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Original Contribution

Discovery of a Novel Alveolate Pathogen Affecting Southern Leopard Frogs in Georgia: Description of the Disease and Host Effects

Andrew K. Davis,[1] Michael J. Yabsley,[1,2] M. Kevin Keel,[2] and John C. Maerz[1]

[1]*D.B. Warnell School of Forestry and Natural Resources, The University of Georgia, Athens, GA 30602, USA*
[2]*Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, The University of Georgia, Athens, GA, USA*

Abstract: In April of 2006, we observed southern leopard frog *(Rana sphenocephala)* tadpoles in a pond in northeast Georgia that were dying from an unknown pathogen. Examination of affected specimens, as well as PCR characterization, revealed that all were infected with a novel alveolate pathogen closely related to freshwater and marine eukaryotic organisms and, to a lesser degree, to members of the genus *Perkinsus*. This pathogen has been documented in numerous mortality events in anuran tadpoles in the United States, although it has not yet been named nor clearly described. We subsequently conducted a systematic survey of this and four other ponds in the same area to document the extent of the pathogen and to describe the nature of infections in leopard frog tadpoles. Of 87 live tadpoles examined, 25% were infected with the alveolate pathogen, based on visual inspection of tadpole liver tissue. Affected tadpoles frequently had enlarged abdomens, swam erratically, and could be captured by hand. All organs of infected tadpoles were infiltrated but typically to a lesser extent than the liver and kidneys, which often had hundreds of thousands of the spherical, 6-μm organisms. Infected tadpoles tended to weigh more than noninfected ones, likely due to the massive organ swelling that coincided with infections. Infected tadpoles did not differ in developmental stage from noninfected tadpoles. Infection prevalence varied widely among ponds, and in one pond, we witnessed a rapid die-off of *R. spenocephala* tadpoles during our surveys, although we did find infected metamorphic frogs. The rapid mortality we observed as well as the vast number of organisms seen in specimens suggests that this pathogen has tremendous transmission potential, and therefore deserves further monitoring and study.

Keywords: *Rana sphenocephala*, southern leopard frogs, amphibian disease, alveolate pathogen

INTRODUCTION

Infectious diseases can play important roles in wildlife populations, from influencing host genetic diversity to causing widespread declines in terrestrial and marine systems (e.g., Harvell et al., 1999; Daszak et al., 2000; Lafferty and Gerber, 2002; Altizer et al., 2003). Amphibians represent a group of organisms that have been particularly impacted by infectious diseases (Daszak et al., 1999). Indeed, reports of large-scale amphibian population declines and the potential role of emerging pathogens in these declines (e.g., Kiesecker, 2002; Lips et al., 2005; Forson and Storfer, 2006; Pounds et al., 2006) have raised awareness of their

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Correspondence to: Andrew K. Davis, e-mail: akdavis@uga.edu

importance among herpetologists and emphasized the need to understand the dynamics of host–pathogen interactions for establishing effective management strategies. From the perspective of the parasites that cause these diseases, self-replication and transmission to new hosts are their ultimate goals, and in doing so some pathogens can be extremely virulent and cause high mortality within their host population. Examples of this include *Cyathocotyle*, *Leyogonimus*, or *Sphaeridiotrema* infections in waterfowl (Huffman, 2005) and the fungus *Batrachochytrium dendrobatidis* in amphibians (e.g., Muths et al., 2003; Lips et al., 2005), although recent evidence suggests a degree of variability in host susceptibility to chytridiomycosis (Hanselmann et al., 2004; Blaustein et al., 2005). At the local population level, pathogen-related mass mortality events have been observed in many amphibian species throughout the United States, and thus far have largely been attributed to ranaviruses and chytridiomycosis (Green et al., 2002).

A recently discovered disease has now been linked to local amphibian mortality events at multiple locations in the eastern United States (Green et al., 2003). The causative agent is a protozoan that has been anecdotally described as "*Perkinsus*-like" because of its similarity to pathogens in the genus *Perkinsus*, although it phylogenetically does not fall within that genus (Green et al., 2003; this study). This agent has not yet been formally named, and while it is unclear how or when this pathogen began affecting amphibians, anecdotal reports of this disease suggest that it affects larval frogs in the genus *Rana* (Green et al., 2003). Infections with this pathogen are said to be characterized by the presence of hundreds of thousands of small, spherical organisms embedded in the tissues of the host, especially in liver tissue (Green et al., 2003), although detailed descriptions and morphology of the pathogen have not yet been published.

In April of 2006, while conducting an unrelated experiment at a pond in northeast Georgia, we observed approximately 10 *Rana sphenocephala* tadpoles that had recently died near the shore of the pond. We collected these tadpoles and conducted postmortem examinations back in the lab. Microscopic examination of organ tissues (via impression smears) revealed the presence of spherical organisms which we suspected were the alveolate pathogen described by Green et al. (2003). Diagnosis of the pathogen was later confirmed using a nested PCR protocol (see Methods) similar to one previously used for other eukaryotes (Medlin et al., 1988; Yabsley et al., 2005).

This initial discovery lead us to initiate a more systematic investigation into the scale and effect of this pathogen in *R. spenocephala* in other ponds in the vicinity of the initial pond. For the next 2 months, we collected and screened tadpoles for the pathogen (via visual inspection of liver smears) from the initial, plus four other ponds, all within 5 km of each other. Here we describe the results of these surveys, examine the effects of the pathogen on the tadpoles in terms of host tissue damage and tadpole mass, determine if tadpoles are affected early or late in development, and provide detailed descriptions of the pathogen, including phylogenetic analysis, gross pathology, and histological observations of the host.

Methods

PCR Identification and Pathogen Classification

To initially characterize the pathogen, DNA was extracted from fresh tissues and subjected to PCR using primers that amplify the 18S rRNA gene from a wide range of protozoan eukaryotic organisms. Nested PCR was conducted using primary primers 5.1 and B (Medlin et al., 1988; Yabsley et al., 2005), followed by a secondary reaction using primers KjF and 3.1 (Kjemtrup et al., 2000; Yabsley et al., 2005) as described. The amplicon was bidirectionally sequenced at the University of Georgia Sequencing and Synthesis Facility using secondary primers and the internal primer RLBHF (Gubbels et al., 1999) and aligned with related organisms from GenBank with the ClustalX program. Phylogenetic analyses were conducted using the MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 program using the neighbor-joining and minimum evolution analyses using the Kimura 2-parameter model and maximum parsimony using a close-neighbor-interchange search.

Pond Surveys

We surveyed five permanent ponds in the University of Georgia's Whitehall Forest, a 700 acre property approximately 5 km south of Athens, GA. Surveys were conducted during April and May, 2006. Surveys involved walking the edge of the pond and dipnetting at haphazard intervals until we captured tadpoles of *R. sphenocephala*. Upon capture, each tadpole was placed in a plastic container with water from the pond for transport to the lab. We continued this until at least 5–20 tadpoles were captured from each

pond. While dipnetting, we also recorded the presence of dead tadpoles near the edge of the ponds. When we first started the surveys, we initially collected several of the dead tadpoles and examined them for the alveolate pathogen using the methods described below. We subsequently found that all were infected. Thereafter, we confined our dipnetting to tadpoles that were alive.

In addition to the pond surveys and during the same time period as the surveys, we intermittently collected *R. sphenocephala* tadpoles from cattle tanks that had been placed at the edge of two of the surveyed ponds earlier in the year, and had been filled with water from the pond. In one set, the tadpoles were purposely raised in the tanks for a separate experiment, and in the other they had accidentally been placed in them when the tanks were filled with water from the pond. There were no predators introduced in either set of tanks and there was no water flow between the tanks and the ponds. Because these tadpoles ($n = 19$ for one set, 18 for the second) were not collected systematically, they were only included in our analysis of the effects of the pathogen on tadpole weight and developmental stage (below).

Tadpole Data and Visual Pathogen Screening

In the lab, all tadpoles were killed by overdose of MS-222. Each was blotted dry and weighed to the nearest 0.01 g, and their developmental stage was assigned according to Gosner (1960). Each body cavity was dissected, and a small (2 × 2 mm) piece of the liver was excised and placed on a clean microscope slide. A second slide was used to smear the liver piece across the length of the bottom slide, ensuring that the entire liver piece was flattened. This ''liver smear'' was examined immediately with a light microscope at 40× magnification for the presence or absence of the protozoan pathogen and, based on this assessment, tadpoles were assigned as infected or not infected. For a subset of infected tadpoles, we conducted histopathology to determine the extent of the tissue infiltration (below).

Histopathology of Tadpoles

Each tadpole was immersed in 10% neutral buffered formalin and allowed to fix for approximately 24–72 hours. Each entire tadpole was then cut into serial sagittal sections. The samples were then arranged in tissue cassettes and dehydrated by passage through serial baths of graded alcohol and xylene. The dehydrated samples were embedded in paraffin, and 5-µm-thick sections were stained by hematoxylin and eosin. Sections of all organs were examined for the presence of pathogens and any associated lesions.

Pathogen Morphology Measurement

On one liver smear of an infected tadpole, we measured the morphological characteristics of the protozoan organisms. For this, we examined the slide via light microscopy under 1000× oil immersion and selected a field of view with large numbers of organisms (>150). We obtained a digital image of this field using a Canon (Lake Success, NY) Powershot G5 digital camera mounted on the microscope. This image was imported into an image analysis program (Fovea Pro, Asheville, NC http://www.reindeergraphics.com), and 50 random organisms were manually outlined and their dimensions measured, after calibration of the software using an image of a stage micrometer. The features measured included length (length of the longest axis, in microns), breadth (length of the axis perpendicular to the longest), two-dimensional surface area (in square microns) and aspect ratio, a measure of roundness, or the ratio of length to breadth.

Data Analysis

As part of this project, we wished to determine the effect of the alveolate infection on tadpole weights. However, tadpole weights are directly related to their developmental stage (Gosner, 1960), so it was necessary to include this variable in our examination of weights. We therefore used analysis-of-covariance to examine the effect of infection (included as a dichotomous variable), and developmental stage (as a continuous variable), and their interaction on log-transformed tadpole weights. For this analysis, we used the combined tadpole data from all locations (i.e., including the experimental tanks). We also wished to know if the pathogen affects tadpoles of certain developmental stages. For this, we used a *t*-test to compare the average development stage of infected and noninfected tadpoles. These analyses were performed using Statistica software (Statistica, 2003).

Results

Pathogen Identification/Taxonomy

Near full-length 18S rRNA gene sequence (1583 bp; GenBank EF675616) of the pathogen from *R. sphenocephala*

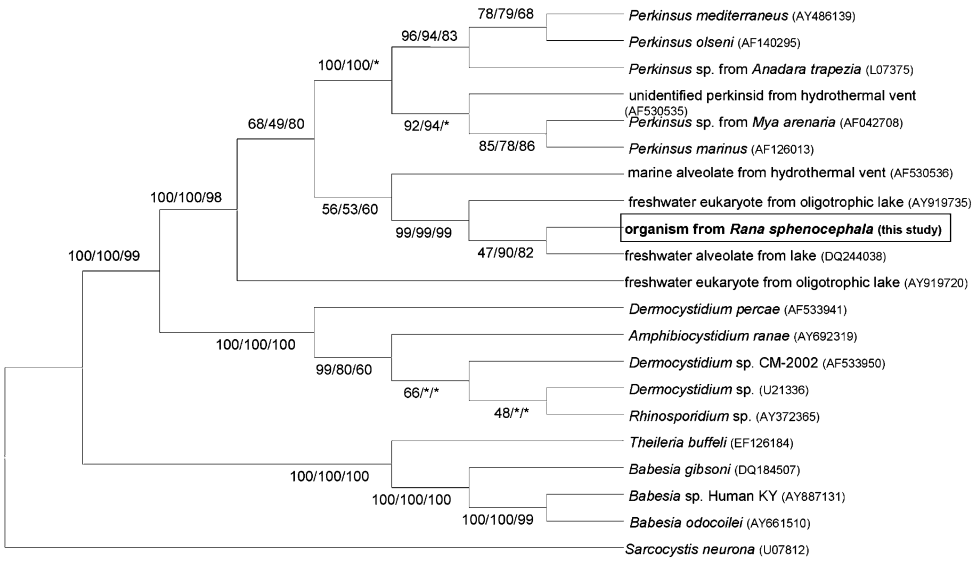

Figure 1. Phylogenetic tree inferred by comparisons of 18S rRNA gene sequences among the pathogen from this study (highlighted by box), related aquatic eukaryotic organisms, *Perkinsus* spp., morphologically similar frog pathogens, and various protozoan parasites. The percentages of 500 bootstrap replications in which groupings appeared are noted above the branches for neighbor-joining, minimum evolution, and maximum parsimony, respectively.

shared the highest identity (88.9%–93.4%) with various uncultured freshwater and marine organisms previously detected during environmental surveys of lakes and hydrothermal vents (Fig. 1). Moreover, alignment of the 1583 bp of the 18S rRNA gene sequence of the organism with related organisms in GenBank, various ranid pathogens morphologically similar to *Perkinsus* (e.g., *Dermocystidium*, *Rhinosporidium*, and *Amphibiocystidium*), and some protozoan parasites (*Babesia*, *Theileria*, and *Sarcocystis*) as an outgroup, resulted in an alignment of 1670 bp in length, of which 1073 characters were invariant, 136 variable characters were parsimony uninformative, and 461 variable characters were parsimony informative.

All three analyses (neighbor-joining, minimum evolution, and maximum parsimony) produced trees with similar topology (Fig. 1) and indicated that the pathogen in this study was related to, but distinct from, eukaryotes previously detected in freshwater lakes. All three analyses placed this clade as a sister clade to the genus *Perkinsus* (although bootstrap values were generally low, 49%–80%). Maximum parsimony analysis failed to resolve all divisions within the genus *Perkinsus*, and both minimum evolution and maximum parsimony failed to resolve relationships in the *Dermocystidium* and *Rhinosporidium* group; however, none of these effected the placement of the pathogen in the group of freshwater eukaryotes.

Infection Characteristics/Morphology

Infected tadpoles appeared bloated and were nearly all lethargic in water. Internally, the organisms were present in all organs examined, although more organisms were noted in the livers than other organs. In fact, infected tadpoles had greatly distended whitish-colored livers (up to 3× normal size) that were easily distinguished from normal livers of uninfected tadpole [A. Davis, personal observation]. Interestingly, while examining the liver tissue for the alveolate pathogen, we noted that some livers contained varying numbers of larval nematodes (Fig. 2A). We later determined that these were larval nematodes in the order Spirurida, based on morphological characteristics, but their larval nature has prevented us from further classification.

In tissue section slides, the livers of affected larvae were typically obliterated by the pathogen, which appeared bright-purple and spherical (Fig. 2A). These spheres were loosely arranged along the remains of the hepatic cords and some were in aggregates that seemed to be limited by the cell membranes of dead hepatocytes. Large veins and some large bile ducts remained, but all other hepatic tissue had been replaced by the pathogen. The organisms also greatly expanded the renal interstitium, and most renal tubules were necrotic and/or attenuated. All other organs were infiltrated by the organism but typically to a lesser extent than the liver and kidneys. The organisms did not invade epithelium of the skin or gastrointestinal tracts, but they were abundant in those organs in the subcutaneous connective tissue and the lamina propria, respectively. A small number of nematode larvae (mentioned previously) were present in bile ducts.

The results of our morphological measurements of the pathogen, as seen in liver smears, are shown in Table 1. From the 50 organisms measured, we calculated an average length of 6.2 μm, and this value only ranged from 5.6 to 6.8 μm. Most were approximately round (in two-dimen-

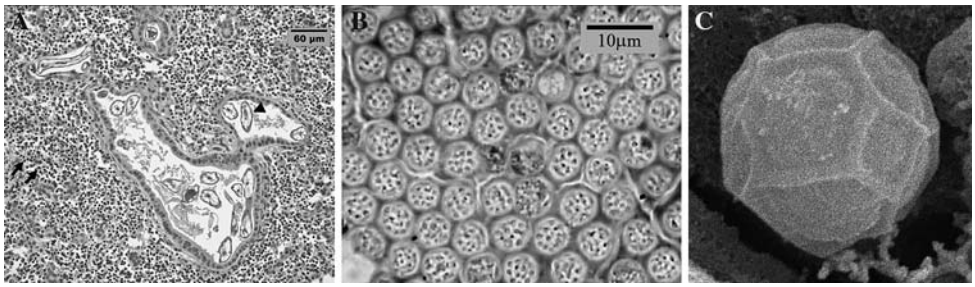

Figure 2. Photomicrograph of *R. sphenocephala* liver showing **(A)** alveolate pathogens present throughout the liver tissue (arrows) and several cross-sections of larval nematodes located within bile ducts (arrowhead); **(B)** alveolate pathogens from liver smear under 1000× magnification; and **(C)** scanning electron microscope image of an alveolate pathogen seen in liver tissue.

Table 1. Summary of Pathogen Measurements[a]

	Average	SD	Minimum	Maximum
Area (μm^2)	25.8	2.7	19.6	32.5
Length (µm)	6.2	0.3	5.6	6.8
Breadth (µm)	5.5	0.4	4.5	6.3
Aspect ratio	1.13	0.07	1.05	1.35

[a]A total of 50 organisms from a *R. spenocephala* tadpole liver smear (i.e., a cytologic prep) were measured via image analysis (see Methods). Area is the two-dimensional surface area of the spherical organisms as seen in the slide preparation. Length refers to the length of the longest axis, breadth is the length of the side perpendicular to the longest axis, and aspect ratio is the ratio of length and breadth.

sional space), as shown by the average aspect ratio of 1.13, and this value did not vary greatly (i.e., SD = 0.07). Thus, the overall impression we gained from our microscopic measurements was that the organism was noticeably uniform in dimension (Fig. 2B). Further, scanning electron microscopy revealed a simple polyhedral surface (Fig. 2C).

Pond Survey Results

A total of 87 *R. sphenocephala* tadpoles were captured from the five ponds, and these tadpoles ranged from Gosner stage 27 to 39. Of these individuals, 22 (25%) were infected with the alveolate pathogen. The prevalence of the pathogen varied between ponds, from 55% in pond 1 to 0% in pond 5 (Fig. 3). Importantly, an observation not necessarily apparent from Figure 3 is that, in pond 1, we witnessed a mass mortality event that occurred during our tadpole collecting. On the day we initially discovered the dead tadpoles in pond 1 (which lead to this investigation), we visually counted over 500 live, seemingly normal, *R. spenocephala* tadpoles near the shore. On return visits to this pond, we

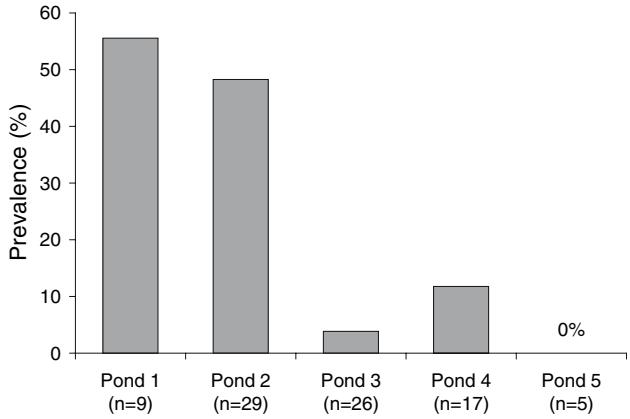

Figure 3. Prevalence of pathogen infections in live *R. sphenocephala* tadpoles from the five ponds surveyed in this study. A total of 87 tadpoles were captured from all five ponds.

observed increased numbers of dead tadpoles (viewed from the pond edge), and fewer live tadpoles, until our final visits revealed no visible (live) leopard frog tadpoles. The overall disease prevalence of 55% in this pond does not reflect this outbreak, since we confined our dipnetting surveys to live individuals. We did not observe any such mass mortality at the four other ponds during our surveys, even in the ponds where we collected infected tadpoles.

Effects on Tadpole Mass and Stage Variation

Our analysis of covariance (weight = infection + developmental stage + infection × stage) using the data from the pond-captured tadpoles, as well as those intermittently captured in mesocosms ($n = 124$), provided no support for the interaction term in explaining tadpole weight ($F_{1,123} = 0.74$, $P = 0.391$). Results from a simplified model with main effects only showed an expected effect of developmental stage ($F_{1,123} = 262.23$, $P < 0.001$) and a nearly significant effect of infection ($F_{1,123} = 3.23$, $P = 0.075$), such that

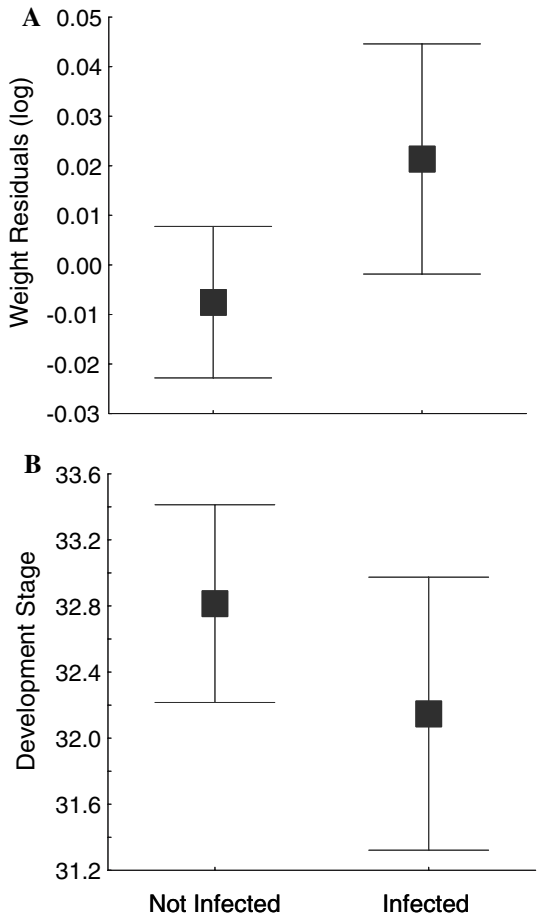

Figure 4. **A:** Effect of infection on tadpole weight residuals (i.e., residuals of a linear regression of log-transformed weight vs. development stage) of all *R. sphenocephala* tadpoles from this study (n = 124). **B:** Average developmental stage of tadpoles infected and not infected with the alveolate pathogen. Shown are the mean and 95% CI for each group.

weight increased with developmental stage and tended to be *higher* among infected tadpoles (Fig. 4A). We found no significant difference in development stage between infected and noninfected tadpoles (t = 1.09, P = 0.277; Fig. 4B).

Observations of Metamorphic Frogs

Although in this study we focused mainly on infections of tadpoles, it is important to point out here that, in the weeks following our surveys, we observed four cases of nearly completed or completely metamorphosed *R. spenocephala* that were infected, based on examinations of liver smears. However, all of these individuals were raised (accidentally) in experimental cattle tanks that were in one of the ponds with a high prevalence (pond 1).

Discussion

The pathogen we discovered at our field site in northeast Georgia and describe here has been diagnosed in other outbreaks of *Rana* species in Florida, Mississippi, Virginia, New Hampshire, and Minnesota (Green et al., 2003), although in these outbreaks the pathogen has thus far been referred to as a "*Perkinsus*-like" organism. In this study, we have refrained from using this term since our phylogenetic analysis of the pathogen showed that it is clearly not a member of the genus *Perkinsus* (Fig. 1), but is more closely related to several other freshwater eukaryotes, none of which have been called "*Perkinsus*-like." Regardless of the name, however, we point out that there currently is little published information about the nature of this disease, including its mode of transmission, range of host species affected, and geographic distribution. While the results of the current study do not address these questions, it is an important first step by providing baseline information about the physical signs of infection in tadpoles and establishing a simple methodology for screening specimens in future work. Further, our descriptions of the pathogen and associated disease should prove useful to other researchers who encounter this disease.

Prevalence of Infections

From our pond surveys, we found a highly variable prevalence of the pathogen (Fig. 3). Moreover, despite the fact that several ponds had infected individuals, in only one pond did we witness a mass mortality event. At this outbreak pond, we observed a gradual buildup of carcasses over the course of several weeks from when we found the first dead individual, until there were few live *R. sphenocephala* tadpoles seen in this pond. This observation, plus the shear number of organisms we observed within the tissues of each infected individual (on the order of hundreds of thousands), speaks to the tremendous transmission potential of this pathogen. However, given that we only witnessed a mass mortality event in one of the five ponds emphasizes the need for a better understanding of the mechanism of transmission and host susceptibility. Moreover, this study highlights the importance of continued monitoring and study of this disease which appears to be widespread across the eastern United States (Green et al., 2003).

Importantly, metamorphic *R. sphenocephala* that were infected with the alveolate pathogen were observed, suggesting that the parasite could be naturally transported to

unaffected ponds by movement of recently metamorphosed frogs. However, it may be that infected individuals do not make it to this stage in natural settings because they are predated when infected. Our cases of infected metamorphs were all reared (accidentally) in experimental cattle tanks that were sitting in the outbreak pond (but in an impermeable container) and in which there were no predators. In the actual pond, we made several observations of tadpoles swimming lethargically, enough so that we were able to capture them by hand, and we later confirmed that these individuals were infected. Indeed, lethargy is a common effect of infection in animals (e.g., Hotchkiss et al., 2005), which can lead to increased predation (Lindström et al., 2003).

An unexpected finding in this study was that tadpoles infected with this pathogen tended to weigh more than noninfected individuals (Fig. 4A). While initially surprising, we suspect that this was most likely due to the fact that the livers of infected tadpoles were at least twice the size of normal livers, which often caused the entire tadpole body to appear bloated when viewed from above or below. Our examinations of other organs besides the livers of infected tadpoles revealed invasions in these as well. This result may have implications for other research projects on *R. spenocephala* tadpoles, where the mass or growth of individuals is used as an indicator of tadpole performance. Researchers should make every effort to identify this infection in their tadpoles to minimize the risk of biasing conclusions drawn from data based on tadpole weights.

Larval Nematodes

We note here that while screening liver tissue smears for the alveolate pathogen, we found that 68% of all tadpoles had larval nematodes (in the order Spirurida) within the liver tissue (Fig. 2A). Furthermore, there was a great range in the degree of nematode infections, with some individuals having over 100 worms in the small liver section we examined. While this finding was tangential to our study of the protozoan agent, it was nonetheless interesting since we do not know of other reports of larval nematodes in liver tissue of *R. sphenocephala*. Various species of spirurid larvae (e.g., *Spiroxys contortus*) have been reported from frogs and tadpoles; however, none matched the location and morphology of the nematodes observed in the present study (Hedrick, 1935; Brandt, 1936; Joy et al., 1996). One previous study reported numerous spirurid larvae in the livers of *R. palustris* tadpoles from North Carolina, but no morphologic characters for these nematodes were provided (Murphy, 1965).

Conclusions

The pathogen described here is an unnamed organism that appears related to uncultured eukaryotes detected from freshwater lake and marine hydrothermal vent water samples. This alveolate pathogen caused a localized outbreak in northeast Georgia that resulted in near-complete mortality of the host species examined *(R. sphenocephala)* within one pond, and was found in high to low numbers in other ponds in the immediate vicinity. Infected tadpoles tended to be lethargic, often appearing bloated, and examination of tissues revealed the presence of hundreds of thousands of small (6.2 μm) spherical organisms in most organs but more so in livers. As this pathogen appears to have only recently been found in freshwater amphibians, we suggest that future research be directed at the mode of transmission, towards a comprehensive list of amphibian species known to be susceptible, and documentation of the geographic range in North America. Given the current research focus on pathogen-related amphibian declines (e.g., Kiesecker, 2002; Hanselmann et al., 2004; Harris et al., 2006; Lampo et al., 2006), this emerging disease should be added to the suite of pathogens of concern and should be monitored closely.

Acknowledgments

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