

Investigating temporal changes and effects of elevation on the prevalence of a rickettsial blood parasite in red-backed salamanders (*Plethodon cinereus*) in Virginia, USA

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Received: 14 April 2016 / Accepted: 7 July 2016
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Abstract Amphibians are host to a number of vector-transmitted blood parasites. One parasite for which there is little information is a recently described intracellular bacteria in the order *Rickettsiales*, found in red-backed salamanders (*Plethodon cinereus*) in the eastern USA. This parasite was observed in 16.7 % of individuals across three states in the summer of 2008. Here, we resampled one of the locations from the 2008 study (Mountain Lake, Virginia, where site-level prevalence was 22 %) to determine if the incidence of infection has changed in 5 years. We also examined red-backed salamanders at seven other nearby sites that varied in elevation (670–1310 m) to ascertain if the pathogen shows any corresponding variation. We collected a total of 113 salamanders across the eight sites. We found no evidence that prevalence of the pathogen has changed; overall prevalence was 12.4 %, which was not significantly different from the 2008 estimate, and prevalence at the Mountain Lake site (28.6 %) also did not differ from the 2008 estimate. When comparing prevalence across elevations, we found that the highest-elevation sites tended to have highest levels of infections; one in five salamanders was infected with rickettsial bacteria at these locations. Ironically, these high-elevation sites appeared to have the highest salamander densities, and the largest salamanders, which usually point to healthy

populations. Since salamander habitats in the Appalachian highlands are predicted to shrink in the future, our results suggest that the remnant, mountaintop populations will remain highly infected with this mysterious parasite.

Keywords Red-backed salamanders · *Plethodon cinereus* · Rickettsia · Elevation · Climate change

Introduction

Amphibians are host to a wide variety of blood-borne parasites, including extracellular and intraerythrocytic parasites, which includes certain protozoa (e.g., *Hepatozoon*; Shutler et al. 2009) and bacteria, the latter being in the order *Rickettsiales* (Desser and Barta 1984; Desser 1987). Bacteria in this order are considered protobacteria, are vector transmitted (usually by ticks and other arthropods; Witter et al. 2016), and are known to cause diseases in their hosts (e.g., Richter et al. 1996; Nicholson et al. 1999; Moore et al. 2000; Apperson et al. 2008). Rickettsia parasites can be readily observed within erythrocytes of affected amphibians using light microscopy; cells with the parasite have distinctive darkly staining vacuoles that contain the bacteria. These bacteria, or at least the vacuoles, have long been observed by researchers studying amphibian blood parasites (Hegner 1921; Rankin 1937; Lehmann 1961; Barta and Desser 1984; Davis and Cecala 2010), although the taxonomy and nomenclature of the bacteria have not always been clear.

In 2008, a study characterized this bacterial parasite in red-backed salamanders (*Plethodon cinereus*, Fig. 1a) in the eastern USA, which included both field surveys of its prevalence and more formal molecular identification (Davis et al. 2009). The phylogenetic analyses indicated the bacterium was a member of the family *Anaplasmatacea* (within the order

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Rickettsiales), although it appeared to be distinct from all other members of that family, and may even represent a new genus (Davis et al. 2009). The field collections involved sampling *P. cinereus* salamanders from four US states, including a site in the Appalachian region, the Mountain Lake Biological Station (MLBS), in Giles Co., Virginia. This high-elevation site (1200 m) is located in the heart of the red-backed salamander range in North America, and because of its cool, moist forest habitat, it has long been considered a hot spot for plethodontid diversity (Bogert 1952; Brown et al. 1977; Wilbur 1998; Grover 2000). At that time (summer 2008), 22 % of red-backed salamanders at the MLBS site harbored the parasite. Across all sites sampled (across four US states), prevalence was 16.7 %.

This prior study of rickettsial bacteria in red-backed salamanders raised a number of questions regarding this enigmatic blood parasite. For example, we know little about its temporal consistency within the salamander population; i.e., is this a pathogen that is currently spreading throughout the salamander range (i.e., increasing in prevalence) or has its prevalence remained constant over time? In addition, the sites where salamanders were collected in 2008 represented a range of elevations, although the effect of elevation was confounded with location in that study (sites were separated across four US

states). Elevation is a known factor influencing the transmission of other pathogens of terrestrial salamanders, such as ranavirus (Sutton et al. 2015). It would therefore be important to know if elevation affects prevalence of the rickettsial parasite as well, especially since recent work showed how global climate change is predicted to reduce plethodontid habitat in the Appalachian Mountains (Milanovich et al. 2010), which will result in only remnant pockets of suitable habitat remaining, primarily at high-elevation sites in the central and southern Appalachian Mountains.

In summary, since much of the biology of the rickettsial parasite in plethodontid salamanders is largely unknown, the current study was undertaken to address two specific questions regarding its prevalence: (1) is its prevalence increasing over time at local sites and (2) does prevalence vary with elevation. If the latter was true, it would suggest an influence of climatic conditions on prevalence. To address these questions, we revisited one of the sites from the 2008 study (MLBS) exactly 5 years later to determine if infection prevalence of the rickettsial bacteria in the local red-backed salamander population has changed in this intervening time. We also collected salamanders at seven nearby sites in the same county, which varied in elevation (670–1310 m), to evaluate the effect of elevation, if any, on prevalence. We hoped that doing so will provide insight into how prevalence across the Appalachian highlands may change in the future, once available salamander habitat becomes limited to high-elevation sites.

Methods

Study sites Collecting sites for this project were in Giles Co., VA, USA. We selected sites based primarily on elevation but also on ease of access and land ownership. There were eight sites in total, including the Mountain Lake location (Table 1). These locations ranged in elevation from 670 to 1310 m. All sites were forested, containing mostly hardwood trees. At each site, we attempted to collect at least 10 salamanders (below). At most sites, all collections were completed within 3–4 h, although it tended to take longer at the lowest elevations.

Capturing and handling salamanders Salamanders were collected by hand by flipping rocks and logs (sometimes pieces of bark). Each captured salamander was immediately anesthetized with a buffered solution of 2 % MS-222, then photographed from above (next to a ruler) using a digital camera mounted to a small stand. It was then decapitated and blood from the heart was siphoned with a heparinized capillary tube. The blood was dripped onto a clean microscope slide, and a second slide was used to make a thin blood film.

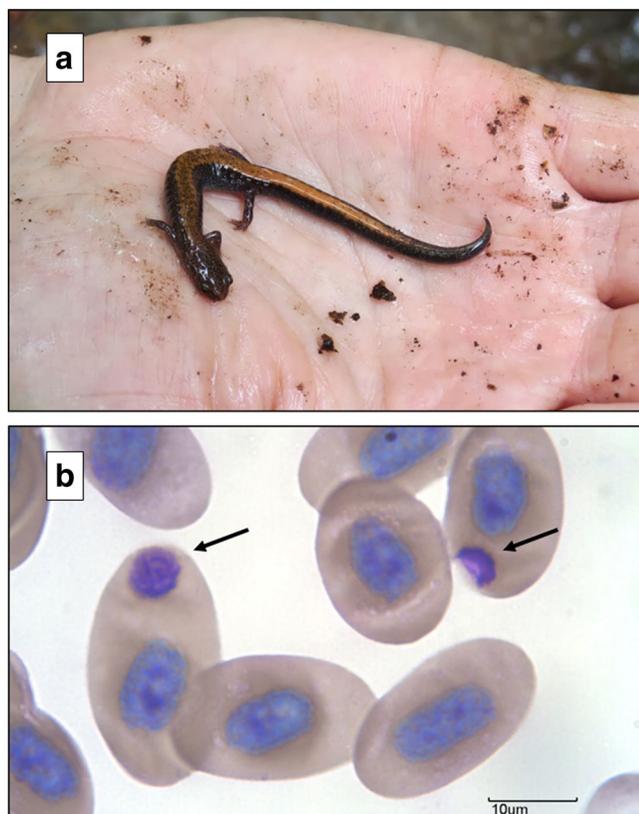


Fig. 1 **a** Red-backed salamander, *Plethodon cinereus*, captured at the Mountain Lake Biological Station, in Giles Co., Virginia. Photograph by Pat Davis. **b** Photomicrograph of red-backed salamander red blood cells with inclusions of the rickettsial bacteria (arrows)

Table 1 Summary of collecting sites for red-backed salamanders and collection results for each site

Location name	Coordinates	Elevation (m)	Elevation group ^a	Number of salamanders	Percent infected
Pandapas Pond	37.283°N, -80.470°W	670	1	10	0.0
Potts Rail Trail	37.467°N, -80.435°W	700	1	16	0.0
Lower White Rock Road	37.438°N, -80.515°W	822	2	14	14.3
White Rocks	37.430°N, -80.492°W	945	2	13	7.7
Route 613	37.424°N, -80.507°W	1000	2	11	0.0
MLBS ^b	37.375°N, -80.522°W	1200	3	21	28.6
Wind Rock	37.412°N, -80.523°W	1250	3	15	20.0
Bald Knob	37.349°N, -80.538°W	1310	3	13	15.4
All sites				113	12.4

All sites were in Giles Co, Virginia (USA), and collections were made during July 2013

^a For one analysis of infection prevalence, sites were pooled into three groups based on elevation

^b Mountain Lake Biological Station, where prevalence in 2008 was 21.7 % (Davis et al. 2009)

When films were dried, they were stored in a plastic slide box until examination (below). The salamander bodies were kept in a cooler and brought to a lab at the Mountain Lake Biological Station. There, they were dissected to determine gender. Note that gender was not determined from 11 salamanders from the MLBS site. From the photographs, we recorded the snout-vent length (SVL, in cm) of all salamanders (Davis and Grayson 2007).

Blood parasite screening Salamander blood films were stained with Giemsa (Camco Quik Stain II) and examined with a light microscope (at $\times 1000$ magnification) by one of us (AKD) for the presence of *Rickettsia* bacteria (Fig. 1b). With Giemsa staining, the bacteria appear as violet or purple circular inclusions within the cytoplasm of erythrocytes, and these inclusions are easily recognized (Davis et al. 2009; Davis and Cecala 2010). Slides were moved in a zigzag fashion across the stage so that most of the blood smear was viewed. At least 50 fields of view were inspected per film. If no inclusions were seen, we classified the animal as uninfected.

Data analysis To determine if infection prevalence has changed at the MLBS site between 2008 (from the Davis et al. 2009 study) and the current study (2013), we compared the frequency of infection (number uninfected and number infected) at this site between both years using chi-squared tests. We further compared infection frequencies from all sites in 2008 to all sites in the current study. Our second question concerned the effect of elevation on prevalence, and for this, we used logistic regression to determine if infection (yes, no) varied by sex, size (SVL), and elevation. Size was a continuous covariate. We initially treated elevation also as a covariate, but we also considered this as a categorical predictor in a second model. For this second model, we pooled the eight

locations into three elevation groups (Table 1) and used these as a categorical predictor. Analyses were conducted using the Statistica 12.0 software package.

Results

For this study, we collected a total of 113 salamanders across the 8 sites (Table 1). The sex ratio was biased toward males; of the 104 salamanders we identified to gender, 87 (84 %) were males and 17 (16 %) were females. From our evaluation of salamander blood smears, the prevalence of the rickettsial bacteria at the Mountain Lake site was 28.6 % (6 of 21 salamanders infected). This is compared to 21.7 % (5 of 23 salamanders infected) from the same site 5 years earlier (Davis et al. 2009). These frequencies were not significantly different ($\chi^2 = 0.273$, $p = 0.601$). Prevalence across all collection sites in 2008 was 16.7 % or 17 out of 102 salamanders. In the current study, prevalence across all elevations was 12.4 % or 14 out of 113 salamanders. These frequencies were also not significantly different ($\chi^2 = 0.795$, $p = 0.372$). Collectively then, these data indicate the prevalence of the rickettsial bacteria in red-backed salamanders has not changed in the 5-year span of these two studies.

In our analysis of elevation, sex was not a significant predictor of rickettsial bacteria infection in the logistic regression model, but body size was (Table 2), with infections being more prevalent in larger salamanders. This is consistent with prior findings (Davis et al. 2009). Moreover, salamanders tended to be larger at the highest elevations (Fig. 2a). We found contrasting effects of elevation, depending on the way we treated elevation in the two models. When considered as a continuous covariate, there was no effect ($p = 0.3275$; Table 2). In contrast, prevalence of infection varied significantly across the three elevation groups ($p = 0.0254$; Table 2); no salamanders were

Table 2 Summary of two logistic regression models used to examine effects of sex, size, and elevation on prevalence of rickettsial infection in red-backed salamanders in Giles Co., Virginia

Model	Predictor	df	Wald	χ^2	p value
1	Sex (categorical)	1	-30.81	0.71	0.3979
	SVL (continuous)	1	-33.52	6.15	0.0131
	Elevation (continuous)	1	-30.93	0.96	0.3275
2	Sex (categorical)	1	-28.77	0.67	0.4124
	SVL (continuous)	1	-31.27	5.69	0.0171
	Elevation group (categorical)	1	-30.93	5.00	0.0254

In model 1, elevation was treated as continuous, and in model 2, all sites were pooled into three groups based on elevation (see Table 1), and this was treated as a categorical predictor. See Fig. 2 for the visual depiction of the effect of elevation group

infected at the lowest elevations, and 22.5 % were infected at the highest elevations (Fig. 2b and Table 1).

Discussion

Results from this study showed that rickettsial infection levels in red-backed salamanders at MLBS had remained remarkably constant over a 5-year time span, which suggests that this pathogen is not currently escalating or intensifying in this local population. Moreover, the similar overall infection levels between 2008 and 2013 across all salamander collections also suggest that this pathogen is consistently present at low levels (12–16 %) throughout the red-backed salamander range. Given this temporal consistency, plus the widespread nature of the pathogen, it stands to reason that this rickettsial agent is a naturally occurring parasite of this salamander, as opposed to a novel, rapidly spreading pathogen.

In evaluating salamanders across multiple elevations, we encountered what appears to be a paradox; salamander populations at the highest elevations appeared to be thriving, yet these contained the highest incidence of infections. Evidence of thriving populations comes from our anecdotal observations made during collecting; at the highest elevations, 10–15 individuals could be collected by one person in 2–3 h at the high sites, but it took two full days to catch the same number at the lowest elevation sites (Davis, unpublished data). Moreover, salamanders at the highest elevations were also about 15 % larger than those at middle and low elevations (Fig. 2a). This is consistent with recent work from other investigators (Peterman et al. 2016). It was ironic then that these high-elevation populations also harbored the highest incidence of infection (Fig. 2b). In retrospect, these two observations may not be unrelated. It may be that lower-elevation populations are more dispersed, leading to fewer opportunities for parasite transmission. Alternatively, the

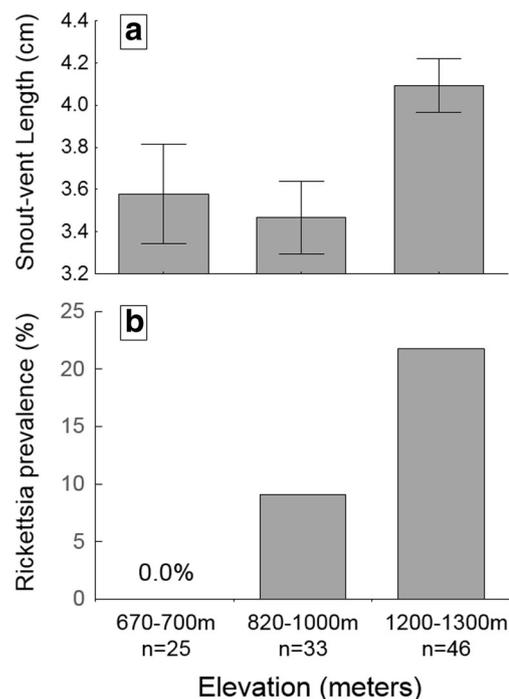


Fig. 2 **a** Comparison of average body size (snout-vent length) of red-backed salamanders at varying elevations in this study. Error bars indicate 95 % confidence intervals. **b** Prevalence of the rickettsial bacteria at varying elevations

smaller-sized, lower-elevation populations could simply be less tolerant of infections; this idea is consistent with the fact that the parasite apparently is only associated with large salamanders (Davis et al. 2009).

Comparisons of parasite infection dynamics across different elevations essentially provide a glimpse into the future for these and other terrestrial, Appalachian salamanders. From this study, and from other investigations of related species (Dodd and Dorazio 2004), it is clear that the cool, moist woodland habitat at the high-elevation peaks provides the ideal environmental conditions for these animals. At lower elevations, the forests become increasingly drier, which is not an ideal condition for terrestrial salamanders (e.g., Grover 1998), and this is consistent with what we observed during collecting. Climate change is expected to constrict the range of most Appalachian salamanders (Milanovich et al. 2010). As this happens, most of the lower-elevation habitats will in theory become drier and uninhabitable, leaving only the higher-elevation sites as isolated refugia. Our data suggest that these sites currently contain the highest incidence of infections, which means that these future remnant populations of red-backed salamanders will likely continue to harbor this mysterious parasite.

Acknowledgments We thank Sonia Altizer and the MLBS Disease Ecology class of 2013 for their assistance with the field and lab work. This project was supported by an Early Career Fellowship from the

Mountain Lake Biological Station to AKD. We thank Joe Milanovich for the helpful comments on the manuscript.

Compliance with ethical standards

Funding This project was supported by an Early Career Fellowship Award from the Mountain Lake Biological Station, given to the lead author (A. Davis).

Conflict of interest All authors declare they have no conflict of interests in this study.

Ethical approval All animals in this project were handled and sacrificed with approval from the Institutional Animal Care and Use Committees of the University of Virginia, and the University of Georgia.

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