Parasitol Res
DOI 10.1007/s00436-010-1869-z

ORIGINAL PAPER

Intraerythrocytic rickettsial inclusions in Ocoee salamanders (*Desmognathus ocoee*): prevalence, morphology, and comparisons with inclusions of *Plethodon cinereus*

Andrew K. Davis · Kristen Cecala

Received: 4 December 2009 / Accepted: 1 April 2010
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Abstract Reports of an unusual intraerythrocytic pathogen in amphibian blood have been made for decades; these pathogens appear as membrane-bound vacuoles within erythrocytes. It is now understood that the pathogen is a *Rickettsia* bacteria, which are obligate intracellular parasites, and most are transmitted by arthropod vectors. In an effort to further understand the host range and characteristics of this pathogen, we examined 20 Ocoee salamanders (*Desmognathus ocoee*) from a site in southwest North Carolina for the presence of rickettsial inclusions and report the general characteristics of infections. Seven individuals (35%) were infected, and this level of prevalence was consistent with all other members of this genus examined to date. In contrast, infections within the genus *Plethodon* tend to occur in less than 10% of individuals, based on review of the literature. The difference could be related to the semi-aquatic nature of *Desmognathus* salamanders versus the completely terrestrial *Plethodon*. It is also possible that both groups are hosts to different types of rickettsial bacteria, since the inclusions found here only vaguely resembled those found recently in red-backed salamanders (*Plethodon cinereus*). Inclusions in Ocoee salamanders measured 4 µm in diameter on average, and most cells (88%) only had one inclusion. Of all infected individuals, inclusions occurred in approximately 3% of erythrocytes. Similar to that found in red-backed salamanders, infected hosts were significantly larger than non-infected. Future investigations into the nature of this unusual parasite of amphibians would help to further our understanding of its life cycle and transmission.

A. K. Davis (✉) · K. Cecala
D.B. Warnell School of Forestry and Natural Resources,
The University of Georgia,
Athens, GA 30602, USA
e-mail: akdavis@uga.edu

Introduction

Salamanders are hosts to a variety of parasites, including helminths (McAllister et al. 2008), arthropods (Westfall et al. 2008), and in particular, intracellular bacteria in the order Rickettsiales (Davis et al. 2009a). All bacteria in this order are obligate, intracellular (usually within erythrocytes) organisms that are transmitted by arthropod vectors (Rikihisa 2006). Most infections can be seen by examining stained blood smears with light microscopy and noting the presence of the characteristic inclusions that are formed within blood cells. Such inclusions have been noted in amphibian blood cells for decades (Hegner 1921; Lehmann 1961; McAllister et al. 1993), although their taxonomy was never clear in earlier studies. They were once thought to be protozoal in origin and given the name *Cytamoeba bacterifera* (Hegner 1921). This designation continued until later investigations using electron microscopy suggested that the organism was a rickettsia-like bacterium that was segregated from the host-cell cytoplasm within a membrane-bound vacuole, and based on this evidence, Desser (1987) argued that it belonged to the genus *Aegyptianella*. However, a recent investigation using gene sequencing of blood from infected red-backed salamanders (*Plethodon cinereus*) indicated that the bacteria, at least in that host, was only distantly related to the *Aegyptianella* genus and in fact belonged in a new, as yet unnamed, genus of *Rickettsia* (Davis et al. 2009a). Interestingly, in that same study, the authors found that the parasite does not affect salamanders randomly; larger salamanders were more affected than smaller ones, which suggests that older individuals are more prone to infections. Clearly, there is still much to be learned from this enigmatic blood parasite (or parasites), such as the extent of its host range and its mode of transmission among amphibians.

Published online: 27 April 2010

While conducting a study of salamander movements within streams in southwest North Carolina, a collection of Ocoee salamanders (*Desmognathus ocoee*) was made (Fig. 1), giving us the opportunity to examine them for rickettsial parasites. Ocoee salamanders are small, semi-aquatic, stream-inhabiting salamanders with a range that covers the southeastern United States (Petranka 1998). To our knowledge, no prior studies have examined this species for rickettsial parasites, although rickettsial inclusions have been found in other members of the *Desmognathus* genus (Rankin 1937; McAllister et al. 1995). In the current study, we describe the results of this (largely descriptive) investigation, including the prevalence of the parasite in this host, its morphology in host cells, as well as some general comparisons with the rickettsial inclusions found recently in red-backed salamanders (Davis et al. 2009a).

Methods

Collecting and processing salamanders

A total of 20 Ocoee salamanders were collected by hand from a headwater stream in the Coweta Hydrologic Laboratory forest near Franklin, North Carolina. This stream was a typical high-elevation headwater stream in the southern Appalachian Mountains with a fully forested catchment. Like other streams at this location, the focal stream was steep with dense over- and under-story vegetation. When salamanders were collected, they were placed individually into 500 ml plastic containers, along with a moist paper towel, and brought to the lab for processing, which was done later in the same week. In the lab, processing of salamanders generally followed Davis et al. (2009b). Briefly, all salamanders were weighed with an electronic balance then killed with an overdose of MS-222. Immediately after death, each salamander was decapitated, and the blood that welled from the heart region was dripped onto a clean microscope slide. A second slide was used to smear the blood across the first slide. All slides were air-dried, then stained with Giemsa.

Fig. 1 Photograph of a typical Ocoee salamander, *D. ocoee*, from northeast Georgia. Photographed by A. Davis

Examining blood smears

To determine the prevalence of rickettsial infections in the collected salamanders, we examined all blood smears with a standard light microscope under ×1,000 (oil immersion) magnification. We examined 50 fields of view per slide and recorded the number of erythrocytes with inclusions for each salamander. At this magnification, fields of view had an average of 71.5 erythrocytes (±9.3 SD), based on examination of ten random fields, so that this amounted to examination of approximately 3,575 cells per salamander.

Measurements of inclusions

For infected salamanders, we randomly selected 12 inclusions per individual and obtained photomicrographs using a digital camera mounted to the microscope. With these images, we obtained measurements of the inclusions using image analysis software, following Davis and Holcomb (2008). These included the area, length, and width of each inclusion.

Results

Prevalence and severity of infection

Rickettsial inclusions were found in 7 (35%) of the 20 Ocoee salamanders we examined from the site in North Carolina. There was a clear size bias in infection prevalence, as infected salamanders were significantly larger, in terms of body mass, than uninfected salamanders (two-sample t test, df=18, $t=-3.77$, $p=0.001$; Fig. 2). The severity of infection varied to some degree among the 7 infected individuals (Table 1). The average number of erythrocytes with inclusions was 109 per 50 fields of view. With an average of 71.5 erythrocytes per field, this translates to approximately 3% of cells. Most affected erythrocytes only contained a single inclusion (88% of the time).

Inclusion morphology

The inclusions found in Ocoee salamanders appeared as rounded structures within erythrocytes and were often clear in color with a dark blue ring (with Giemsa staining; Fig. 3, top). In other cases, they appeared faint blue throughout

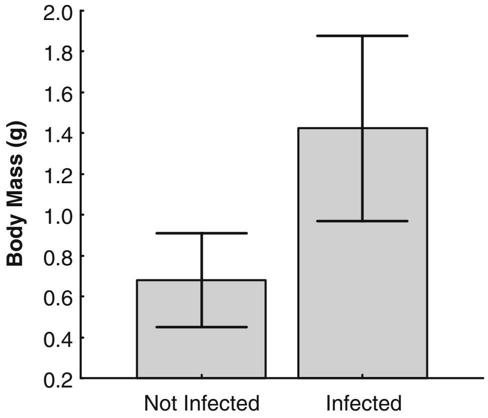

Fig. 2 Average body mass of Ocoee salamanders with ($n=7$) and without ($n=13$) rickettsial infections. *Error bars* represent 95% confidence intervals

with a granular-like interior. These are both in contrast to the appearance of inclusions found in red-backed salamanders (Davis et al. 2009a), which were usually violet in color after Giemsa staining and with no visible interior structure (Fig. 3, bottom). The Ocoee salamander inclusions were most commonly situated in a polar region of the cell, and most did not displace the nucleus. Based on measurement of 84 inclusions (12 per individual), the average length of inclusions in Ocoee salamanders was approximately 4 µm, while the area was approximately 11 µm^2 (Table 1). For comparison, inclusions in red-backed salamanders averaged 6.2 µm in length and 23.7 µm^2 in area (A.K. Davis, unpublished data).

Rickettsia infections of other salamanders

To place the prevalence of infections within Ocoee salamanders into context, we tabulated the published records of similar infections within other salamanders in this genus as well as in the genus *Plethodon* (Table 2). Rickettsial infections have been observed in at least three other *Desmognathus* species and four *Plethodon* species (although in the earlier cases, the taxonomy was not clear). We note here that Rankin (1937) actually examined several other salamander species within these genera, although in these cases, the number of salamanders examined was less than 10, and we felt that the prevalence estimates from these samples would not be representative of those species. Interestingly, tabulating the prevalence estimates from the literature, along with that found in Ocoee salamanders, revealed an interesting pattern, in that all *Desmognathus* species appeared to be much more frequently infected with rickettsial bacteria than the *Plethodon* species. In fact, statistical comparison of average prevalence values between these groups showed a highly significant difference (two-sample *t* test, df=7, $t=5.91$, $p<0.001$).

Discussion

The prevalence of rickettsial infection found in Ocoee salamanders of 35% was highly consistent with prevalence estimates for other members of this genus (at least from collections made in the southern United States), which ranged from 24% to 35%, and averaging 30% (Table 2). In contrast, the average rickettsial prevalence for all *Plethodon* salamanders examined to date was only 8%. In other words, approximately one out of every three *Desmognathus* salamanders appears to harbor rickettsial infections while only one out of every 12 *Plethodon* does. This difference is perhaps one of the most interesting findings of the current study, as it begs the question why. The main difference between these two groups of salamanders is their life cycle. All salamanders in the genus *Desmognathus* are semi-aquatic, in that they breed in shallow streams, but live

Table 1 Summary of rickettsial characteristics in the seven infected Ocoee salamanders found in this study, shown in no particular order

Salamander number	Body mass (g)	Infection severity	Single inclusions (%)[a]	Average inclusion length (µm)	Average inclusion width (µm)	Average inclusion area (µm^2)
1	1.39	152.0 (4.3%)	88.2	3.82	3.34	9.86
2	1.23	26.3 (0.7%)	95.2	5.63	4.92	21.19
3	1.02	110.0 (3.1%)	84.3	4.40	3.77	12.60
4	1.01	231.4 (6.5%)	78.0	3.56	3.05	8.69
5	1.81	9.3 (0.3%)	95.0	2.47	2.04	3.97
6	2.35	207.6 (5.8%)	84.1	3.58	2.96	8.31
7	1.16	26.8 (0.7%)	90.9	4.68	3.84	13.91
Average	1.42	109.1 (3.1%)	88.0	4.02	3.42	11.22

Infection severity shown as number of inclusions per 50 fields of view at ×1,000 and as percentage of erythrocytes in parentheses (see "Methods" for calculation)

[a] Of all affected erythrocytes, the percentage containing a single inclusion

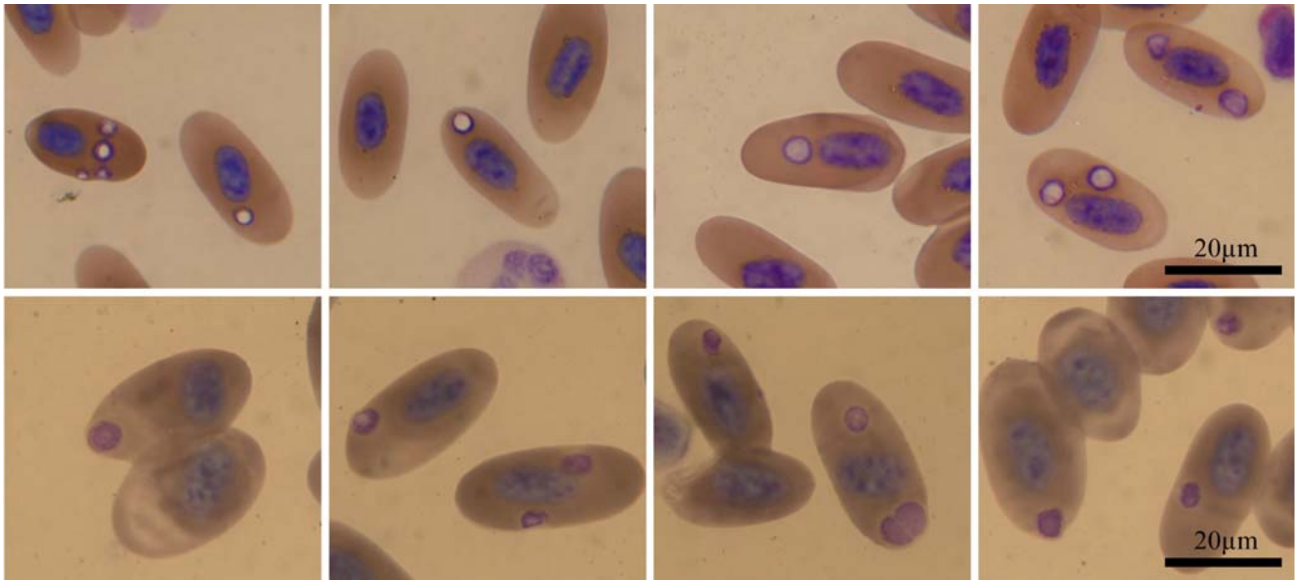

Fig. 3 Photomicrographs of rickettsial inclusions found in *D. ocoee* (*top*) in the current study and in *P. cinereus* (*bottom*) from previous investigation (Davis et al. 2009a)

terrestrially otherwise (Petranka 1998). Salamanders in the *Plethodon* genus are completely terrestrial, even laying their eggs on land. Because of this, Davis et al. (2009a) speculated that the *Rickettsia* bacteria in *P. cinereus* was likely transmitted by a terrestrial arthropod vector, such as mites that live in leaf litter. However, if this is the case, then why would the completely terrestrial genus (which would have more opportunities for contact with the vector) have a *lower* prevalence of rickettsial infections than the semi-terrestrial genus?

The difference in rickettsial infection prevalence between *Desmognathus* and *Plethodon* salamanders could be explained if the two genera contract different species of rickettsial bacteria that each have different vectors and life cycles. If so, then perhaps the *Desmognathus* variety is transmitted by an aquatic vector, while the *Plethodon* by a terrestrial one. Consistent with the idea of two rickettsia types, the general morphology of inclusions in Ocoee salamanders did appear to be different than that of red-backed salamanders, in terms of staining characteristics and size (Fig. 3). Of course, this entire line of thought would need to be verified using gene-sequencing approaches, which was not done here. An alternative explanation is that the rickettsial bacterium is the same within both genera, but the two groups are different in terms of susceptibility. To answer this question, we suspect that more detailed investigation into the host's immune reaction to the pathogen is warranted.

The characteristics of inclusions described here are consistent with an earlier investigation of the pathogen in

Table 2 Prevalence of rickettsial inclusions reported in salamanders in the genus *Desmognathus* and *Plethodon*

Species	Location	Number	Prevalence (%)	Reference
Desmognathus ocoee	North Carolina	20	35.0	This study
Desmognathus brimleyorum	Arkansas	41	24.0	(McAllister et al. 1995)
Desmognathus fuscus	North Carolina	219	28.3	(Rankin 1937)
Desmognathus quadramaculatus	North Carolina	46	34.7	(Rankin 1937)
Plethodon cinereus	Eastern US (NY, PA, VA)	105	16.7	(Davis et al. 2009a)
Plethodon cinereus	North Carolina	74	8.3	(Rankin 1937)
Plethodon glutinosis	North Carolina	119	6.9	(Rankin 1937)
Plethodon metcafi	North Carolina	18	0.0	(Rankin 1937)
Plethodon albagula	Arkansas	37	8.0	(McAllister et al. 1993)
Average prevalence of all *Desmognathus*			30.0	
Average prevalence of all *Plethodon*			8.0[a]	

[a] Significantly different (two-sample *t* test, df=7, t=5.91, p<0.001)

other salamanders, although then, it was called *C. bacterifera* (Lehmann 1961). In this study of four salamander species from California (*Aneides flavipunctatus*, *Aneides lugubris*, *Batrachoseps attenuates*, and *Dicamptodon ensatus*), inclusions averaging 4.7 µm in diameter were reported, and the incidence of single inclusions within cells was 71–93%. In addition, the percentage of cells infected ranged from 2.0–11.3% across all four species. This is compared to an average inclusion diameter of 4.0 µm in Ocoee salamanders, where the percentage of single inclusions was 88%, and the infection severity was approximately 3% of erythrocytes (Table 1). It would have been interesting to compare the population prevalence of rickettsial infections from the four species examined in California to that found here, although despite the title given to that paper, that information was not reported (Lehmann 1961).

Similar to that found with red-backed salamanders (Davis et al. 2009a), infected Ocoee salamanders were significantly larger than non-infected ones (Fig. 2). This consistency may not be a coincidence, as McAllister et al. (1993) reported a similar trend (though not statistically examined) of 37 *Plethodon albagula* examined in Arkansas, the mean snout-vent length was 53.9 mm (±0.2 SE), but the three individuals with rickettsial infections had snout-vent lengths between 66 and 75 mm. Thus, it appears that this is a consistent pattern across all salamanders that are susceptible to rickettsial infections, and it may indicate the role of host age in the transmission cycle of the pathogen. Older (and therefore larger) salamanders would have had more opportunities to contract the infection over their lifetime, regardless of the vector. However, this presumes that the infection is long-lasting within salamanders (or that older individuals are repeatedly exposed to the pathogen), which is something that has not yet been established. Alternatively, the size difference could also indicate some preference by the vector(s) for larger hosts, or perhaps younger individuals that contract the infection suffer higher mortality from it (than larger salamanders do), leading to the appearance of a size bias. In any case, given that this pattern has been found in three different salamander species, it is clearly something that should be considered in future studies of other salamanders.

Finally, we suggest that future investigations into this pathogen of amphibians would be fruitful areas of research. Based on the results gathered here, interesting questions to address would include if larger salamanders have different reactions to the pathogen than smaller ones, which could possibly be tested experimentally, and if the prevalence is similar among other members of the genus *Desmognathus*. Furthermore, is there a seasonal trend in prevalence, especially among the *Desmognathus* salamanders, which alternate between terrestrial and aquatic lifestyles during the year? Clearly, despite a long history of reports of its occurrence, we still have a long way to go before we can fully understand the nature of this enigmatic pathogen of amphibians.

Acknowledgements We thank the members of the Warnell field techniques class 2009 for their assistance in collecting salamanders. Allie Robillard helped in the sampling of parasites. Salamanders were collected as part of a separate project and with the approval of the University of Georgia's animal care and use committee.

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