

## Original Contribution

# New Findings from an Old Pathogen: Intraerythrocytic Bacteria (Family Anaplasmatacea) in Red-Backed Salamanders *Plethodon cinereus*

Andrew K. Davis,<sup>1</sup> Jayna L. DeVore,<sup>1</sup> Joseph R. Milanovich,<sup>1</sup> Kristen Cecala,<sup>1</sup> John C. Maerz,<sup>1</sup> and Michael J. Yabsley<sup>1,2</sup>

<sup>1</sup>D.B. Warnell School of Forestry and Natural Resources, The University of Georgia, Athens, GA 30602

<sup>2</sup>Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602

**Abstract:** During a recent study of red-backed salamanders (*Plethodon cinereus*), we discovered an intraerythrocytic organism typified by violet-staining, intracellular inclusions, consistent with descriptions of *Cytamoeba* or *Aegyptianella* (bacteria). Here we characterize its taxonomic status using molecular techniques and ask basic questions about its nature. Blood smears from 102 salamanders were examined from Pennsylvania, New York, and Virginia to determine prevalence, and whole blood from several infected animals was tested using a PCR which targets the 16S rRNA gene of bacteria. Phylogenetic analysis of partial 16S rRNA gene sequence (1201 bp) indicated this organism was in the order Rickettsiales and is likely a member of the family Anaplasmatacea. The organism differed from currently described taxa and was clearly differentiated from *Aegyptianella pullorum* of birds and “*Candidatus Hemobacterium ranarum*” (formally *A. ranarum*) of frogs. Of all salamanders, 17 (16.7%) were infected and these were significantly larger (snout–vent length) and had higher body condition scores than uninfected ones, and males were more likely to be infected than females. Erythrocytes affected by the pathogen were 5% larger than unaffected ones, but otherwise similar in morphology. Infected animals tended to have a greater number of circulating white blood cells, based on estimates from smears, indicating a nonspecific response to the pathogen by the innate immune system. Given its phylogenetic position, this pathogen is likely transmitted by an arthropod vector, and the male-biased prevalence strongly implicates trombiculid mites, which also live in leaf litter and affect male salamanders more so than females.

**Key words:** Anaplasmatacea, *Cytamoeba*, *Aegyptianella*, red-backed salamander, *Plethodon cinereus*

## INTRODUCTION

Amphibians are hosts to a large number of parasites, some of which cause mass mortalities and population declines

(e.g., Daszak et al., 1999; Muths et al., 2003; Davis et al., 2007), while others, such as blood parasites, appear to have little outward effect on the hosts (Barta and Dessler, 1984; Werner, 1993; Stenberg and Bowerman, 2008). For over a century, researchers studying blood parasites of ectothermic animals have reported the presence of strange, globular, violet-staining, intracellular inclusions in the

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

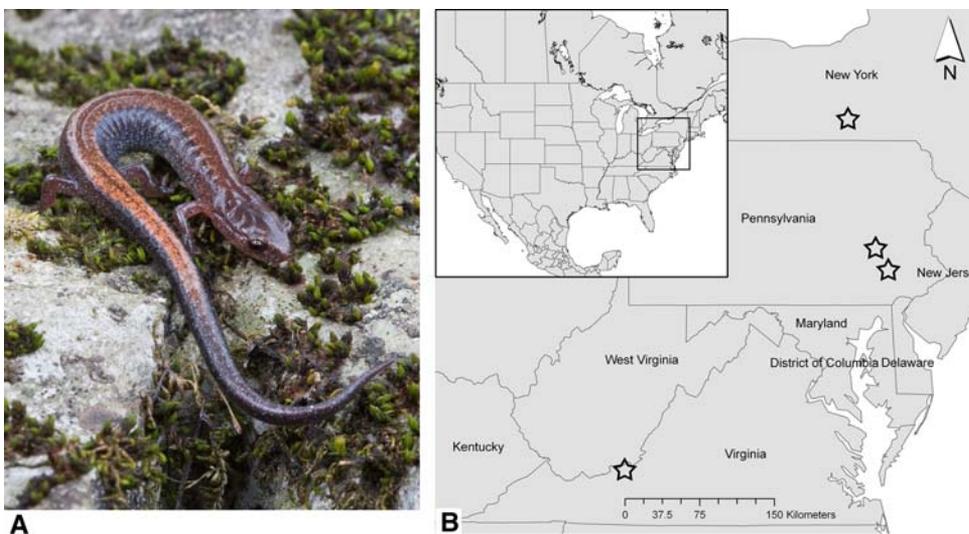
erythrocytes of a variety of frogs, salamanders, and even some turtles (Hegner, 1921; Brumpt and Lavier, 1935; Rankin, 1937; Lehmann, 1961; McAllister et al., 1995), with no reports of negative effects on the hosts. These parasites were first described by Labbé (1894) and originally called *Cytamoeba bacterifera*. They appear as a violet or purple membrane-bound structure within red blood cells, and have rod-shaped granules visible inside some inclusions. Initially, the parasite was believed to be an amoeba (Hegner, 1921; Lehmann, 1961, 1964), but ultrastructural studies have since demonstrated the “organism” is a membrane-bound, violet-staining valvule containing numerous bacteria (Desser, 1987).

The taxonomic status of this pathogen is not clear. Morphologically, the organisms and inclusion are consistent with members of the family Anaplasmataceae (order Rickettsiales), for which all known species are all obligate, intracellular bacteria transmitted by vectors (Rikihisa, 2006). At one point this organism of frogs, as well as other morphologically similar organisms (*Tunetella emydis*) from turtles, were all transferred to the genus *Aegyptianella*, due to similarities with *A. pullorum* of birds (Gothe and Kreier, 1978; Desser, 1987). Recently though, molecular characterization of an *Aegyptianella*-like organism from a bullfrog (*Rana catesbeiana*) from Canada indicated that the bacteria were actually members of the family Flavobacteriaceae (Zhang and Rikihisa, 2004). Clearly, there exist large gaps in our knowledge of these intracellular organisms, as evidenced by their uncertain taxonomic status, but also with respect to their ecology.

Perhaps the most well-known aspect of this organism is its geographic distribution. This organism, at least organisms

consistent with *Aegyptianella*, appears to have a wide host range distributed across a wide geographic area. Early studies cited by Hegner (1921) reported a parasite meeting this description in amphibians from Africa and Brazil. Werner (1993) and Chutmongkonkul et al. (2006) reported infected amphibians from China and Thailand, while Desser (1987) and Barta and Desser (1984) describe the organism in frogs from Ontario, Canada. Desser and Barta (1989) also report it from ranid frogs in Corsica. It has been reported from a number of places in the United States, including in salamanders from California (Lehmann, 1961, 1964), Arkansas and Oklahoma (McAllister et al., 1993, 1995), and North Carolina (Rankin, 1937). Finally, Babudieri (1972) reported a violet-staining, “mycoplasma-like” inclusion in red blood cells of the Italian plethodontid salamander, *Hydromantes italicus*, but the descriptions of the organism are consistent with the *Aegyptianella*-like organism.

During a recent hematological investigation of red blood cells from plethodontid salamanders (red-backed salamander, *Plethodon cinereus*; Figure 1A) in Pennsylvania (Davis et al., 2009), numerous violet-staining inclusions were observed in certain individuals which we suspected were the *Aegyptianella* parasites reported in frogs (Desser, 1987; Desser and Barta, 1989) and salamanders (McAllister et al., 1993, 1995). Given the knowledge gaps regarding this organism, we conducted the present study in an effort to further understand this parasite. Our goals in this work were to: 1) report its prevalence in two Pennsylvania sites, as well as in sites in New York and Virginia; 2) report results from our own phylogenetic analyses of the organism; 3) evaluate whether there are sex- or size-related patterns of infection rates; 4) determine the effects of the



**Figure 1.** Photograph of typical red-backed salamander (A) and map of collecting sites for this study. (B) From north to south, sites were Arnot Forest, NY, Hawk Mountain Sanctuary, PA, Hopewell Furnace National Historical Park, PA, and Mountain Lake Biological Station, VA. Salamander photo taken by Bill Peterman in Jefferson Co, IN.

parasite on salamander body condition; 5) compare cellular morphology of affected and unaffected erythrocytes; and 6) examine associations between the presence of the parasite and the host's innate immune system (via white blood cell counts).

## METHODS

### Collecting Salamanders

Salamanders were hand-captured from four sites: Hawk Mountain Sanctuary, PA (HMS), a hillslope site at ~1200 ft elevation on the eastern edge of the Valley and Ridge physiographic region; Hopewell Furnace National Historic Park, PA (HFP), a low aspect site at ~400 ft elevation in the Piedmont Lowlands; Arnot Forest, NY (ARN) at ~1100 ft elevation in the southern New York section of the Appalachian Plateau; and Mountain Lake Biological Station, VA (MLB) at ~3,800 ft elevation also in the Appalachian Plateau (Figure 1B). HMS and HFP were sampled in October 2007, ARN in May 2008, and MLB in June 2008. Salamanders were collected by hand from under rocks and logs, and held in containers with moist leaf litter in coolers until processed (all within 5 days of capture).

### Processing Salamanders

In the lab, processing and obtaining blood samples from salamanders generally followed established procedures (Davis and Maerz, 2008a, b; Davis et al., 2009). Briefly, individuals were anesthetized by wrapping in a cloth soaked with a 1% solution of buffered MS-222. Thereafter, we recorded the snout-vent length (SVL) of each individual, its weight was obtained with an electronic balance, and it was decapitated. Blood from the exposed heart region was dripped onto a clean microscope slide, and a second slide was used to smear the blood across the first. For a subset of individuals, an additional sample of whole blood was collected and stored at 4°C. Finally, the body cavity was dissected open and sex determined based on reproductive organs. Three individuals were juveniles and were not assigned to sex.

### Examining Blood Smears

All smears were air-dried and stained with Giemsa. They were then examined at 200× with a standard light microscope for the presence of intraerythrocytic blood

parasites. Slides were scanned in a standard zigzag fashion, covering all parts of the smear, until at least 75 fields of view were examined. When infected salamanders were identified, the severity of infection was quantified by counting the number of infected erythrocytes per field of view from 100 fields at 1000× (oil). At this magnification, fields of view in this study had an average of 30 erythrocytes ( $\pm 6$  SD, based on examination of 50 fields from five salamanders), and with this value the number of infected cells per 100 erythrocytes was calculated as the infection severity score. In addition, a running tally of white blood cells were counted in each field of view examined (at 1000×) following Davis and Maerz (2008a, b). This parameter was considered an index of the innate immune activity of each individual (Forson and Storfer, 2006).

### Erythrocyte Evaluation and Measurement

In a subset of infected individuals ( $n = 5$ ), we compared basic erythrocyte morphology (i.e., size and shape) of affected and unaffected cells. Using image analysis (Davis, 2008), we measured cell area (in  $\mu^2$ ) as a measure of size, and aspect ratio (length/width) as a measure of shape, in 25 infected and 25 uninfected erythrocytes from each salamander, for a total of 125 infected and 125 noninfected erythrocytes. We also made qualitative observations of the staining characteristics, nuclear position, and overall appearance of affected and unaffected cells.

### Molecular Analyses

For PCR, DNA was extracted from 10  $\mu$ l of whole blood of two blood-smear positive salamanders using the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol. Amplification of bacterial 16S rRNA gene was conducted as described (Persing, 1996). Briefly, 5  $\mu$ l of DNA was added to 20  $\mu$ l of a master mix containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.4 mM each dNTP (Promega, Madison, WI), 2 units Taq DNA Polymerase (Promega), and 0.8  $\mu$ M of primers 8F and 1448R. Samples were incubated at 94°C for 2 minutes before 40 cycles of 94°C for 45 seconds, 45 for 2 minutes, and 72 for 2 minutes. A final extension of 72 for 5 minutes was conducted. Amplified products were separated in 2% agarose gels, stained with ethidium bromide, and visualized with UV light. Amplicons of both samples were purified with a Gel Extraction Kit (Qiagen, Chatsworth, CA) and

independently bi-directionally sequenced at Clemson University Molecular Genomics Institute (Clemson, SC).

Sequences obtained from the two infected red-backed salamanders in this study and from other bacteria stored in GenBank were aligned using the multisequence alignment ClustalX program (Thompson et al., 1994). Phylogenetic analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 3 program (Kumar et al., 2004) using the neighbor-joining and minimum evolution algorithms using the Kimura 2-parameter model and maximum parsimony using a heuristic search.

## Data Analysis

We used logistic regression analysis to determine factors influencing infection with the rickettsial agent. Using all salamanders of known sex ( $n = 102$ ), the analysis included gender, collecting site, and body length (SVL) as independent variables, along with all two-way interaction terms. Infection (yes or no) was the response variable for this analysis. Statistical examination of infection severity was hindered by the small number of infected individuals (17, see Results). Thus, severity of infection was compared among collecting sites using one-way analysis-of-variance, using only the infected individuals. For this analysis, we pooled the two collecting sites in Pennsylvania because only one parasitized individual was found at the HFP site (see Results). Infection severity was compared between sexes using a two-sample  $t$ -test. To examine the effects of infection on general salamander health, we created a “body condition” variable by retaining the residuals of a linear regression of mass on body length (Todd and Davis, 2007), and this was then used as the response variable in a three-way ANOVA where sex, site, and infection (yes, no) were explanatory variables. All two-way interactions were also included in this model.

To compare erythrocyte morphology between affected and unaffected cells of the subset of infected salamanders (5), we used two-way ANOVA (this time with the erythrocyte as the unit of replication), where cell area or aspect ratio were the response variables, cell infection was an explanatory variable (infected, not infected), and salamander ID was a blocking variable, which accounted for natural variation in cellular morphology among salamanders (Davis et al., 2009).

For examination of the effects of the pathogen on innate immunity, the counts of white blood cells per field of view were transformed into number of cells per 2000

erythrocytes, based on the average number of erythrocytes per field of view (30). This variable was then used in comparisons of white blood cell numbers between all infected (17) and noninfected (85) salamanders (two-sample  $t$ -test), as well as for relating white blood cell numbers to levels of infection (Pearson correlation) in infected salamanders. Analyses were conducted using Statistica 6.1 software (Statistica, 2003).

## RESULTS

---

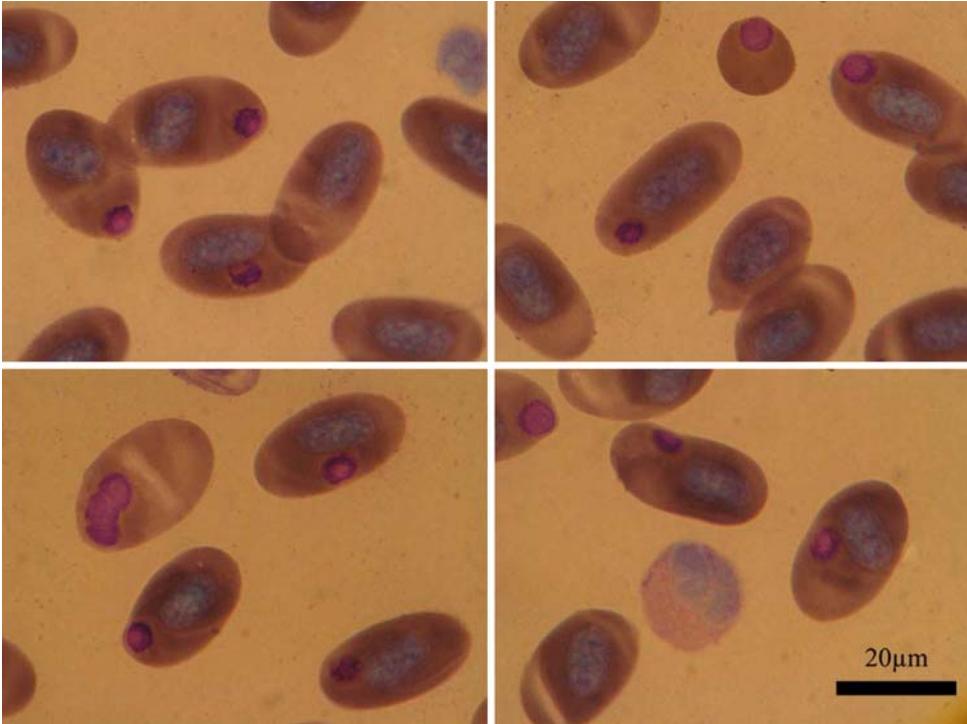
### General Observations

With Giemsa-staining, the intracellular organism appeared as a violet-colored inclusion within the cytoplasm of erythrocytes, usually (but not always) situated in a polar region of the cell (Figure 2). Cells with more than one inclusion were also observed. Measurements via image analysis (following Davis and Holcomb, 2008) of 32 haphazardly selected inclusions indicated they were 13.0  $\mu\text{m}$  in length on average ( $\pm 3.2$  SD), and 10.2  $\mu\text{m}$  in width ( $\pm 2.0$  SD). Inclusions were also observed within anucleated cells (Figure 2), which are common in Plethodontid salamanders (Villolobos et al., 1988).

### Molecular Analysis

The sequences obtained from two infected salamander blood samples were identical and 1201 bp in length. Alignment of the 16S rRNA gene sequence of this salamander Rickettsial agent with related bacteria resulted in an alignment 1287 bp in length, of which, 511 of 627 variable characters were parsimony informative. Neighbor joining, minimum evolution, and maximum parsimony analyses provided phylogenetic trees of similar topology, and all have high support for the inclusion of the red-backed salamander agent in the order Rickettsiales and family Anaplasmataceae in a clade separate from currently designated taxa (Figure 3). The only discrepancy in the trees was the placement of the “*C. Midichloria mitochondrii*” clade. Neighbor joining and minimum evolution analyses indicated that this group was in a sister clade to the *Rickettsial/Orientia* clade, while maximum parsimony analysis indicated it was ancestral to the Anaplasmataceae, as has been previously indicated (Sassera et al., 2006).

In our initial molecular analysis, we excluded *A. pullorum* because the only available sequence from this species was 573 bp in length. Including *A. pullorum* and deleting



**Figure 2.** Photomicrographs of the intraerythrocytic rickettsial agent in red-backed salamanders in this study. The parasite was occasionally observed in anucleated cells (upper right, lower left). Lower right panel shows a white blood cell (neutrophil).

nonoverlapping bases resulted in an alignment of 625 bp, of which, 311 were variable and 246 were parsimony informative. All three analyses placed the red-backed salamander (RBS) rickettsial organism in the same position, but the relationships had lower bootstrap support compared with the analyses using the longer sequence. These analyses did indicate that *A. pullorum* was most closely related to the *Anaplasma* spp., as previously reported (Rikihisa et al., 2003), and that the salamander rickettsial agent was only distantly related to *A. pullorum*.

### Prevalence

Of the 102 salamanders of known age examined in this study, 17 (16.7%) were infected with the rickettsial agent (Table 1). Prevalence within collecting sites varied significantly ( $P = 0.028$ ; Table 2) with the highest number of infected individuals (32%) found in the New York site. Comparing prevalence between males and females revealed that males were much more likely to be infected than females (25.9% vs. 6.3% overall; Table 1), and this sex-related difference was significant ( $P = 0.005$ ; Table 2). There was also a significant effect of body size ( $P = 0.018$ ; Table 2) on the probability of infection, as infected salamanders tended to be larger than uninfected ones (Figure 4A), although this trend was more apparent in

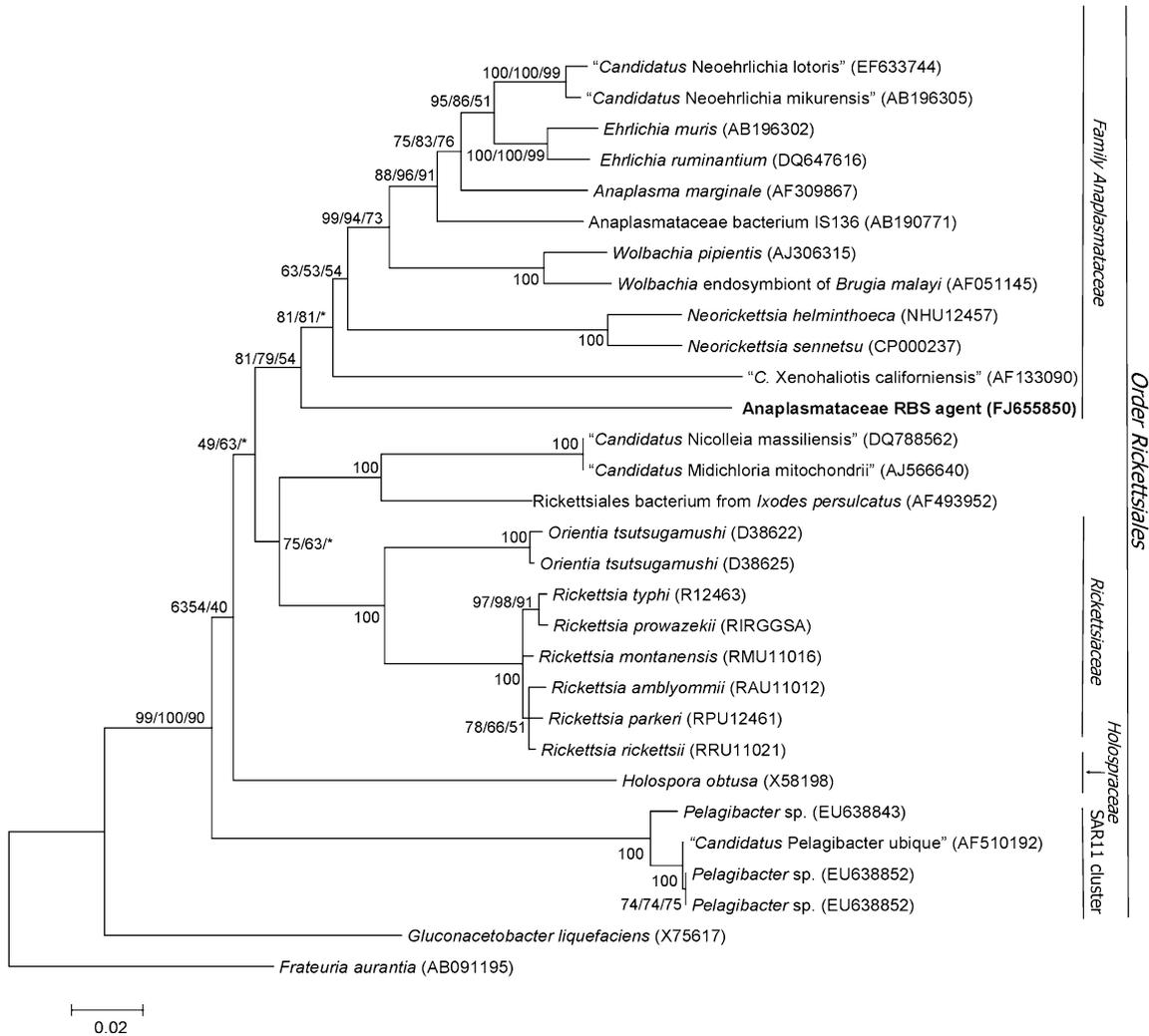
males than females. We point out that this trend was significant even after the effect of sex was accounted for in the model (Table 2).

### Infection Severity

Of the 17 infected individuals, comparison of infection severity across the New York, Pennsylvania (pooled), and Virginia sites revealed no significant variation ( $F_{2,16} = 0.97$ ,  $P = 0.403$ ). There was also no difference in average infection severity between male and female salamanders ( $t = -0.48$ ,  $P = 0.660$ ). On average, 5.2% of erythrocytes ( $\pm 5.3$  SD) were infected across all infected salamanders in this study, with severity levels ranging from 0.03% to 17.0% of erythrocytes.

### Effects of Infection on Body Condition

There was a significant effect of parasitism on salamander body condition ( $P = 0.050$ ; Table 3), though not in the direction we would have predicted for a typical parasite infection. Infected salamanders had *higher* body condition score than noninfected salamanders, and this was true for both males and females (Figure 4B). Indeed, the nonsignificant interaction of sex\*parasite in the ANOVA model (Table 3) indicates this effect was similar for both sexes.



**Figure 3.** Phylogenetic tree inferred by comparison of partial 16S rRNA gene sequences of the red-backed salamander (RBS) rickettsial agent from this study (bold text) and related bacteria. The percentages of 500 bootstrap replications in which groupings appeared are noted above the branches for neighbor-joining analysis, minimum evolution, and maximum parsimony, respectively.

**Table 1.** Prevalence (%) of the Intraerythrocytic Rickettsial Agent across All Sites and Gender Groups<sup>a</sup>

Site	Females	(n)	Males	(n)	Both sexes	(n)
ARN	22.2	(9)	37.5	(16)	32.0	(25)
HMS	0.0	(15)	25.0	(12)	11.1	(27)
HFP	0.0	(11)	6.3	(16)	3.7	(27)
MLB	7.7	(13)	40.0	(10)	21.7	(23)
All Sites	6.3	(48)	25.9	(54)	16.7	(102)

ARN, Arnot Forest, NY; HMS, Hawk Mountain Sanctuary, PA; HFP, Hopewell Furnace National Historic Park, PA; MLB, Mountain Lake Biological Station, VA.

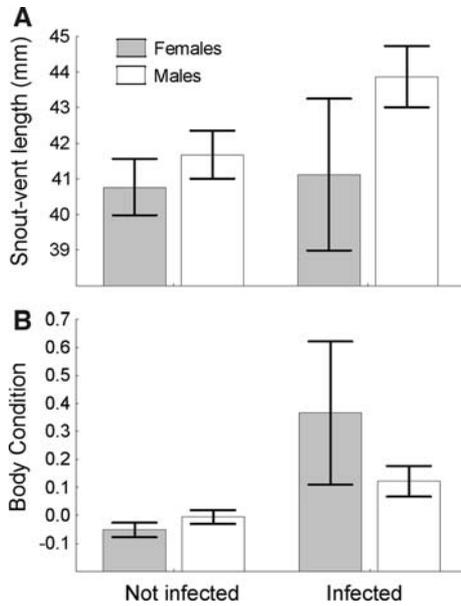
<sup>a</sup>Three salamanders were not assigned to sex because of juvenile status and are not included in this table.

**Table 2.** Results of Logistic Regression Analysis Examining Factors Influencing Infection with the Intraerythrocytic Rickettsial Agent, Using All Salamanders of Known Sex<sup>a</sup>

Variable	df	Log-likelihood	$\chi^2$	P
Site	3	-41.41	9.09	0.028
Sex	1	-37.48	7.85	0.005
SVL	1	-34.68	5.56	0.018
Site*Sex	2	-33.24	2.87	0.237
Site*SVL	3	-33.08	0.34	0.952
Sex*SVL	1	-32.98	0.19	0.660

SVL, snout-vent length.

<sup>a</sup>n = 102.



**Figure 4.** Average (A) snout-vent length ( $\pm 1$  SE) and (B) body condition of salamanders with ( $n = 17$ ) and without ( $n = 88$ ) the rickettsial agent, grouped by sexes.

**Table 3.** Results of ANOVA Examining Factors Influencing Salamander Body Condition<sup>a</sup>

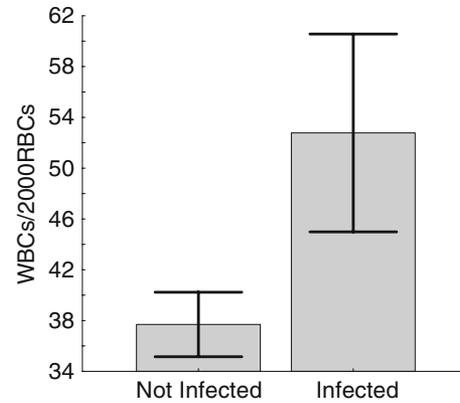
Variable	df	MS	F	P
Site	3	0.26	13.61	<0.001
Sex	1	0.01	0.38	0.541
Parasite	1	0.07	3.92	0.050
Site*Sex	3	0.06	3.36	0.022
Site*Parasite	3	0.02	1.19	0.318
Sex*Parasite	1	0.04	2.05	0.156
Error	89	0.02		
Total	101			

SVL, snout-vent length.

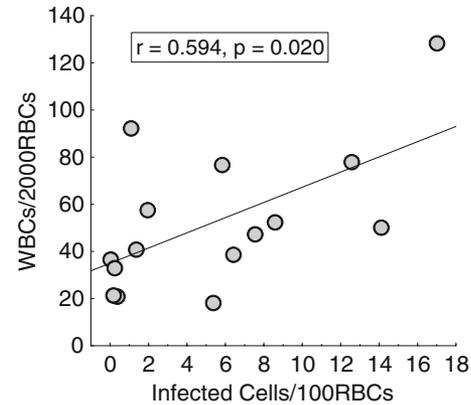
<sup>a</sup>Residuals of mass-SVL regression. "Parasite" was a binomial infection variable (infected or not infected with the rickettsial agent). Collecting sites were in New York, Virginia, and two in Pennsylvania.

### Effects of the Parasite on Erythrocyte Morphology

Parasitized red blood cells were significantly larger in two-dimensional area than nonparasitized cells ( $F_{1,244} = 9.07$ ,  $P = 0.003$ ), though this difference was small; the average area of parasitized cells was  $348 \mu\text{m}^2 (\pm 66 \text{ SD})$  while that of nonparasitized cells was  $332 \mu\text{m}^2 (\pm 57 \text{ SD})$ . Thus, parasitized erythrocytes were approximately 5% larger than nonparasitized. Meanwhile, the overall shape of parasitized cells (aspect ratio) was not significantly different than nonparasitized ( $F_{1,244} = 0.01$ ,  $P = 0.907$ ); the mean ratio of



**Figure 5.** Average white blood cell counts ( $\pm 1$  SE, expressed as number of white blood cells per 2000 red blood cells) of salamanders with and without the rickettsial agent.



**Figure 6.** Relationship between infection severity (number of infected cells out of 100) and white blood cell counts in 17 infected salamanders.

parasitized cells was  $1.90 (\pm 0.27 \text{ SD})$ , and the mean of nonparasitized cells was  $1.90 (\pm 0.28 \text{ SD})$ . Qualitatively, the general appearance of parasitized cells was not noticeably different from unaffected cells: the nuclear position was similar in both groups (i.e., the parasite did not alter the nucleus position), and the staining characteristics appeared similar.

### White Blood Cell Counts

Comparison of average white blood cells counts of infected and noninfected salamanders showed significantly higher numbers of white blood cells in infected individuals ( $t = 2.26$ ,  $P = 0.026$ ; Figure 5). Moreover, among all infected individuals, there was a significant positive relationship between the severity of infection and white blood cell count ( $r = 0.594$ ,  $P = 0.020$ ; Figure 6).

## DISCUSSION

---

The results of this study provide several new insights into the nature of this frequently observed, blood-borne, bacterial organism of ectotherms, starting with its taxonomy. While the bacterial organism we examined is certainly not new to science, our molecular characterization of its DNA does indicate that it should be considered a new genus of bacteria within the order Rickettsiales, family Anaplasmataceae. We do not know, however, if the particular organism we examined is the same as that previously referred to as *Cytamoeba* and *Aegyptianella* in other amphibians (e.g., Rankin, 1937; Lehmann, 1961; Desser, 1987). If so, it is possible we have merely updated its taxonomy with the present study. On the other hand, this organism may indeed represent an undescribed bacterial species. We lean toward the former, although given the number of hosts reported, the seemingly global distribution, and the prior discrepancies in taxonomy reported, it is very possible that there exist multiple varieties of these morphologically similar, intraerythrocytic agents, and here we have simply categorized the one affecting Plethodontid salamanders in the eastern United States.

Morphologically similar organisms have been detected in numerous species of salamanders including *Plethodon cinereus* from North Carolina (Rankin, 1937) and *Desmognathus* spp., *Plethodon* spp., *Ambystoma* spp., *Aneides* spp., *Batrachoseps attenuatus*, *Dicamptodon ensatus*, *Eurycea guttolineata*, and *Notophthalmus viridescens*, from regions of Arkansas, North Carolina, Oklahoma, and California (Rankin, 1937; Lehmann, 1961; McAllister et al., 1993, 1995). Comparison of prevalence across these host species and geographic regions is difficult because we do not know if the organisms are the same based solely on morphology. As this study shows, molecular characterization of these organisms from different salamander species and geographic regions is needed to better understand the ecology of this organism or group of organisms.

In all prior reports of either *Cytamoeba* and *Aegyptianella* in other amphibians, negative effects on the host were never reported (Rankin, 1937; Lehmann, 1961; McAllister et al., 1993, 1995). Consistent with this, our results showed that infected salamanders actually had higher body condition scores than those not infected. Thus there was no outward sign of negative health effects to the hosts. In terms of the hosts' blood cells, we also found no evidence of negative effects on host erythrocytes. In contrast however,

infection by this organism appears to activate the white blood cell component of the host immune system. Infected salamanders had significantly more white blood cells than uninfected, and white blood cells increased in abundance with increasing severity of infection. General increases in white blood cells in animals is an indication of a nonspecific innate immune reaction (Jain, 1986), and if this is indeed the result of the rickettsial agent in the salamanders, it is not consistent with results from other intracellular organisms in this group. In a similar evaluation of hematological response of chickens with, and without, infection by *Aegyptianella pullorum*, Nazifi et al. (2008) found no evidence of an innate immune (i.e., white blood cell) response; however, less than 1% of red blood cells were infected in that study. Humans infected with the bacteria, *Ehrlichia chaffeensis*, which infects white rather than red blood cells, have fewer white cells than uninfected individuals (Hamilton et al., 2004).

While we do not know the transmission cycle of this rickettsial bacteria among red-backed salamanders, we can draw inferences based on its taxonomic position, because many other members of Anaplasmataceae are transmitted by arthropod vectors (Rikihisa, 2006). For example, *Ehrlichia chaffeensis* is transmitted by ticks to mammals such as coyotes (Kocan et al., 2000), deer (Paddock and Yabsley, 2007), and humans (Hamilton et al., 2004). If we assume that this organism is also vectored by an arthropod, then this pathogen is particularly intriguing. Unlike other animals which may be regularly exposed to biting insects or ectoparasites, plethodontid salamanders spend the majority of their time underground and under moist surface retreats (Feder, 1983). Therefore, salamanders would need to acquire this organism from soil- or leaf litter-dwelling vectors during the limited periods of time when they are surface active. Several lines of evidence from our study suggest the vector is indeed related to surface activity. First, transmission of this pathogen appears to be male-biased; male salamanders were four times more likely to be infected than females. This may be due to the fact that males of this species are generally more surface-active than females during the summer [Maerz, unpublished data]. Females spend much of the summer months (June–August) below ground or in logs attending to their clutches. Second, we found that infected salamanders were generally larger than uninfected salamanders. Larger salamanders lose water at a slower rate than smaller salamanders, and water loss is the primary determinant of surface activity in plethodontids

(Feder, 1983). Therefore, larger salamanders in general may be more surface active than smaller individuals.

As for potential vectors of this pathogen, we suggest the trombiculid mite *Hannemania dunni* is a likely candidate (Brown et al., 2006; Westfall et al., 2008). It is an ectoparasite that inhabits forest leaf litter and is known to infect a large number of salamanders across a wide geographic range (Rankin, 1937; Anthony et al., 1994; Westfall et al., 2008). In fact, it is the *only* ectoparasite ever reported for *Plethodon* spp., arthropod or otherwise, despite meticulous searching (Rankin, 1937). Larval trombiculid mites invade the skin of their hosts, which can cause very conspicuous lesions (Brown et al., 2006). While we did not see these lesions on any of the animals collected here, they could have easily been infected by mites at some prior time in their life. Furthermore, trombiculid mite infections in plethodontid salamanders are known to be male-biased (Anthony et al., 1994), which is consistent with the male-biased rickettsial infection found here. Given this consistency in prevalence, the overlapping habitat (leaf litter), the fact that host tissues are invaded, and the fact that no other arthropod has been reported affecting plethodontid salamanders (Rankin, 1937), it is very likely that trombiculid mites are the vector for this bacterial pathogen.

Given that we detected the organism in all populations of red-backed salamanders we sampled, and morphologically similar organisms have been reported from salamanders in other parts of the United States (Rankin, 1937; Lehmann, 1961), it is clear that this organism is broadly established. Because of this broad occurrence and the clear link to immune responses in salamanders, we suggest this organism warrants additional attention. Priority questions include determining if trombiculid mites are indeed the vector, and what is the cost to the host of the immune reaction. Also, do hosts eventually clear the pathogen from their blood? Do other tissues or organs become infected? Finally, is this intracellular pathogen the same species of bacteria as that reported from frogs (and reported as *Aegyptianella*) (Desser, 1987)? Clearly, there are many questions raised by this study and, hopefully, they will stimulate new investigations into this “old” pathogen.

## ACKNOWLEDGMENTS

Sonia Altizer provided blood samples from Virginia salamanders and provided helpful discussions on the project. AKD was supported by the D.B. Warnell School

of Forestry and Natural Resources during this project. Tissues were collected from salamanders collected and sacrificed for a separate project covered under IACUC AUP#A2006-10041, and supported by NSF-DEB DEB05 42974 to JCM.

## REFERENCES

- Anthony CD, Mendelson JR, Simons RR (1994) Differential parasitism by sex on plethodontid salamanders and histological evidence for structural damage to the nasolabial groove. *American Midland Naturalist* 132:302–307
- Babudieri B (1972) Mycoplasma-like organism, parasite of red blood cells of an amphibian, *Hydromantes italicus* (*Spelerpes fuscus*). *Infection and Immunity* 6:77–82
- Barta JR, Desser SS (1984) Blood parasites of amphibians from Algonquin Park, Ontario. *Journal of Wildlife Diseases* 20:180–189
- Brown JD, Keel MK, Yabsley MJ, Thigpen T, Maerz JC (2006) Clinical challenge. *Journal of Zoo and Wildlife Medicine* 37:571–573
- Brumpt E, Lavie C (1935) Sur un piroplasmide nouveau parasite de Tortue, *Tunetella emydis* n. g. n. sp. *Annales de Parasitologie Humaine et Comparee (Paris)* 13:544–550
- Chutmongkonkul M, Khonsue W, Pariyanonth P (2006) Blood parasites of six species of wild amphibians from Khun Mae Kuang forest area, Thailand. *Proceedings from the 2nd Symposium on Asian Zoo and Wildlife Medicine and the 1st Workshop on Zoo and Wildlife Pathology*: 48
- Daszak P, Berger L, Cunningham AA, Hyatt AD, Green DE, Speare R (1999) Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases* 5:735–748
- Davis AK (2008) Ontogenetic changes in erythrocyte morphology in larval mole salamanders, *Ambystoma talpoideum*, measured with image analysis. *Comparative Clinical Pathology* 17:23–28
- Davis AK, Holcomb KL (2008) Intraerythrocytic inclusion bodies in painted turtles (*Chrysemys picta picta*) with measurements of affected cells. *Comparative Clinical Pathology* 17:51–54
- Davis AK, Maerz JC (2008a) Comparison of hematological stress indicators in recently captured and captive paedomorphic mole salamanders, *Ambystoma talpoideum*. *Copeia* 2008:613–617
- Davis AK, Maerz JC (2008b) Sex-related differences in hematological stress indices of breeding, paedomorphic mole salamanders. *Journal of Herpetology* 42:197–201
- Davis AK, Yabsley MJ, Keel K, Maerz JC (2007) Discovery of a novel alveolate pathogen affecting southern leopard frogs in Georgia: description of the disease and host effects. *EcoHealth* 4:310–317
- Davis AK, Milanovich JR, DeVore JL, Maerz JC (2009) An investigation of factors influencing erythrocyte morphology of red-backed salamanders. *Animal Biology* 59:201–209
- Desser SS (1987) *Aegyptianella ranarum* sp. n. (Rickettsiales, Anaplasmataceae): ultrastructure and prevalence in frogs from Ontario. *Journal of Wildlife Diseases* 23:52–59
- Desser SS, Barta JR (1989) The morphological features of *Aegyptianella bacterifera*: an intraerythrocytic rickettsia of frogs from Corsica. *Journal of Wildlife Diseases* 25:313–318

- Feder ME (1983) Integrating the ecology and physiology of plethodontid salamanders. *Herpetologica* 39:291–310
- Forson D, Storfer A (2006) Atrazine increases ranavirus susceptibility in the tiger salamander, *Ambystoma tigrinum*. *Ecological Applications* 16:2325–2332
- Gothe R, Kreier JD (1978) Aegyptianella, Eperythrozoon and Haemobartonella. In: *Parasitic Protozoa*, Vol 4, Kreier JD (editor), San Diego, CA: Academic Press, pp 251–294
- Hamilton KS, Standaert SM, Kinney MC (2004) Characteristic peripheral blood findings in human ehrlichiosis. *Modern Pathology* 17:512–517
- Hegner RW (1921) *Cytamoeba bacterifera* in the red blood cells of the frog. *Journal of Parasitology* 7:157–161
- Jain NC (1986) *Schalm's Veterinary Hematology*, Philadelphia: Lea & Febiger
- Kocan A, Levesque GC, Whitworth LC, Murphy GL, Ewing SA, Barker RW (2000) Naturally occurring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. *Emerging Infectious Diseases* 6:477–480
- Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5:150–163
- Labbé DL (1894) Recherches zoologiques et biologiques sur les parasites endoglobulaires du sang des vertébrés. *Archives de Zoologie Experimentale et Generale* 3:1–258
- Lehmann DL (1961) *Cytamoeba bacterifera* Labbe, 1894 I. Morphology and host incidence of the parasite in California. *Journal of Protozoology* 8:29–33
- Lehmann DL (1964) Cytological and cytochemical studies on *Cytamoeba bacterifera* Labbé, 1894. *Parasitology* 54:121–124
- McAllister CT, Upton SJ, Trauth SE (1993) Endoparasites of western slimy salamanders, *Plethodon albagula* (Caudata, Plethodontidae), from Arkansas. *Journal of the Helminthological Society of Washington* 60:124–126
- McAllister CT, Bursley CR, Upton SJ, Trauth SE, Conn DB (1995) Parasites of *Desmognathus brimleyorum* (Caudata, Plethodontidae) from the Ouachita mountains of Arkansas and Oklahoma. *Journal of the Helminthological Society of Washington* 62:150–156
- Muths E, Corn PS, Pessier AP, Green DE (2003) Evidence for disease-related amphibian decline in Colorado. *Biological Conservation* 110:357–365
- Nazifi S, Razavi SM, Yavari F, Rajaifar M, Bazayr E, Esmailnejad Z (2008) Evaluation of hematological values in indigenous chickens infected with *Plasmodium gallinaceum* and *Aegyptianella pullorum*. *Comparative Clinical Pathology* 17:145–148
- Paddock CD, Yabsley MJ (2007) Ecological havoc, the rise of white-tailed deer, and the emergence of *Amblyomma americanum*-associated zoonoses in the United States. In: *Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission*, MacKenzie JS, Childs JE, Richt JA (editors), Berlin: Springer-Verlag Berlin, pp 289–324
- Persing DH (1996) *PCR Protocols for Emerging Infectious Diseases. A Supplement to Diagnostic Molecular Microbiology: Principles and Applications*, Washington, DC: ASM Press
- Rankin JS (1937) An ecological study of parasites of some North Carolina salamanders. *Ecological Monographs* 7:169–269
- Rikihisa Y (2006) New findings on members of the family Anaplasmataceae of veterinary importance. *Annals of the New York Academy of Sciences* 1078:438–445
- Rikihisa Y, Zhang C, Christensen BM (2003) Molecular characterization of *Aegyptianella pullorum* (Rickettsiales, Anaplasmataceae). *Journal of Clinical Microbiology* 41:5294–5297
- Sassera D, Beninati T, Bandi C, Bouman EAP, Sacchi L, Fabbi M, et al. (2006) 'Candidatus Midichloria mitochondrii', an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle. *International Journal of Systematic and Evolutionary Microbiology* 56:2535–2540
- Statistica (2003) Statistica version 6.1, Tulsa, OK: Statsoft Inc.
- Stenberg PL, Bowerman WJ (2008) Hemoparasites in Oregon spotted frogs (*Rana pretiosa*) from central Oregon, USA. *Journal of Wildlife Diseases* 44:464–468
- Thompson JD, Higgins DG, Gibson TJ, Clustal W (1994) Improving and sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22:4673–4680
- Todd BD, Davis AK (2007) Sexual dichromatism in the marbled salamander, *Ambystoma opacum*. *Canadian Journal of Zoology* 85:1008–1013
- Villolobos M, Leo P, Sessions SK, Kezer J (1988) Enuclated erythrocytes in plethodontid salamanders. *Herpetologica* 44:243–250
- Werner JK (1993) Blood parasites of amphibians from Sichuan Province, Peoples Republic of China. *Journal of Parasitology* 79:356–363
- Westfall MC, Cecala KK, Price SJ, Dorcas ME (2008) Patterns of trombiculid mite (*Hannemania dunnii*) parasitism among plethodontid salamanders in the western Piedmont of North Carolina. *Journal of Parasitology* 94:631–634
- Zhang C, Rikihisa Y (2004) Proposal to transfer 'Aegyptianella ranarum', an intracellular bacterium of frog red blood cells, to the family Flavobacteriaceae as 'Candidatus Hemobacterium ranarum' comb. nov. *Environmental Microbiology* 6:568–573