

**RH: CHYTRID IN PLETHODONTID SALAMANDERS**

*BATRACHOCHYTRIUM DENDROBATIDIS* IN A CAPTIVE COLLECTION OF GREEN  
SALAMANDERS (*ANEIDES AENEUS*), LONG-TAILED SALAMANDERS (*EURYCEA*  
5 *LONGICAUDA*), AND TWO-LINED SALAMANDERS (*EURYCEA BISLINEATA*)

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25     Abstract: A chytridiomycosis outbreak from *Batrachochytrium dendrobatidis* (*Bd*) in a  
mixed species plethodontid salamander exhibit resulted in four green salamander (*Aneides*  
*aeneus*) deaths. One green salamander died prior to treatment and three died during treatment  
with daily 0.005% itraconazole baths. All salamanders had evidence of severe *Bd* infections via  
cytology, histopathology, and/or PCR at the time of death. Ten long-tailed salamanders (*Eurycea*  
30 *longicauda*) and one two-lined salamander (*Eurycea bislineata*) that shared the enclosure were  
initially negative for *Bd* on quantitative PCR, but were prophylactically treated with daily 0.01%  
itraconazole baths for 11 days. Post-treatment testing yielded eight long-tailed salamanders and  
one two-lined salamander positive for *Bd* with low gene equivalents. All salamanders were  
negative following two to three treatment courses, and there were no additional mortalities. The  
35 difference in mortality and fungal load suggested that genus *Aneides* salamanders may be more  
susceptible to *Bd* than genus *Eurycea* salamanders.

*Key words:* *Aneides aeneus*, *Batrachochytrium dendrobatidis*, chytrid, itraconazole,  
40 Plethodontidae, salamander.

## BRIEF COMMUNICATION

A green salamander (*Aneides aeneus*) in a multi-species plethodontid salamander exhibit housing four green salamanders, ten long-tailed salamanders (*Eurycea longicauda*), and one two-lined (*Eurycea bislineata*) salamander was found dead with no premonitory signs (day 0). The enclosure measured 2.1x0.6x1.2 meters and was constructed of fiberglass overlaid with hydrostone. The substrate consisted of live Java moss and the exhibit was planted with mostly fake plants. Environmental conditions were: air temperature (21-22 °C), humidity (60-75%), and water temperature (18 °C). On day 3, the remaining green salamanders reportedly exhibited abnormal behavior, sitting in open exhibit areas versus hiding in rockwork. A group swab of the three salamanders was collected and submitted to the Amphibian Disease Laboratory at the San Diego Zoo Institute for Conservation Research for quantitative PCR (qPCR) to detect *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*).<sup>4,11</sup> On day 10, test results indicated the three green salamanders were positive for *Bd* and negative for *Bsal*. One salamander had poor righting reflex and abnormal shaky movement. Treatment was initiated for all green salamanders on day 11.

Treatment prescribed consisted of an itraconazole bath (Sporanox, Janssen Pharmaceutica N.V., Beerse, Belgium, diluted with carbon-filtered water to 0.005%, 5 min bath s.i.d. for 11 days) delivered individually in plastic tanks lined with damp paper towels. Tanks were maintained in a quarantine room and soaked in 0.6% bleach solution x 1 min, rinsed, and air-dried between treatments.<sup>2</sup> Dedicated personal protective equipment (scrubs, footwear, gloves) was employed.

All three green salamanders died during treatment after receiving one, four, and nine days of treatment, respectively. Cytology of the integument was performed on the salamander that died

70 after one day of treatment with abundant chytrid organisms identified (Figure 1). Histopathologic findings of all four deceased green salamanders were similar and included dermatitis and hyperkeratosis with visible chytrid organisms and chytridiomycosis as the determined cause of death. However, minimal inflammation was evident in the initial salamander (Figures 2-3).

Prior to the death of the last green salamander, individual testing of all exhibit animals  
75 yielded a positive result on qPCR for *Bd* of this individual and one long-tailed salamander at 100,000 and 1-10 gene equivalents (GE), respectively. All other individuals tested negative. Testing was completed in house using similar methods as the San Diego lab. The ventral surface of each salamander was swabbed back and forth ten times, and each foot was swabbed five times. A Qiagen DNeasy Blood and Tissue Kit (Germantown, MD 20874, USA) was used for  
80 DNA extraction following the manufacturer's protocol. Extracted DNA was assayed in duplicate for *Bd* using a previously described qPCR using CFX96 real-time system (Bio-Rad Laboratories, Hercules, CA 94547, USA).<sup>4</sup>

After the death of the last green salamander, all ten long-tailed salamanders and the remaining two-lined salamander were prescribed itraconazole baths at an increased dose of 0.01% (5 min  
85 bath s.i.d. for 11 days) based on history of exposure to positive animals. Treatment was initiated between days 17 and 28 depending on when the animal was caught out of the exhibit. Following the treatment courses (day 43), the long-tailed salamanders and two-lined salamander were swabbed for qPCR to detect *Bd*, with eight long-tailed salamanders and the two-lined salamander testing positive at 1-100 GE. All eleven animals underwent a second course of treatment as  
90 described above. Quantitative PCR performed at the end of treatment (day 63) identified one positive long-tailed salamander (10 GE). This individual underwent a third treatment course, after which all eleven salamanders were swabbed again for qPCR (day 85) and found no *Bd*.

Thus, all remaining salamanders were negative via one to three PCR tests, and were returned to the exhibit following disinfection. All plants and soil were discarded; water was replaced with  
95 10% bleach solution for ten minutes; clean water was replaced; and exhibit temperature was raised to 35 °C for 48 hr. The water was drained, and the exhibit was air-dried for two weeks prior to replacing substrate. At follow-up on day 358, there were no *Eurycea* mortalities and no subsequent clinical signs were detected.

The source of *Bd* was not determined in these cases. There were no other known *Bd* positive  
100 animals in the collection at the time of the initial case. All live plants in the exhibit were rinsed and submerged in 10% bleach solution for two minutes and then thoroughly rinsed prior to being placed on exhibit. All exhibit salamanders were new to the collection and included both wild-caught and captive-bred animals. All underwent a 30-day quarantine period prior to being released into the exhibit. Individual or pooled swabs collected during quarantine or preshipment  
105 exams and tested for *Bd* via either qPCR at San Diego or in-house were all negative. However, use of a single testing event and pooled samples may have precluded detection of low-level infections.<sup>16</sup> This may also explain how some salamanders initially negative were later found positive for *Bd*. Based on this and literature reports, repeated testing to confirm negative findings is strongly encouraged and recommended.<sup>16</sup>

110 The only other salamanders that were previously housed in the exhibit but had been moved were red-backed salamanders (*Plethodon cinereus*) and red salamanders (*Pseudotriton ruber*). All of these salamanders tested negative for *Bd* during quarantine and were confirmed negative again on qPCR after the positive individuals were identified in the exhibit.

*Batrachochytrium dendrobatidis* is associated with declines in wild salamanders in the  
115 neotropics, and has been detected in about half of the salamander species sampled for *Bd*.<sup>5,14</sup> In

the United States, however, multiple surveys have revealed that *Bd* occurs at low prevalence and intensity in wild salamander populations.<sup>9,10,12,13</sup> Susceptibility of native U.S. species in experimental exposure trials appears to vary, depending on the species, the initial dose of the fungus, experimental temperature, and skin bacteria.<sup>3,6,18</sup> The population-level effects of *Bd* in wild salamanders remain unclear.

The 100% mortality of the four green salamanders in comparison to the 0% mortality of the combined *Eurycea* species and the higher fungal loads detected in the green salamanders suggests that green salamanders (and possibly the genus *Aneides*) may be more susceptible to *Bd*. Though premonitory clinical signs were subtle or absent, high fungal loads detected in the green salamanders indicate that supportive treatments during antifungal therapy may have been beneficial.<sup>1</sup> However, treatment failures were unlikely due to reinfection, as strict biosecurity protocols instituted during treatment were consistent with previously reported successful protocols.<sup>2</sup>

Green salamanders are listed as “Endangered” in Indiana, Ohio, Maryland, and Mississippi, “Threatened” in Pennsylvania, “Protected” in Georgia, “Rare” in North Carolina, and “Of Special Concern” in South Carolina and West Virginia.<sup>15</sup> They have experienced a 98% decline in relative abundance since the 1970’s, and a causative agent of these declines has not been definitely identified.<sup>7</sup> The terrestrial behavior of this species is thought to be an attribute that could limit their exposure to *Bd*, which is a water-borne pathogen.<sup>7</sup> However, this may also lead to naivety to the pathogen and increase susceptibility when exposed. In the wild, relatively few reports are recorded in this species. No records of *Bd* in green salamanders were reported in the global *Bd* database, but a single green salamander has been reported positive on PCR for *Bd* documented from Transylvania county, North Carolina, (1 out of 21 individuals).<sup>12,14</sup>

There are also few records of *Bd* in long-tailed salamanders in the wild. Two long-tailed  
140 salamanders (of 9 individuals) were reported as PCR positive for *Bd* in the global *Bd* database,  
and a single publication reported *Bd* via PCR testing in a long-tailed salamander from Wise  
County, Virginia.<sup>8,14</sup> Treatment cleared confirmed infections in the ten long-tailed salamanders  
present in the exhibit during the outbreak in this case, though it is important to note that no  
clinical signs were noted and there were no mortalities in this species. The long-tailed  
145 salamanders in this case received a higher dose of itraconazole than the green salamanders. The  
dose protocol was increased after post-mortem cytology and histopathology of the green  
salamanders showed the continued presence of high loads of *Bd* during treatment. Despite the  
different treatment protocols, the low chytrid loads and the lack of mortality in the long-tailed  
salamanders may indicate that this species is less susceptible to chytridiomycosis than the green  
150 salamanders.

Lastly, there have been few reports of two-lined salamanders positive for *Bd* in the wild. Two  
(of 26 individuals) PCR positive animals were reported in the global *Bd* database, and 5 out of 28  
museum samples of two-lined salamanders tested positive for *Bd*.<sup>14,17</sup> The one two-lined  
salamander present in the exhibit during the outbreak in this case never showed clinical signs,  
155 carried a low fungal load, and was successfully cleared of infection based on qPCR. The  
outbreak described here indicates that green salamanders, genus *Aneides*, may be more  
susceptible to chytridiomycosis than salamanders of the genus *Eurycea* and *Bd* should be further  
investigated as a potential causative agent of population declines in this species.

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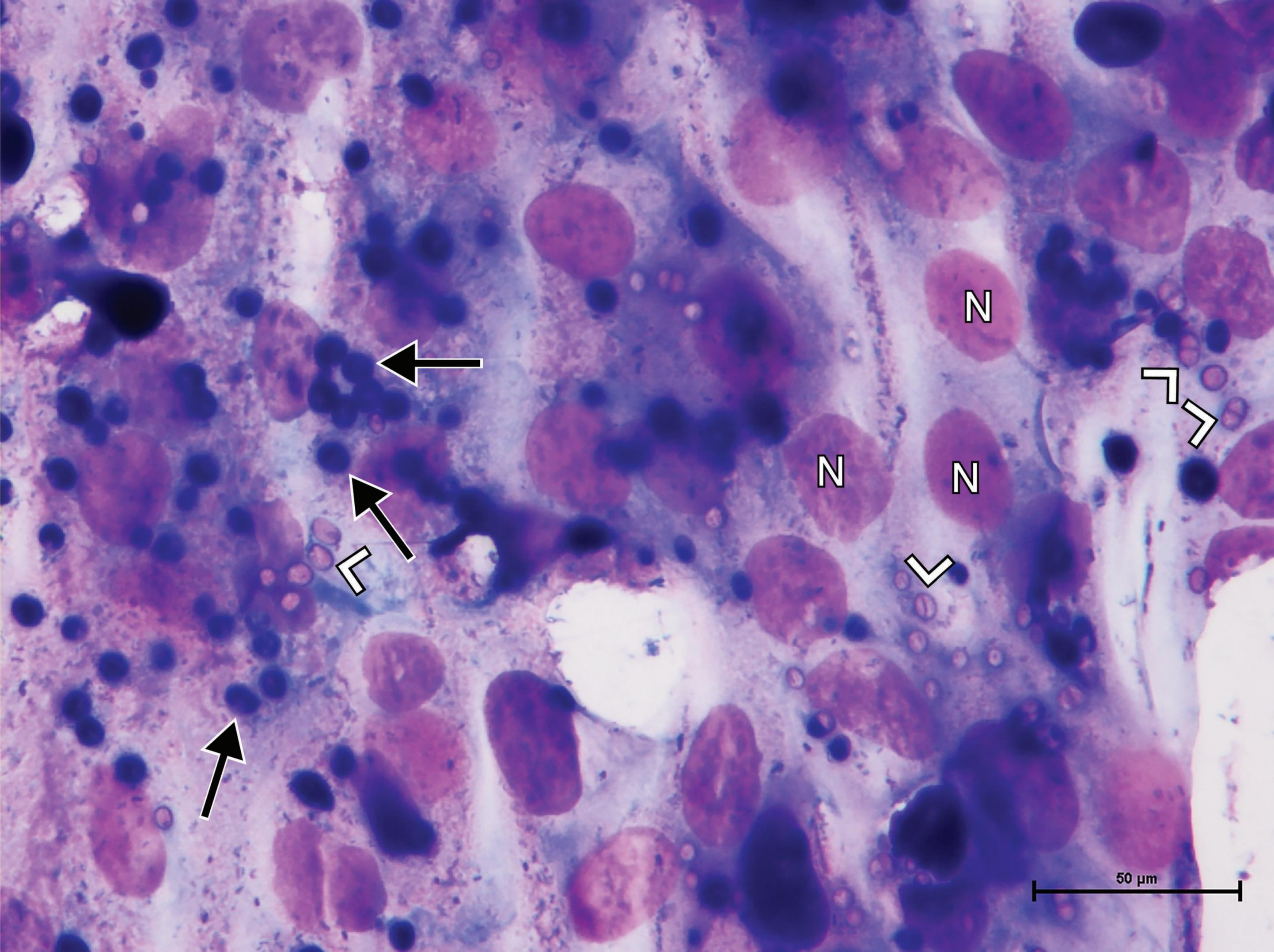
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## FIGURE CAPTIONS

Figure 1: Cytology (Diff Quick stain) of exfoliated skin (400x) from a green salamander (*Aneides aeneus*). Numerous dark staining round to oval chytrid structures (arrow) along with  
255 paler, sometimes septate, empty thalli (chevrons). Normal nuclei of squamous cells (N).  
Bacterial overgrowth and debris are also present in the background.

Figure 2: Hematoxylin and Eosin stained section of skin (400x) from the foot of a green salamander (*Aneides aeneus*) with no premonitory clinical signs showing exfoliating  
260 hyperkeratosis , numerous chytrid structures (arrows), and no significant inflammation.

Figure 3: Hematoxylin and Eosin stained section of colonic contents (400x) from a green salamander (*Aneides aeneus*) representing aggregated layers of partially digested skin shed.  
Numerous structures typical of chytrid remain visible including some with prominent discharge  
265 papillae (D).

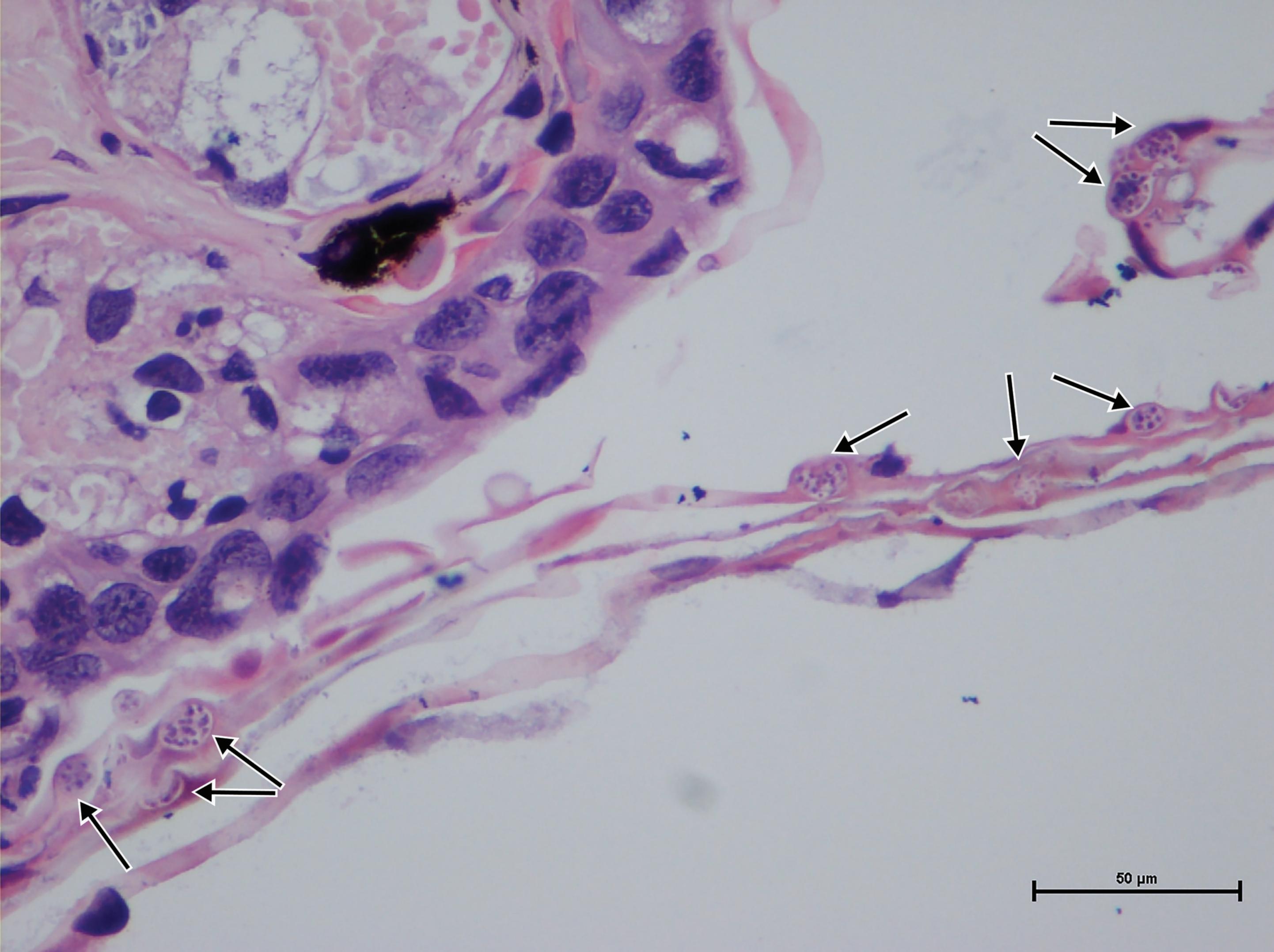


N

N

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50  $\mu$ m



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