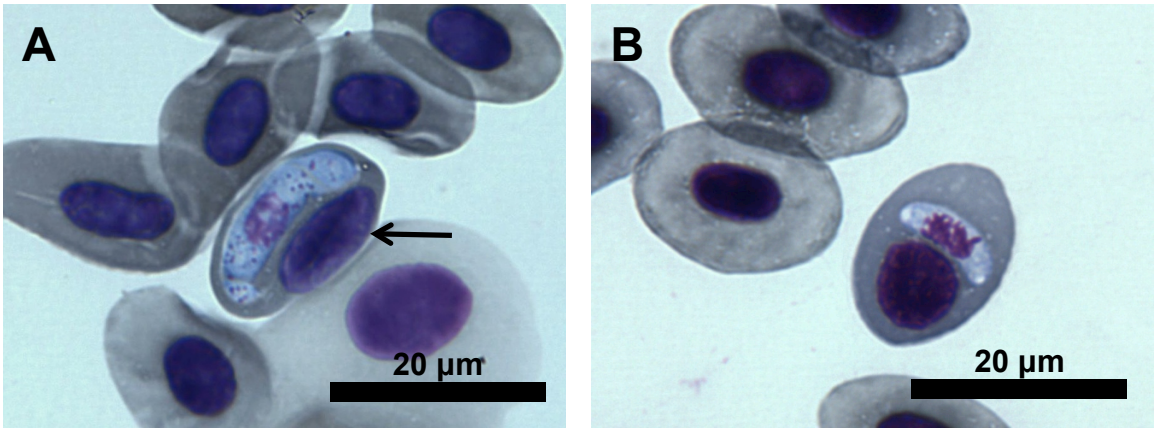


**Figure 1**. Photomicrographs of *Hepatozoon catesbianae* infecting red blood cells in Giemsa stained blood smears from naturally infected southern leopard frogs and bullfrogs in Payne Co., Oklahoma. **A**. Gamont of *H. catesbianae*, note displaced yet intact host cell nucleus characteristic of the species (black arrow). **B**. Merozoite of *H. catesbianae*, note parasite cell size, about half as large as the gamont stage.

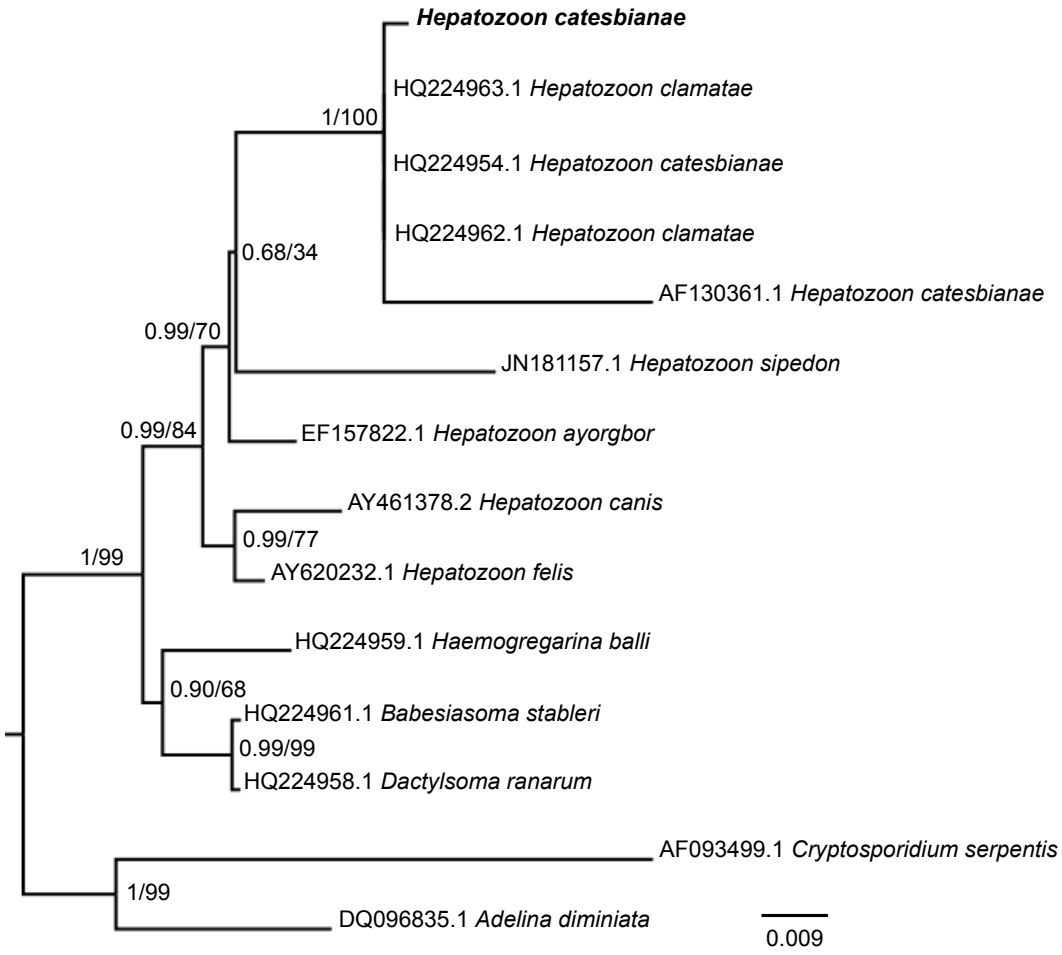


**Figure 2.** Phylogeny of selected apicomplexan 18s rRNA gene sequences. –Ln = 2616.40. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values, respectively. *Hepatozoon catesbianae* (bold) was added in this study.

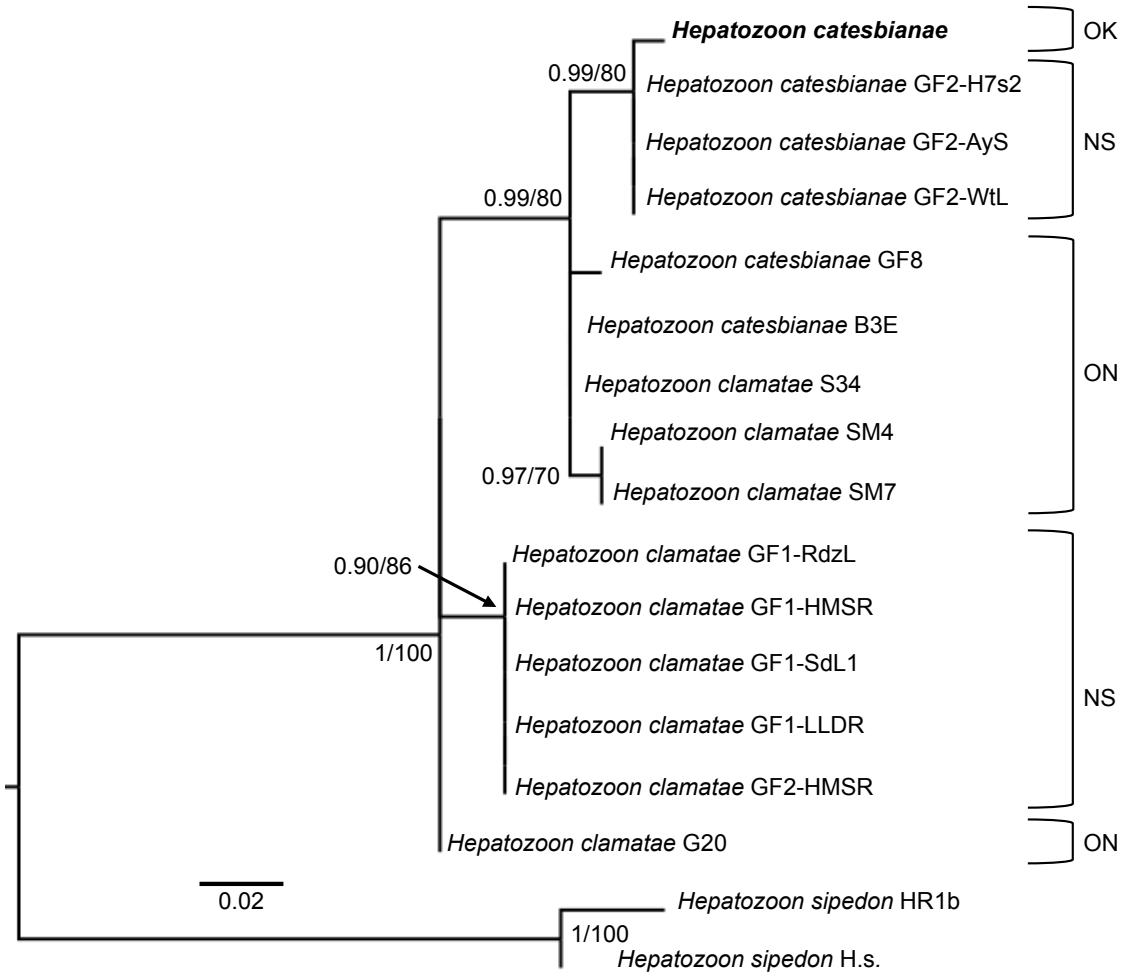


**Figure 3.** Phylogeny of *Hepatozoon* ITS-1 gene sequences. –Ln = 447.60. Values at nodes represent Bayesian posterior probabilities and ML bootstrap values, respectively. Letters at right indicate isolate locality. OK = Oklahoma, USA. NS = Nova Scotia, Canada. ON = Ontario, Canada. *Hepatozoon catesbianae* (bold) was added in this study.

Table I. Apicomplexans sampled for phylogenetic analysis of the 18s rRNA gene, their hosts, and geographic origins.

| Taxa | Host Species | Geographic origin | Accession Number | Length (bp) |
|---|---|---|---|---|
| *Adelina dimidiata* | Centipede *Scolopendra cingulata* | Melnik, Bulgaria | DQ096835.1 | 1924 |
| *Babesiasoma stableri* | Mink frogs *Lithobates septentrionalis* | Algonquin Park, Ontario, Canada | HQ224961.1 | 1649 |
| *Cryptosporidium serpent* | Rat snake *Pantherophis emoryi* | Kansas, USA | AF093499.1 | 1743 |
| *Dactylsoma ranarum* | Edible frogs *Pelophylax* kl. *esculentus* | Fium'Orbo River, Corsica, France | HQ224958.1 | 1810 |
| *Haemogregarina balli* | Common snapping turtle *Chelydra serpentina serpentina* | Algonquin Park, Ontario, Canada | HQ224959.1 | 1817 |
| *Hepatozoon ayorgbor* | Ball python *Python regius* | Ghana | EF157822.1 | 1773 |
| *Hepatozoon canis* | Domestic dog *Canis familiaris* | Spain | AY461378.2 | 1762 |
| *Hepatozoon catesbianae* | Bullfrog *Lithobates catesbeianus* | Algonquin Park, Ontario, Canada | AF130361.1 | 1824 |
| *Hepatozoon catesbianae* | True frogs *Lithobates* spp. | Payne Co., Oklahoma, USA | TBA | 923 |
| *Hepatozoon catesbianae* | Bullfrog *Lithobates catesbeianus* | Algonquin Park, Ontario, Canada | HQ224954.1 | 1810 |
| *Hepatozoon clamatae* | Green frogs *Lithobates clamitans* | Speed River, Ontario, Canada | HQ224962.1 | 1655 |
| *Hepatozoon clamatae* | Green frogs *Lithobates clamitans* | Birge Mills, Ontario, Canada | HQ224963.1 | 1655 |
| *Hepatozoon felis* | Domestic cat *Felis catus* | Spain | AY620232.1 | 1771 |
| *Hepatozoon sipedon* | Northern water snake *Nerodia sipedon sipedon* | Lake Opinicon, Ontario, Canada | JN181157.1 | 1804 |

Table II. *Hepatozoon* species sampled for phylogenetic analysis of the ITS-1 sequences, their hosts, geographic origins, and sources.

| Taxa | Designation | Host Species | Geographic origin | Accession # or source | Length (bp) |
|---|---|---|---|---|---|
| *Hepatozoon catesbianae* | | Bullfrog *Lithobates catesbeianus* and southern leopard frog *Lithobates sphenocephalus* | Payne County, Oklahoma, USA | This study | 191 |
| *Hepatozoon clamatae* | GF1-RdzL | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856588 | 129 |
| *Hepatozoon clamatae* | GF1-HMSR | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856584 | 129 |
| *Hepatozoon clamatae* | GF1-SdL1 | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856587 | 129 |
| *Hepatozoon clamatae* | GF2-LLDR | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856586 | 129 |
| *Hepatozoon clamatae* | GF2-HMSR | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856585 | 129 |
| *Hepatozoon catesbianae* | GF2-H7s2 | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856591 | 129 |
| *Hepatozoon catesbianae* | GF2-AyS | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856590 | 129 |
| *Hepatozoon catesbianae* | GF2-WtL | Green frog *Lithobates clamitans* | Nova Scotia, Canada | DQ856589 | 129 |
| *Hepatozoon catesbianae* | GF8 | Bullfrog *Lithobates catesbeianus* and green frog *Lithobates clamitans* | Algonquin Park, Ontario, Canada | Kim et al. 1998 | 211 |
| *Hepatozoon catesbianae* | B3E | Bullfrog *Lithobates catesbeianus* and green frog *Lithobates clamitans* | Algonquin Park, Ontario, Canada | Kim et al. 1998 | 211 |
| *Hepatozoon clamatae* | G20 | Bullfrog *Lithobates catesbeianus* and green frog *Lithobates clamitans* | Algonquin Park, Ontario, Canada | Kim et al. 1998 | 211 |
| *Hepatozoon clamatae* | S34 | Bullfrog *Lithobates catesbeianus* and green frog *Lithobates clamitans* | Algonquin Park, Ontario, Canada | Kim et al. 1998 | 211 |
| *Hepatozoon clamatae* | SM4 | Northern leopard frog *Lithobates pipiens* | Halliburton Co., Ontario, Canada | Kim et al. 1998 | 211 |
| *Hepatozoon clamatae* | SM7 | Northern leopard frog *Lithobates pipiens* | Halliburton Co., Ontario, Canada | Kim et al. 1998 | 211 |
| *Hepatozoon sipedon* | HR1b | Northern ribbon snake *Thamnophis sauritus septentrionalis* | Coldwater, Simcoe County, Canada | AF110249 | 309 |
| *Hepatozoon sipedon* | H.s. | Northern water snake *Nerodia sipedon sipedon* | Queen's University Biological S., Frontenac County, Canada | AF110244 | 309 |

Table III. Morphological characteristics of intracellular gamonts of the 5 *Hepatozoon* species infecting North American anurans and new locality record from Oklahoma.

| Species | Type Host | Other reported hosts | Gamont Length Mean ± SD or range (µm) | Gamont Width Mean ± SD or range (µm) | Effect on Host Nucleus | Reference |
|---|---|---|---|---|---|---|
| *H. catesbianae* | Bullfrog | Green Frog | 22.09 ± 1.22 | 5.19 ± 0.52 | Displaced, Non-Fragmented | Desser et al. 1995 |
| *H. clamatae* | Green frog | Bullfrog, Northern leopard frog | 23.7 ± 1.7 | 5.1 ± 0.7 | Displaced, Fragmented | Kim et al. 1998 |
| *H. sonomae* | Yellow-Legged Frog | None | 24.3 (Max) | 7.0 (Max) | Displaced, Non-Fragmented | Lehman, 1959b |
| *H. boyli* | Yellow-Legged Frog | None | 12.3 (11.4-15.4) | 4.9 (4.2-5.6) | Displaced, Non-Fragmented | Lehman, 1959a |
| *H. aurorae* | Northern Red-legged Frog | None | 17.9 (15.1-24.5) | 5.2 (4.8-7.0) | Displaced, Non-Fragmented | Lehman, 1960 |
| *H. catesbianae*[a] | Bullfrog | None | 22.5 ± 1.52 | 5.0 ± 0.69 | Displaced, Non-Fragmented | This Study |
| *H. catesbianae*[a] | Southern leopard frog[b] | None | 22.5 ± 1.94 | 4.84 ± 0.88 | Displaced, Non-Fragmented | This Study |

[a] New locality record: Teal Ridge, Payne County, Oklahoma (this study)

[b] New host record

# CONCLUSIONS

This work is the first study to report blood protozoans infecting amphibians in Oklahoma. Six species/morphotypes of blood protozoans infected southern leopard frogs (*Lithobates sphenocephalus*) and bullfrogs (*L. catesbeianus*) and conformed to previous descriptions of (1) *Hepatozoon catesbianae*, (2) *Trypanosoma ranarum*, (3) *T. schmidti*, (4) *T. loricatum*, (5) *T. rotatorium*, and (6) *T. chattoni*. Southern leopard frogs are new host records for 5 species/morphotypes of blood protozoans, including *T. ranarum*, *T. rotatorium*, *T. chattoni*, *T. loricatum*, and *H. catesbianae*. Additionally, bullfrogs are new host records for 2 species/morphotypes of blood protozoans, including *T. schmidti* and *T. loricatum*.

My study adds new molecular and morphological data for the blood parasites found in this study. Additionally, phylogenetic analyses of the blood parasites in this study revealed that the trypanosome morphotypes are genetically distinct and the Oklahoma strain of *H. catesbianae* formed a polytomy with previous sequences of *H. catesbianae* and *H. clamatae* from Canada. Broader sampling of blood parasite species/morphotypes with additional genetic markers is necessary to resolve the phylogenetic relationships within these blood protozoans and to better support trypanosome species identifications.

My study is also one of the first surveys for potential vectors of amphibian blood protozoa at a site containing confirmed infected amphibians. The potential vectors identified at Teal Ridge in Oklahoma include one species of turtle leech (*Placobdella rugosa*) and 2 species of mosquitoes that are known to feed on amphibians (*Culex erraticus* and *Uranotaenia sapphirina*). The vectors and life cycles of the blood protozoa found in this study have yet to be elucidated. However, the results of my vector surveys and the blood protozoan prevalence and species richness data across multiple amphibian life stages provide the necessary data for future studies of potential vector candidates for transmission of amphibian trypanosomes. I hope my study provides insight for other parasitologists to examine the potential vectors and life cycles of these neglected amphibian parasites.

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