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Prevalence of external and internal parasites of four species of freshwater turtles (*Trachemys scripta*, *Chrysemys picta*, *Chelydra serpentina*, and *Sternotherus odoratus*) in the piedmont of North Carolina, USA

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Abstract

Reptile hematology monitoring can provide important indicators of overall individual and ecosystem health of reptile populations. Freshwater turtles are commonly encountered in semi-urban systems across the southeastern United States, making them ideal to assess wetland health and study parasite ecology. We characterized internal and external parasites of four species of aquatic turtles, Painted turtles (*Chrysemys picta*), Yellow-bellied slider turtles (*Trachemys scripta*), Common snapping turtles (*Chelydra serpentina*), and musk turtles (*Sternotherus odoratus*) found in a semi-urban campus pond in Wingate, North Carolina. We found overall low prevalence of both external parasites (11.8%) in all turtles samples and internal parasites, or parasitemia in blood from a subset of turtles sampled (only in *C. serpentina* and *S. odoratus*), which varied across species with the most common intra-erythrocyte vacuoles morphologically consistent and similar to *Chelonoplasma* and *Sauroplasma*. Interestingly, we utilized a simple approach for identification of external leech parasites down to species using DNA barcoding and report on the diversity of leeches found on turtles, an often understudied area of turtle research. This data provides baseline estimates for both blood and parasite load of leeches belonging to Glossiphoniidae in wild piedmont freshwater turtles.

Keywords: Hematocrit, Testudines, turtle parasites, blood parameters, DNA barcoding

1. Introduction

Reptiles inhabiting freshwater ecosystems can be susceptible to a wide range of internal and external parasites which can impact their overall health and function (Zelmer and Platt 2008) [35]. Moreover, parasites of reptiles and amphibians can negatively impact individuals by reducing hemoglobin concentrations, reducing oxygen delivery to tissue and reduced reproduction and physiological behavior (Brown *et al.* 1994, Bower *et al.* 2018) [5, 3]. Hemogregarine parasites are internal parasites found across most turtle species (Mcarthur *et al.* 2004, Telford 2009) [20, 32], however little is known regarding prevalence across regions as their role in wild animals is not completely understood (Calil *et al.* 2017) [7]. Hirudinea leeches are common ectoparasites of reptiles which can be vectors for blood hemogregarine parasites (Siddall and Desser 1992) [30]. Therefore, assessing the relationship between turtle demography and parasites is important as a factor of environmental ecosystem health.

In some cases, identification of leeches commonly encountered on turtles such as *Placobdella parasitica* and *Placobdella ornata*, presents challenges and may be overlooked by turtle biologist (Davy *et al.* 2009) [9] possibly due to the difficulty in visual identification and classification (McManus and Bowles 1996) [21]. More recent methodologies for resolving identification of external turtle parasites include DNA barcoding, which allows taxonomic resolution typically down to species, based on a standardized DNA region unique to species (Valentini *et al.* 2009) [33]. In this study, we report on the prevalence of internal and external parasites found in a semi-urban pond in North Carolina across four freshwater turtle species. We also aimed to investigate the utility of an emerging genetic technology (DNA barcoding) for identification of leeches down to species.

2. Materials and Methods

2.1 Study area and field collection of external parasites

A total of 102 turtles were collected between 9/28/18 and 9/30/18 from a small pond on Wingate University Campus, Wingate North Carolina, USA (coordinates 34°59'11"N, 80°25'45.6"W). Out of these 102 individuals, 12 were recaptured during the sample period resulting in 90 unique individuals. These unique turtles include thirty-one painted turtles (*Chrysemys picta*) 20 M, 11 F, forty-four yellow bellied sliders (*Trachemys scripta*) 24 M, 20 F, seven musk turtles (*Sternotherus odoratus*) 5 M, 2 F, and eight of snapping turtles (*Chelydra serpentina*) 6 M, 2 F. Turtles were captured using baited hoop traps as part of ongoing mark recapture study, with all individuals receiving permanent marks (unique identifiers) on their marginal scutes (Cagle 1939)^[6]. All turtles were identified down to species, sexed, weighed, measured, and examined for external parasites (presence of leeches). For each leech, the location and total number of leeches on the turtle was noted and leeches were measured to the nearest mm straight length. Representative samples from leeches found across all species (eight total: four from *C. picta*, two from *C. serpentina*, one from *T. scripta*, and one from *S. odoratus*) were DNA barcoded using the Lifescanner species identification kit. Sequences obtained were identified using the nucleotide sequence BLAST (Basic local alignment search tool) option on the National Center for Biotechnology Information website (blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2 Blood Sample Preparation and Analysis

Blood was collected from a subsample of each species of turtles we captured (N = 32) representing ten painted turtles, fourteen yellow-bellied sliders, seven snapping turtles, and one musk turtle. We collected blood from every turtle with a leech present and also from turtles of the same species to use as a control to examine the relationship between external parasites and potential internal blood parasites (Leech turtles = 9; Control turtles = 23). We collected blood from the dorsal coccygeal vein in the tail using 1 ml graduated syringe (Sigma Aldrich) and 26 G needle (BD Precision Glide). We collected ~ 0.3-0.4cc of blood, to ensure the amount of blood collected was under 0.5 ml per 100 g, following Arikan and Cicek 2014^[2], yet adequate for hematocrit and slide preparation. Two blood slides were prepared per turtle using standard techniques (Perpinan *et al.* 2006, Nardini *et al.* 2013, Eatwell 2014)^[24, 22, 11]. Slides were stained in Giemsa stain (Sigma Aldrich). Two hematocrit tubes per turtle were prepared using standard micro-hematocrit 7.55 cm capillary tubes. All slides and hematocrit tube were prepared within five minutes of blood collection. Sealed capillary tubes were placed in Grafco 410E Hematocrit centrifuge and spun for 2 minutes at 12,000 rpm. Each hematocrit tube was interpreted using the include Grafco reading scale to obtain the percentage of red blood cells and averaged per individual turtle.

Stained slides were examined using a Wolfe light microscope under 400-1000x magnification. A Motic Instruments MotiCam X3 digital microscope camera was used to initially examine slides for blood cell type (Sykes and Klaphake 2008) and presence of blood parasites. For each slide, three to five images were taken from different location on the slide using the MotiConnect application. This ensured we captured multiple blood cells from each slide view, which contained on

average ~100 cells. We used a modified protocol from Fudge 2000, Stacy *et al.* 2011, and Garrido and Perez-Mellado 2013^[12, 29, 15], to identify and characterize blood cell types and presence of parasites by scanning multiple portions of each blood slide until we counted 1,000 total cells, with two slides counted per individual turtle. To count blood cell types, we used a XP-Pen StarG640 drawing tablet connected to a computer. During counts, each blood cell type was marked to ensure each individual cell was counted only once. The most representative and highest quality image was used to count blood cell types for each individual turtle using a minimum of one image per slide (two slides per turtle). If images contained less than 100 total blood cells, we used multiple images from the same slide. Percentage of blood cell types (erythrocytes, leukocytes, thrombocytes, and unknown) was averaged for individual turtles. Prevalence of internal parasites (parasitemia) was estimated by examining all slides and enumerating any potential parasite presence according to Telford 2009^[32] and Davis and Sterrett 2011^[8]. We compared hematocrit values across species using a Kruskal-Wallis test in program R after excluding the single musk turtle blood sample.

3. Results

A total of 12 turtles out of 102 turtles sampled had leeches (11.8%). Among species, snapping turtles had the highest prevalence of leeches (37.5%), followed by painted turtles (16.1%), with yellow bellied slider and musk turtle prevalence of 6.8% and 14.2%, respectively. Leeches were present on the head, carapace, and plastron for all turtles. All leeches successfully sequenced for DNA barcoding (average sequence length used for identification of 525.3) were all identified with 93.4% similarity to be *Placobdella parasitica*, a commonly encountered external aquatic chelonian parasite (Brooks *et al.* 1990)^[4], with the exception of one leech collected from a *S. odoratus*, which was confirmed to be *Helobdella robusta* with 99.1% similarity. Average leech size across turtle species was 9.78 mm total length, with a slight variation in average leech size collected from turtles, including Painted (9.6 mm), Slider (10.3 mm), Snapper (11.2 mm), and Musk (7.5 mm) turtles.

Blood cell parameters varied slightly across species from blood slide but were largely consistent (Table 1). We noted some erythrocytes contained basophilic inclusions commonly observed in Chelonians (Stacy *et al.* 2011)^[29]. However, we did observe a significant difference between species for hematocrit (Kruskal-Wallis H = 21.45, P < 0.001). The hematocrit of *C. serpentina* was higher than other species with a corresponding higher percentage of leukocytes also observed for the same species.

We detected structures consist with hemogregarine parasites and piroplasm parasites of erythrocytes with inclusions characterized by pigmented round and irregular granules and vacuoles or intra-erythrocytic signet rings morphologically similar to *Chelonoplasma* and *Sauroplasma* (McArthur *et al.* 2004, Telford 2009, Alhaboubi *et al.* 2017)^[20, 32, 1] in one musk turtle, one yellow bellied slider, and four snapping turtles (Figure 1). These consisted primarily of small intra-erythrocyte vacuoles. We did not detect any blood parasites in any painted turtles. We did not statistically compare across turtle species based on small number of turtles with blood parasites.

Table 1: Blood Parameters and parasitemia prevalence (# hemoparasitized individuals/total for turtles used in blood analysis) across species examined in this study. *n* refers to subsample used in blood analysis, *N* refers to field sample size. Table heading abbreviations for parameters estimated from blood slides are as follows: RBC [red blood cells or erythrocytes], WBC [white blood cells or leukocytes], TBC [Thrombocytes], and UBC [Unknown blood cell type].

Species	N	n	Hematocrit	RBC%	WBC%	TBC%	UBC%	Leech %	Blood Parasitemia %
<i>T. scripta</i>	44	14	16.5	91.1	4.6	3.2	1.1	6.8%	0.7%
<i>C. picta</i>	31	10	18	91.6	4.4	2.3	1.7	16.1%	0.0%
<i>C. serpentina</i>	8	7	29.9	90.2	5.5	1.8	2.5	37.5%	57.1%
<i>S. odoratus</i>	7	1	15	95.3	3.8	0.9	0.0	14.2%	100%

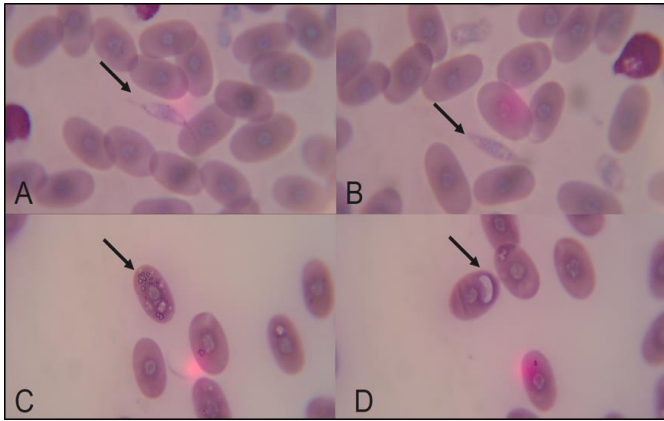


Fig 1: Blood slide examples showing intracellular parasite in *Chelydra serpentina* (A & B), and *Sauroplasma* sp. parasite (C) and hemogregarine parasite (D) from *Sternotherus odoratus*.

4. Discussion

We found overall low prevalence of parasitemia across all turtle blood smears examined with the exception of bottom dwelling turtle species. These results are similar to other studies on parasites of turtles which found large variation across species (Readel *et al.* 2008) [26] with infection levels of internal parasites low or under 2-5% (Davis and Sterrett 2011, McAllister *et al.* 2016) [8, 18]. All individual turtles sampled appeared healthy, both control and turtles with leeches. It is unclear what effect leech prevalence has on turtles, as it is unclear how long leeches remain on individual turtles and are not as high as noted in other studies (Brooks *et al.* 1990) [4]. It is possible painted and yellow bellied slider turtles, which are often seen at the study site basking, elevate their body temperature to rid external parasites (McAuliffe 1977) [19]. Consequently, the common snapping turtle and musk turtle typically are bottom dwellers which do not bask and may be more prone to parasite infection (Readel *et al.* 2008) [26]. The common snapping turtle is a larger bottom dwelling species, which may provide greater likelihood of leech colonization due to its large size. Alternatively, leeches may move between host and only be present on turtles for short periods or lower in specific seasons (Dodd 1988) [10], having a varied effect on the prevalence of internal parasites, however further research is needed.

While most hematocrit measured in our study are within the expected range found for most turtle species (Mader 1996, Warren and Jackson 2017, Savo *et al.* 2018) [17, 34, 28], we did find elevated hematocrit levels for *C. serpentina*, highlighting a potential link between presence of parasite load and hematocrit. This has been observed for hemogregarine parasites and their host two leeches *Placobdella parasitica* and *Placobdella ornata* found on *C. serpentina* (Patterson and Desser 1976) [23]. Alternatively, this could represent variation among turtle species in standard hematocrit values. Parasites such as *Sauroplasma* sp. have previously been documented in the *Podocnemis expansa*, or Arrau turtle (Picelli *et al.* 2016)

[25] with few published reports on presence in lizards, (Halla *et al.* 2014) [16], with very little known across reptiles and limited observations in turtles. Moreover, the piroplasmid parasites we observed, include *Sauroplasma* which are morphologically similar to the poorly defined *Chelonoplasma* in turtles (Frye 1991, McArthur *et al.* 2004) [13, 20] and *Serpentoplasma* (Sajjadi and Javanbakht 2017) [27], an understudied group of piroplasmid reptile parasites as their pathology is unknown (Frye *et al.* 1999) [14]. These piroplasmid parasites form small inclusions with chromatin granules often associated with a vacuole (Telford 2009) [32], and warrant further study in turtles.

5. Conclusion

This study provides important baseline information on parasites of common freshwater turtles in the piedmont of North Carolina. In addition, this study highlights a new methodology for utilizing DNA barcoding to identify external reptile parasites, which may illuminate cryptic diversity and confirm the presence of more than one species of leech at pond sites, as we identified in our study. Therefore, we recommend conservation managers augment their monitoring programs of wild and captive populations to investigate any potential cryptic biodiversity of parasites often overlooked in field studies of reptiles using DNA barcoding, along with continued monitoring of both internal and external parasites.

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