Vol. 84: 1–7, 2009
doi: 10.3354/dao02026

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 9

Experimental infection of North American plethodontid salamanders with the fungus *Batrachochytrium dendrobatidis*

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ABSTRACT: The presence in the southeastern USA of *Batrachochytrium dendrobatidis*, a fungal pathogen of amphibians, is a potential threat to the diverse salamander assemblages in the region. In this study, we tested the susceptibility of plethodontid salamanders to infection with *B. dendrobatidis*. We experimentally infected one terrestrial species (*Plethodon metcalfi*) and one stream-dwelling species (*Desmognathus monticola*). Mortality of *P. metcalfi* due to *B. dendrobatidis* infection was 41.7% and was higher at 8°C (75%) than at 16°C (8.3%). *B. dendrobatidis* did not cause any mortality in *D. monticola*. Infected salamanders exhibited few of the clinical signs associated with *B. dendrobatidis* infection; however, they exhibited histologic signs of disease. Our results suggest that *Plethodon* species in the southeastern USA are at risk of becoming infected with *B. dendrobatidis* and developing chytridiomycosis. However, some animals may have survived with or cleared the infection. Additional studies are required to determine whether chytridiomycosis is a significant factor in declines of plethodontid salamanders.

KEY WORDS: Chytridiomycosis · *Batrachochytrium dendrobatidis* · Salamander · Amphibian · Mortality

INTRODUCTION

The Appalachian Highlands region of eastern North America hosts 38 species of endemic plethodontid salamanders, more than any other region in the world (Duellman & Sweet 1999). Many of these species have restricted ranges, often on mountain tops, in essence rendering them island populations surrounded by inhospitable lowlands (MacArthur & Wilson 1967). Endemic species on islands are vulnerable to population declines that result from disease introduction (Warner 1968). The presence in the southeastern USA of *Batrachochytrium dendrobatidis*, a fungal pathogen of amphibians, is a potential threat to the region's diverse salamander assemblages (Rothermel et al. 2008). *B. dendrobatidis* has been detected in wild populations of amphibians in at least 2 sites in the southern Appalachian Mountains, one in northeastern Georgia and one in western North Carolina (Rothermel et al. 2008). Investigations of disease susceptibility and population trends of plethodontid salamanders in the Appalachian Highlands are urgently needed, given recent reports of their declines in this region (Highton 2005) and in the Mesoamerican Highlands (Lips & Donnelly 2005).

Batrachochytrium dendrobatidis causes chytridiomycosis, an often fatal disease that has caused amphibian population declines in Panama, Costa Rica, Australia and western North America (Berger et al. 1998, Lips 1999, Muths et al. 2003, Lips et al. 2006, Rachowicz et al. 2006) and a species extinction in Australia (Schloegel et al. 2006). Infection with *B. dendro-*

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batidis is limited to the keratinized epithelium in the skin of postmetamorphic animals and the oral disc of anuran larvae (Berger et al. 1998). Death has been attributed to disruption of cutaneous physiologic functions, such as osmoregulation (Voyles et al. 2007). Although a small number of wild-caught plethodontid salamanders have been found infected with *B. dendrobatidis* (Pasmans et al. 2004, Cummer et al. 2005, Byrne et al. 2008), their susceptibility to infection has not been thoroughly investigated. In this study, we tested the susceptibility of plethodontid salamanders to infection with *B. dendrobatidis*. We experimentally infected one terrestrial species, the southern gray-cheeked salamander *Plethodon metcalfi*, and one stream-dwelling species, the seal salamander *Desmognathus monticola*. *P. metcalfi* inhabits the forest floor, seeking refuge in leaf litter, under logs or in small underground tunnels during the day. *D. monticola* typically inhabits rocky, coolwater streams and sometimes frequents crevices in wet rock faces (Petranka 1998). Both species are locally common and, therefore, were suitable model species for our initial investigation of the disease risk to plethodontid salamanders.

MATERIALS AND METHODS

Salamander collection and maintenance. Adult *Plethodon metcalfi* and *Desmognathus monticola* were captured by hand during diurnal and nocturnal searches of stream and forest sites within 6 km of Highlands, North Carolina from April 20 to 23, 2006. Salamanders were housed individually in 415 ml GladWare® (Clorox) plastic containers with perforated lids in 2 environmental chambers at 12°C for 7 wk. Prior to the experiment, each salamander was screened for *Batrachochytrium dendrobatidis* infection by a PCR-based assay (see 'Diagnostic procedures'). Salamanders were fed pinhead crickets dusted with calcium supplement powder (JurassiPet™) every 5 d, provided with wet, unbleached paper towels for moisture and cover and maintained on a 12:12 h light:dark schedule.

Preparation of the fungus inoculum. Zoospores of the type isolate *Batrachochytrium dendrobatidis* (JEL197) (Longcore et al. 1999) were used for the inoculum. This isolate was first isolated from a dead blue poison dart frog *Dendrobates azureus* infected with *B. dendrobatidis* and held in captivity. Since its isolation in 1997, JEL197 had been serially passed *in vitro* 28 times. The fungus was maintained at 23°C in tryptone broth (10 g tryptone, 1 l double distilled water [ddH_2O]). To harvest zoospores, cultures were transferred to tryptone agar (10 g tryptone, 10 g agar, 1 l ddH_2O). After 7 d of growth, when the maximum number of fungal zoospores was being produced, the agar plates were flooded with sterile ddH_2O and allowed to sit for 30 min. The ddH_2O in which the zoospores had collected was combined to form the inoculum. To determine zoospore concentration in the inoculum, 100 µl of the inoculum was added to 100 µl Lugol solution (Sigma-Aldrich) in a microcentrifuge tube. Lugol solution kills and stains zoospores. Microscopic counts were made with a hemocytometer. The inoculum volume was adjusted to a concentration of 3.56×10^5 zoospores ml^{-1}. Each salamander was exposed to 1.07×10^7 zoospores in 30 ml, a volume sufficient to submerge the venter (see 'Experimental design').

Experimental design. Salamanders were randomly assigned to one of the following treatment groups: 8°C/exposed, 8°C/unexposed, 16°C/exposed and 16°C/unexposed (n = 12 for each species per treatment). The experiment was conducted at 2 temperatures because temperature is known to affect growth of *Batrachochytrium dendrobatidis*. The low temperature (8°C) is below the suitable growth range for isolate JEL197 of *B. dendrobatidis* of 10 to 25°C on tryptone/gelatin hydrolysate/lactose (TGhL) agar culture and the high temperature (16°C) is within this range. Temperatures were below the maximal growth range of 17 to 25°C (Piotrowski et al. 2004). The 2 temperatures chosen are representative of the relatively cool microhabitats *Plethodon metcalfi* and *Desmognathus monticola* inhabit and, thus, well within the thermal tolerance range for these species (Spotila 1972). The salamanders were arranged in 2 incubators according to a randomized complete block design with incubator shelf as the block. Each block contained an equal number of replicates of each species-by-disease treatment.

Thirty milliliters of inoculum were added to the exposed treatment containers and 30 ml of sterile water to the unexposed treatment containers. After 8 h the inoculum and water were drained from the containers and replaced with wet paper towels. Salamanders in the exposed treatment were only exposed to *Batrachochytrium dendrobatidis* once. Treatment groups were not indicated on the containers, which ensured unbiased observation of salamanders for clinical signs of disease, such as lethargy, skin sloughing, inappetence (Nichols et al. 2001) and other abnormal behavioral and physical changes. Severely moribund animals were euthanized with MS-222 (tricaine methanesulfonate, Sigma-Aldrich). Prior to the start of the experiment we decided that the experiment would be ended if (1) no clinical signs of disease or mortality were observed in the first 30 d or (2) after observing clinical signs of disease or mortality, no additional signs of disease or mortality were observed for at least 60 d. The experiment was terminated at Day 189 and all surviving salamanders were euthanized with MS-222.

Diagnostic procedures. Histologic examination of a single foot was performed on every salamander after natural death or euthanasia to look for evidence of *Batrachochytrium dendrobatidis* infection. The foot was fixed in 10% neutral buffered formalin, processed and embedded in paraffin, sectioned and stained with hematoxylin and eosin. Slides were observed by light microscopy under 400× magnification for characteristic *B. dendrobatidis* sporangia in the epidermis (Longcore et al. 1999, Berger et al. 2005). In addition, 8 salamanders representing a range of experimental outcomes (Table 1) were selected for histologic evaluation of whole body sections that included skin and internal organs to better define the distribution of *B. dendrobatidis* infection and to search for evidence of other underlying diseases that could contribute to mortality. Histologic evaluation was performed blind to infection status and outcome. After fixation in 10% neutral buffered formalin, the carcasses were demineralized by immersion for 6 h in a hydrochloric acid solution. After demineralization, the body and tail were serially cross-sectioned at 3 mm intervals in the transverse plane and the head was bisected in the median plane with all of the resultant sections processed for histology as described for the feet. Tissues examined histologically in these sections included skin, brain, spinal cord, heart, skeletal muscle, bone and bone marrow, liver (including subcapsular hematopoietic tissue), spleen, kidney, gonad, stomach, small and large intestines and pancreas.

PCR assays were used for infection screening prior to the experiment and when histologic results were inconclusive. For the pre-experiment PCR assays, the ventral skin of each salamander was swabbed 30 times with a cotton-tipped cleaning stick (Puritan Medical Products). For the post-experiment PCR assays, the whole right hind foot was removed. Skin swabs and feet were preserved in 70% ethanol and submitted to Pisces Molecular (Boulder, Colorado) for standard PCR assay for the presence of *Batrachochytrium dendrobatidis* using primers developed by Annis et al. (2004). DNA from *Rhizophydium haynaldii*, a chytrid fungus, was used as a negative control and water was used to detect sample contamination. Each sample was run only once in the assay. Swabs in ethanol were prepared by vortexing the sample tube, transferring the ethanol to a clean tube, spinning down for 3 min and removing the supernatant. Feet were prepared by cutting off the toes and placing them in a clean tube. QIAGEN® ATL+PK tissue lysis buffer was added to prepared swabs and feet and incubated at 55°C for 60 min. The DNeasy® Blood and Tissue Kit (QIAGEN) protocol was used for the rest of the procedure.

Data analysis. All data were analyzed using SAS Software 9.1 (SAS Institute). Mortality rates were compared using Fisher's exact test (PROC FREQ). The effect of salamander experimental location on mortality was determined using Cox regression analysis (PROC PHREG). A hazard ratio (HR) >1 indicates increased risk of mortality due to the disease as a result of the factor tested. Differences in initial body mass between salamanders exposed to *Batrachochytrium dendrobatidis* that died versus those that survived were analyzed using a *t*-test (PROC TTEST).

RESULTS

Mortality

All salamanders tested negative by PCR for *Batrachochytrium dendrobatidis* infection at the onset of the study. Overall, mortality was significantly higher for salamanders exposed to *B. dendrobatidis* zoospores

Table 1. *Desmognathus monticola* and *Plethodon metcalfi*. Salamanders examined comprehensively by histology. Salamanders that survived for the duration of the experiment were euthanized. All salamanders listed were exposed to *Batrachochytrium dendrobatidis* (*Bd*). ID = individual salamander identification number; na = not applicable; PCR not conducted because initial histology of feet (not shown here) was conclusive

ID	Temperature (°C)	Histologic examination	PCR result	Outcome	Days post-infection
Desmognathus monticola					
DM1	8	Moderate lesions consistent with *Bd*; *Bd* thalli absent	–	Died	40
DM11	8	No lesions	+	Euthanized	189
DM13	16	No lesions	na	Euthanized	189
Plethodon metcalfi					
PM1	8	Lesions consistent with *Bd*; *Bd* thalli present	na	Died	18
PM5	8	Lesions consistent with *Bd*; *Bd* thalli present	na	Died	31
PM6	8	Lesions consistent with *Bd*; *Bd* thalli present	+	Died	32
PM10	8	Mild lesions consistent with *Bd*; *Bd* thalli absent	na	Died	43
PM19	16	No lesions; *Bd* thalli absent	–	Euthanized	189

(31.3%) than for unexposed salamanders (4.2%) (p = 0.00086). Of the *Plethodon metcalfi* exposed to *B. dendrobatidis*, 41.7% died due to *B. dendrobatidis* infection and 16.7% died due to unknown causes. Initial body weight of exposed *P. metcalfi* did not affect mortality ($t = 0.64$, p = 0.53). No unexposed *P. metcalfi* died. Mortality of *P. metcalfi* due to *B. dendrobatidis* was higher at 8°C (75%, n = 9) than at 16°C (8.3%, n = 1). Mortality of *P. metcalfi* due to unknown causes was also higher at 8°C (25%, n = 3) than at 16°C (8.3%, n = 1), but not substantially. The mean time to death of *P. metcalfi* infected with *B. dendrobatidis* was 36.0 d (Fig. 1). Incubator shelf (HR = 0.871, p = 0.5439) had no effect on mortality.

No *Desmognathus monticola* that died before termination of the experiment had PCR evidence of *Batrachochytrium dendrobatidis* infection. Only one surviving *D. monticola* in the exposed treatment group was infected, according to PCR assay (DM11, Table 1). A low level of mortality was observed in *D. monticola* due to unknown causes (unexposed group: 8.33%, n = 2; exposed group: 4.17%, n = 1), and only at 8°C.

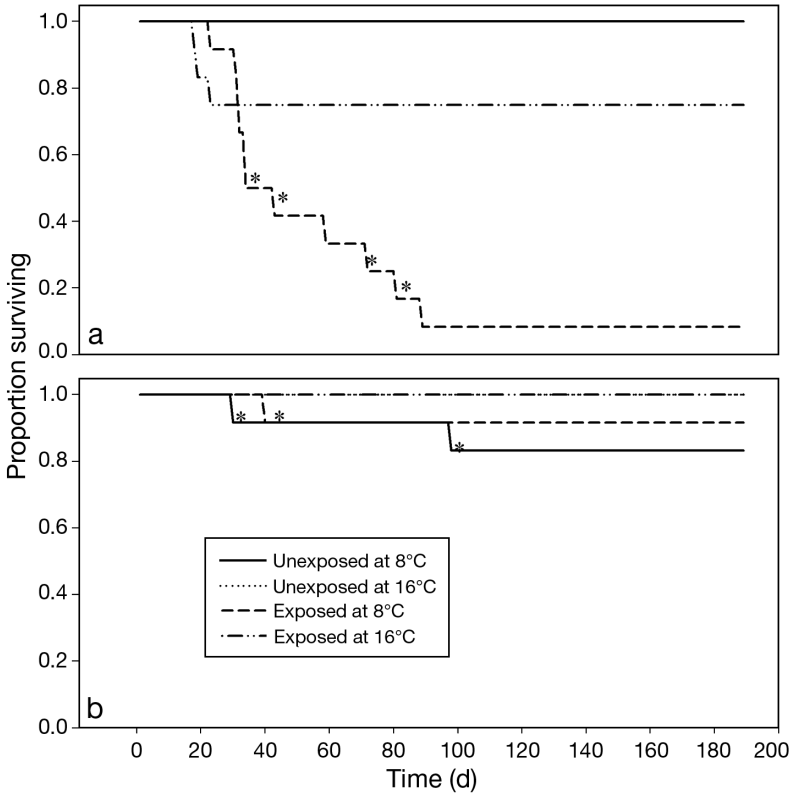

Fig. 1. Survival curves for (a) *Plethodon metcalfi* and (b) *Desmognathus monticola* after exposure to *Batrachochytrium dendrobatidis* or sterile water. Unexposed *P. metcalfi* 8°C and 16°C lines are overlaid because both groups experienced complete survival. Unexposed and exposed *D. monticola* 16°C lines are overlaid because both groups experienced complete survival. Asterisks (*) indicate salamander deaths due to unknown causes

Signs of disease

The most common physical abnormality observed in the course of the study was redness of the ventral surface of the digits, feet and occasionally chin and tail. However, this type of abnormality was observed in both exposed (n = 29) and unexposed (n = 24) salamanders. There was no evidence of red leg disease. Infected salamanders exhibited few of the clinical signs associated with *Batrachochytrium dendrobatidis* infection in other studies of salamanders (Davidson et al. 2003, Pasmans et al. 2004, Cummer et al. 2005) or anurans (Nichols et al. 2001, Berger et al. 2004, Rachowicz & Vredenburg 2004, Carey et al. 2006) such as excessively shedding (sloughing) skin or changes in skin pigmentation. Only 1 infected *Plethodon metcalfi* was noted to have excessive shedding skin at the time of death. Two infected *P. metcalfi* autotomized part or all of their tails 1 to 2 d before death with no other signs of disease.

In contrast, histologic examination of the feet of *Plethodon metcalfi* exposed to *Batrachochytrium dendrobatidis* revealed multifocal moderate parakeratotic hyperkeratosis with myriad intracellular chytrid thalli consistent with *B. dendrobatidis* infection (Fig. 2a). The feet of unexposed *P. metcalfi* and all *Desmognathus monticola* appeared normal. Three *P. metcalfi* (PM1, PM5, PM6; Table 1) exposed to *B. dendrobatidis* and examined comprehensively by histology had similar lesions consistent with *B. dendrobatidis* infection. Lesions in these animals were most evident in the skin of the ventral and lateral body. In portions of the body cranial to the pelvic region, lesions were minimal to mild and chytrid thalli were present multifocally in small numbers (Fig. 2b). In the body caudal to the pelvis, lesions were mild to moderate with higher numbers of chytrid thalli (Fig. 2c) similar to the pattern observed in the feet. Examination of other organs did not reveal evidence of significant disease processes. A single *P. metcalfi* (PM1; Table 1) had small numbers of encysted nematode parasites in the submucosa of the stomach that were interpreted as an incidental finding. In 5 additional salamanders exposed to *B. dendrobatidis* (PM10, PM19, DM13, DM11, DM1; Table 1), no *B. dendrobatidis* thalli could be identified by histologic examination. In one of these animals (DM1; Table 1), there was moderate parakeratotic hyperkeratosis and epidermal hyperplasia in the skin of the body caudal to the pelvis similar to that

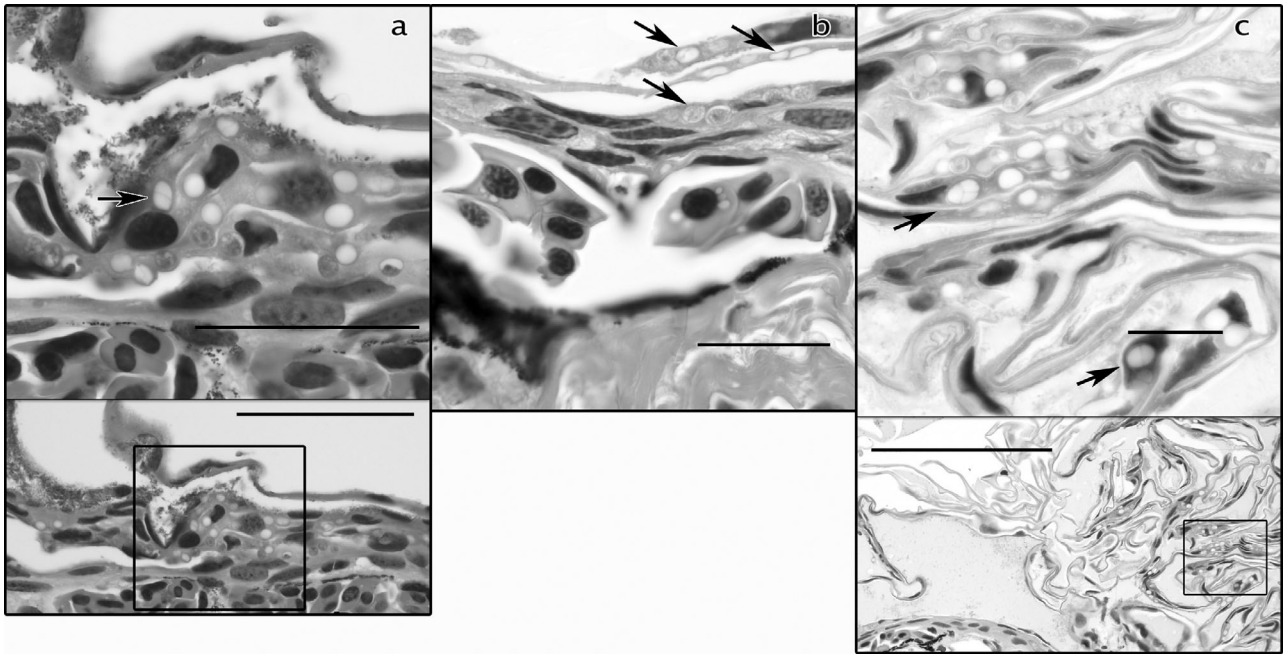

Fig. 2. *Batrachochytrium dendrobatidis* infecting *Plethodon metcalfi*. (a) Histologic section of skin from the foot. The top panel shows detail of thalli including an internally septate thallus (arrow; colonial thallus characteristic of *B. dendrobatidis*). Thalli in this image are empty, having discharged their zoospores. Scale bar = 50 µm. The bottom panel shows the area represented in the top panel (within the box) and the surrounding tissue with moderate parakeratotic hyperkeratosis and intracellular thalli of *B. dendrobatidis* (arrows). Scale bar = 100 µm. (b) Histologic section of skin from the lateral body. There is mild focal hyperkeratosis with small numbers of intracellular chytrid thalli (arrows). Scale bar = 200 µm. (c) Histologic section of skin from the tail. The top panel shows internally septate colonial thalli (arrows). Scale bar = 20 µm. The bottom panel shows the area represented in the top panel (within the box) and the surrounding tissue with moderate parakeratotic hyperkeratosis and intracellular *B. dendrobatidis* thalli. Scale bar = 200 µm. All sections were stained with hematoxylin and eosin

observed in animals with identifiable *B. dendrobatidis* thalli. A second animal (PM10; Table 1) had minimal to mild parakeratotic hyperkeratosis in multiple sections from skin from throughout the body. These 2 salamanders (DM1, PM10; Table 1) died before the termination of the experiment and were considered negative for *B. dendrobatidis* infection due to a negative PCR result and the absence of *B. dendrobatidis* thalli in the histologic samples observed. The 3 remaining exposed animals (PM19, DM13, DM11; Table 1) had histologically normal skin. No evidence of significant underlying disease processes was observed in these animals.

DISCUSSION

This is the first study to test experimentally the susceptibility of plethodontid salamanders to infection with *Batrachochytrium dendrobatidis* and to the development of chytridiomycosis. The mortality rate of *Plethodon metcalfi* due to chytridiomycosis was low compared with the mortality rate of this disease in dendrobatids (Nichols et al. 2001) and bufonids (Carey et al. 2006), but was substantially higher than the mortality rates due to unknown causes. One *Desmognathus monticola* (DM11) survived with *B. dendrobatidis* infection for 189 d and remained asymptomatic throughout this time. The infection was detected by PCR, but no *B. dendrobatidis* thalli were observed by histologic examination. This salamander may have been clearing the infection, as Davidson et al. (2003) observed in *Ambystoma tigrinum*. It also may have been persisting with a low-level infection, as do *Rana catesbeiana* (Daszak et al. 2004). The remaining *D. monticola* exposed to *B. dendrobatidis* may have become infected with *B. dendrobatidis* but cleared the infection, a phenomenon that could not be observed in the study because salamanders were tested only at the beginning and end of the experiment. Alternatively, these *D. monticola* may have been resistant to infection altogether. Both species experienced low levels of mortality unrelated to *B. dendrobatidis* infection, which may be attributed to stress from captivity.

The histologic lesions observed on the body of *Plethodon metcalfi* were similar to those observed in *Ambystoma tigrinum* (Davidson et al. 2003). These lesions differed from the lethal diffuse lesions seen in many anurans because there was less hyperkeratosis

and more multifocal distribution of thalli. In contrast, the distribution of heavier levels of infection in the body caudal to the pelvis, feet and tail in the experimental cases was similar to that described for a single naturally infected *P. neomexicanus* (Cummer et al. 2005). Future research should address differences between caudates and anurans that could contribute to different disease responses in these 2 amphibian groups.

Multiple factors can influence the susceptibility of individual species to development of chytridiomycosis, including differences in host behavior, innate defenses such as skin peptides, and skin bacterial flora. Time spent in water, basking or in contact with other amphibians could affect the probability of transmission of *Batrachochytrium dendrobatidis* (Rowley & Alford 2007); however, it is unlikely host behavior caused the difference in infection susceptibility observed in this study since both species were maintained under the same conditions, which inhibited behavioral response. Skin peptide profiles differ substantially among amphibian species (Woodhams et al. 2006), which could account for the difference in susceptibility between *Plethodon metcalfi* and *Desmognathus monticola*. In addition, Harris et al. (2006) observed variation in the species of cutaneous bacteria isolated from *P. cinereus* and *Hemidactylium scutatum* and their effectiveness in inhibiting *B. dendrobatidis* growth. Differences in abundance of bacteria that inhibit *B. dendrobatidis* probably exist between individual hosts (Woodhams et al. 2007a) and between host populations (Woodhams et al. 2007b).

Disease models would be more robust if they incorporated accurate removal rates. Infection period, or time to death from pathogen exposure, is a critical element of disease dynamics. According to the susceptible/infected/recovered (SIR) model for epidemics, the removal rate (γ) is inversely proportional to the infection period and affects the turnover of infected organisms due to death or recovery (Anderson & May 1991). Mean time to death of exposed *Plethodon metcalfi* (36.0 d) was similar to that of other experimentally infected amphibians (mean time to death = 41.8 d in *Rana muscosa*, Rachowicz & Vredenburg 2004; 40.0 d in *Mixophyes fasciolatus*, Berger et al. 2004). However, it is difficult to compare infection period and mortality rates between experiments that use different isolates of *Batrachochytrium dendrobatidis*, exposure times, doses, inoculum concentrations and temperatures.

Plethodon metcalfi exposed to *Batrachochytrium dendrobatidis* experienced higher mortality at 8°C than at 16°C. However, Piotrowski et al. (2004) observed highest growth of *B. dendrobatidis* in cultures at 17°C, with growth slowing at 10°C. Species-specific differences in expression of cutaneous antimicrobial peptides could account for the discrepancy between our results and the *in vitro* study of *B. dendrobatidis* by Piotrowski et al. (2004). For instance, *Rana sylvatica* held at 5°C do not secrete antimicrobial peptides on their skin, but do at higher temperatures (Matutte et al. 2000). However, peptides isolated from *R. pipiens* were more effective at 10°C than at 22°C in an *in vitro* assay of *B. dendrobatidis* growth inhibition (Rollins-Smith et al. 2002). Salamanders could experience seasonal variation in cutaneous microflora, which would translate to susceptibility varying according to temperature. More studies should test the effects of temperature on susceptibility, including realistic fluctuating temperature regimes.

The terrestrial nature of *Plethodon* salamanders does not eliminate their risk of *Batrachochytrium dendrobatidis* infection. *B. dendrobatidis* is an aquatic fungus; however, like other Chytridiomycetes, it probably can survive in moist substrate (Johnson & Speare 2005). Aquatic salamanders traveling away from streams could spread *B. dendrobatidis* to terrestrial habitats of *Plethodon* spp. In the Nantahala National Forest, North Carolina, adult *Desmognathus monticola* travel up to 8.6 m away from streams during nocturnal movements (Crawford & Semlitsch 2007). As we have demonstrated, *D. monticola* can be infected with *B. dendrobatidis* and, thus, could serve as carriers of the pathogen.

Our results suggest that *Plethodon* species in the southeastern USA are at risk of becoming infected with *Batrachochytrium dendrobatidis* and developing chytridiomycosis. However, some salamanders may survive with or clear infection if given enough time, similar to *Ambystoma tigrinum* (Davidson et al. 2003). Additional studies are required to determine whether chytridiomycosis is likely to be a significant factor in declines of plethodontid salamanders.

Acknowledgements. We thank S. Rothermel for help with salamander collection, L. Hardeman for animal care, and H. Vazquez for lab development. This research was funded by the Amphibian Research and Monitoring Initiative of US Geological Survey, through a Declining Amphibian Population Task Force Seed Grant to B.B.R. and V.M.V, and by a Consortium for Conservation Medicine grant to V.M.V. Proposal preparation and fieldwork were supported by Savannah River Ecology Laboratory (under Financial Assistance Award DE-FC09-96SR18-546 between University of Georgia and US Department of Energy). Salamander collection was conducted under a US Forest Service permit (authorization HIG651801) and a North Carolina Wildlife Resources Commission 2006 Wildlife Collecting License (permit no. 964). Laboratory procedures were approved by the University of Georgia's Animal Care and Use Committee (Animal Welfare Assurance no. A3437-01).

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Submitted: May 5, 2008; Accepted: December 22, 2008
Proofs received from author(s): February 26, 2009