

# Host invasion by *Batrachochytrium dendrobatidis*: fungal and epidermal ultrastructure in model anurans

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**ABSTRACT:** The chytridiomycete fungus *Batrachochytrium dendrobatidis* (*Bd*) colonizes mouthparts of amphibian larvae and superficial epidermis of post-metamorphic amphibians, causing the disease chytridiomycosis. Fungal growth within host cells has been documented by light and transmission electron microscopy; however, entry of the fungus into host cells has not. Our objective was to document how *Bd* enters host cells in the wood frog *Lithobates sylvaticus*, a species at high mortality risk for chytridiomycosis, and the bullfrog *L. catesbeianus*, a species at low mortality risk for chytridiomycosis. We inoculated frogs and documented infection with transmission electron microscopy. Zoospores encysted on the skin surface and produced morphologically similar germination tubes in both host species that penetrated host cell membranes and enabled transfer of zoospore contents into host cells. Documenting fungal and epidermal ultrastructure during host invasion furthers our understanding of *Bd* development and the pathogenesis of chytridiomycosis.

**KEY WORDS:** Chytridiomycosis · Morphology · Transmission electron microscopy · *Lithobates catesbeianus* · *Lithobates sylvaticus*

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## INTRODUCTION

*Batrachochytrium dendrobatidis* (hereafter *Bd*) is a chytridiomycete fungus that causes the potentially lethal amphibian disease chytridiomycosis (Berger et al. 1998, Nichols et al. 1998, Longcore et al. 1999, Pessier et al. 1999). *Bd* compromises tissue of amphibians by colonizing keratin-containing cells of larval mouthparts and superficial epidermis of metamorphosed individuals (Berger et al. 1998). Whether these keratin-rich cells meet structural or nutritive requirements of *Bd* is not known (Berger et al. 2005). Amphibian epidermis is a stratified epithelium that is continuously replaced by its basal stratum (stratum germinativum). Daughter cells of these columnar basal cells move outward, becoming cuboidal or polyhedral in the intermediate stratum (stratum spinosum) and then gradually flattening in the replacement stratum (stratum granulosum) into a cornified

sheet in the corneal stratum (stratum corneum) that is routinely shed (Parakkal & Matoltsy 1964, Farquhar & Palade 1965, Alibardi 2001). Although it seems that all post-metamorphic anurans and urodeles are capable of hosting *Bd* in the outermost epidermal cell layers, host response (i.e. morbidity and mortality) varies by species, population, and individual (e.g. Lips 1999, Daszak et al. 2004). Reasons for this variation are unresolved, although differences in skin microbiota (Harris et al. 2006), innate and adaptive immune mechanisms (Rollins-Smith & Conlon 2005, Ramsey et al. 2010, Savage & Zamudio 2011), environmental factors (Gahl et al. 2011), and behavior (Rowley & Alford 2007, Richards-Zawacki 2010) may play a role.

Clinical signs of chytridiomycosis in post-metamorphic individuals include buildup and erosion of corneal cells, swelling in epidermis, damaged nuclei, and altered cytoplasm (Berger et al. 2005). Macro-

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scopically, excessive skin sloughing, lethargy, anorexia, loss of righting reflex, and abnormal posture may be observed (Nichols et al. 2001, Daszak et al. 2004) but often not until immediately before death (Bradley et al. 2002, Parker et al. 2002). Proximate cause of death is inhibition of electrolyte transport across epidermis and subsequent disruption of cardiac electrical activity (Voyles et al. 2009).

The asexual life cycle of *Bd* consists of a motile zoospore stage and a stationary thallus stage (Berger et al. 2005). Zoospores are flagellated and are not bounded by a cell wall. After a motile period, usually lasting  $\leq 24$  h, a zoospore encysts (i.e. attaches to a substrate, retracts the flagellum, and forms a wall of chitin around the spore body). Morphology after encystment depends on substrate type. On nutrient media, an encysted zoospore develops into a single or colonial (internally divided) zoosporangium (endogenous growth); root-like rhizoids originating from 1 or more points on the zoosporangium may be numerous (Longcore et al. 1999). In an amphibian host, cellular contents of a superficially encysted zoospore leave the cyst and enter a host cell (exogenous growth), inside which a single or colonial zoosporangium develops; rhizoids are usually less abundant than on nutrient media (Berger et al. 2005). The specifics of amphibian host invasion by *Bd* (i.e. the morphological process by which zoospore contents are transferred from a cyst into a host cell) have not been reported. In other chytrid species with known developmental morphologies (saprophytes and pathogens of plants, algae, protists, and invertebrates), endobiotic (i.e. intracellular) growth of a thallus is initiated when a protrusion of the zoospore cyst wall elongates into a germination tube that penetrates host tissue. Cyst cytoplasm and organelles are transferred inside host tissue through the tube, and 1 or more zoosporangia develop where cyst contents have accumulated in the tube. Branching rhizoids may originate from the tube or walls of zoosporangia (Barr & Désaulniers 1987, Longcore 1995, Longcore et al. 1995). Maturation of an endobiotic *Bd* zoosporangium culminates in cleaving of zoosporangial contents into zoospores, formation of 1 or more zoospore-discharge papillae leading to the surface of the host or into intercellular spaces, and release of zoospores (Berger et al. 2005). *Bd* is the only chytrid known to parasitize vertebrate hosts (Longcore et al. 1999) and is one of few endobiotic members of order Rhizophydiales (Letcher et al. 2006).

Nonlethal *Bd* infections in the bullfrog *Lithobates catesbeianus* are well-documented (Daszak et al. 2004). In contrast, the wood frog *L. sylvaticus* can die

from chytridiomycosis at young post-metamorphic stages under laboratory conditions (Searle et al. 2011, Gahl et al. 2012). We used bullfrogs and wood frogs as representative species with low and high mortality risk for chytridiomycosis, respectively. Our objective was to document by electron microscopy how *Bd* invades epidermal cells of these species. Documenting the morphology of this fungus during infection of frog tissue fills a critical gap in our knowledge of *Bd* development and sheds light on the pathogenesis of chytridiomycosis.

## MATERIALS AND METHODS

### Study animals and husbandry

We collected 7 bullfrogs from the lower Penobscot River watershed, Maine, USA, in May 2010. We used them in another experimental *Bd* infection study until July 2010 (Greenspan et al. 2012); animals previously used as controls remained controls. Treatment bullfrogs had not been inoculated for several weeks and tested negative for *Bd* infection in 2 of our most recent *Bd*-specific real-time Taqman (Applied Biosystems) polymerase chain reaction tests following Boyle et al. (2004) and Retallick et al. (2006). We could not age bullfrogs, but they were probably metamorphs and young juveniles because masses at capture were within the range for individuals from these age classes sampled in Rhode Island, USA ([www.uri.edu/cels/nrs/paton/amphibs](http://www.uri.edu/cels/nrs/paton/amphibs)). In July 2010, we obtained 10 newly metamorphosed wood frogs raised from eggs at the University of Maine.

We housed bullfrogs individually and wood frogs in pairs in plastic containers (43.2 × 28.3 × 16.5 cm; Sterilite) that were tilted to create a pool of aged tap water on 1 side and a terrestrial area lined with moist paper towels on the other side. We refreshed water and paper towels weekly. We fed bullfrogs live mealworms or crickets every third day; wood frogs received live flightless fruit flies daily. Laboratory conditions were 20 ± 2°C with a 12:12 h light:dark cycle. Euthanasia was performed by placing animals in a solution of tricaine methanesulfonate (1 g l<sup>-1</sup> for wood frogs and 10 g l<sup>-1</sup> for bullfrogs; Western Chemical) buffered with an equal amount of sodium bicarbonate.

### *Bd* cultures and inoculations

We cultured *Bd* (strain JEL423) in 1% tryptone broth in 125 ml flasks. Two flasks, which were trans-

ferred after 3 mo, functioned as stock cultures from which pairs of fresh cultures were made bimonthly. For each passage, we transferred 1 ml of active culture to 75 ml of fresh broth. New cultures were incubated at 23°C for several days and then maintained at 4°C. To obtain zoospores for inoculations, 9 cm Petri dishes (2 per animal to be inoculated) containing 1% tryptone in 1% agar were each inoculated with 1 ml of cultured broth. Plates were dried in a laminar flow hood and incubated at 23°C for approximately 4 d before the inoculations. On the day of inoculation, we confirmed with a compound microscope the presence of live zoospores on the plates and added up to 4 ml of sterile distilled water to each plate to form a zoospore suspension. We then combined the liquid contents of each plate in a sterile beaker and refrigerated the beaker with a cover at 4°C until immediately before inoculation (up to 1 h later). We enumerated zoospores by diluting a portion of suspension with an equal amount of Lugol solution (Sigma) and then counting zoospores in the diluted sample with a hemocytometer (e.g. Vazquez et al. 2009).

Bullfrogs were inoculated between July and October 2010. Wood frogs were inoculated in September 2010. We inoculated 6 bullfrogs in individual 500 ml plastic containers and 8 wood frogs in individual 118 ml plastic containers. One bullfrog and 2 wood frogs provided uninfected tissue for comparative purposes. To inoculate frogs, we filled each container with enough aged tap water to cover the bottom of the container (approximately 17 ml for bullfrogs and 12 ml for wood frogs), placed each frog into a container, added 1 to 8 ml of zoospore suspension, and gently agitated each container. We inoculated bullfrogs with  $8 \times 10^7$  to  $2 \times 10^8$  zoospores per frog and wood frogs with  $1 \times 10^7$  to  $2 \times 10^7$  zoospores per frog (Table 1). Frogs were left in inoculant baths for 12 to 24 h and euthanized up to 5 d later (Table 1). We chose amounts of inoculum and inoculation durations to maximize infection in both species yet avoid overburdening wood frogs. We varied times to euthanasia to improve the chances of observing different fungal stages of the host invasion process.

### Transmission electron microscopy

We fixed interdigital skin from all frogs and inguinal abdominal skin from all wood frogs and 1 bullfrog (no. 1). Tissue preparation methods were modified from Longcore (1992). Tissue was extracted immediately after euthanasia and placed in 1.5%

Table 1. *Lithobates catesbeianus* and *L. sylvaticus*. Six juvenile bullfrogs and 8 recently metamorphosed wood frogs were inoculated with *Batrachochytrium dendrobatidis* (*Bd*) to obtain infected tissue for electron microscopy. *Bd* inoculum concentration, inoculation duration, and time to euthanasia varied between species and among individuals. Uninfected (control) tissue came from 1 bullfrog (no. 6) and 2 wood frogs (nos. 5 and 6). The start of inoculation occurred at Time 0

Individual	Inoculum (no. zoospores frog <sup>-1</sup> )	Inoculation duration (h)	Time to euthanasia (h)
<b><i>L. catesbeianus</i></b>			
1	$8 \times 10^7$	24	48
2, 3	$8 \times 10^7$	12	12
4, 5	$1 \times 10^8$	24	24
7	$2 \times 10^8$	24	36
<b><i>L. sylvaticus</i></b>			
1, 2	$2 \times 10^7$	12	60
3, 4	$2 \times 10^7$	12	132
7, 8	$1 \times 10^7$	12	36
9, 10	$1 \times 10^7$	12	84

glutaraldehyde buffered with 0.1 M s-collidine containing 6% sucrose. Fixed samples were kept at room temperature for 2 h and then refrigerated overnight at 4°C. Samples were washed 6 times in 0.05 M s-collidine buffer, post-fixed in 1% osmium tetroxide in 0.1 M s-collidine for 1 h, and washed twice in buffer followed by distilled water. We dehydrated samples in a graded acetone series and embedded skin pieces (approximately 0.5–1 mm<sup>2</sup>) in Epon-Araldite epoxy resin blocks (median number of interdigital tissue blocks per frog = 16; median number of abdominal tissue blocks per frog = 5). Semithin sections (600 nm) were cut with glass knives, transferred to glass slides, dried at 60°C, and stained with 1% toluidine blue in 1% sodium borate for 1 min. These were viewed with a compound microscope to find infected areas. Thin serial sections (80 nm) were cut with a diamond knife, transferred to carbon-coated, Pioloform-covered slot grids, stained with uranyl acetate and lead citrate, viewed using a Philips CM10 transmission electron microscope at 100 kV, and photographed with a Gatan Bioscan camera.

We measured parts of *Bd* thalli in micrographs with ImageJ software. To validate identifications of zoospore cysts and germination tubes, we compared shape, size, and general appearance of these features to those of thalli of other isolates of exogenous chytrid fungi in light micrographs of live wet-mounted specimens (Longcore 1995, J. E. Longcore unpubl. data).

## RESULTS

By electron microscopy, we observed encysted zoospores (Figs. 1 & 2) on the surface of bullfrog epidermis and fungal germination tubes (see Figs. 1 to 10) in superficial epidermis of both hosts. Zoospore cysts were ovoid (Figs. 1 & 2). Germination tubes were unbranched, curvilinear, and usually extended through more than 1 cell layer (Figs. 3 & 4). Septa (Figs. 5 to 10) formed at germination tube–zoosporangium junctions, and zoosporangia sometimes bulged near these junctions (Figs. 5 to 7). Diameters of zoospore cysts and germination tubes of *Bd* were smaller than those of other exogenous chytrid fungi; features of *Bd* were most similar in size to those of another member of Rhizophydiales (JEL 326; Table 2). The curvilinear shape of germination tubes made entire tubes difficult to image, but when seen attached to zoosporangia, their orientation suggested that they intersected the epidermal surface (Figs. 3 to 5, 8 & 10). Thalli with germination tubes in the plane of section were 1 or 2 cell layers deep in bullfrogs and 1 to 3 cell layers deep in wood frogs. Encystment to septum formation in a bullfrog occurred within as little as 12 h after inoculation. Rhizoids extending from zoosporangia were seen rarely by electron microscopy in 2 heavily infected wood frogs that were euthanized 60 h and 132 h post-inoculation, respectively (nos. 1 and 4; Figs. 11 & 12); rhizoids were not observed in bullfrog tissue, but all bullfrogs were euthanized 12 to 48 h post-inoculation. Rhizoids pointed toward the dermis and did not have septa, distinguishing them from germination tubes.

Host cytoplasm surrounding or near thalli sometimes differed in electron density or texture from other cell regions. Alterations to host cytoplasm included apparent condensing (Fig. 2) and displacement or reorganization of cellular contents (Figs. 4, 5 & 10). Uninfected epidermis from inoculated frogs resembled epidermis from frogs not inoculated. Disruption of the epidermal maturation cycle was evident by light and electron microscopy in heavily infected areas of tissue from both species, but heavy infection was much rarer and more localized in bullfrogs (Fig. 13) than in wood frogs (Fig. 14). Evidence of this disruption was buildup of keratinized cells (Fig. 13) and erosion in which sloughing exposed incompletely keratinized epidermal surface (Fig. 14).

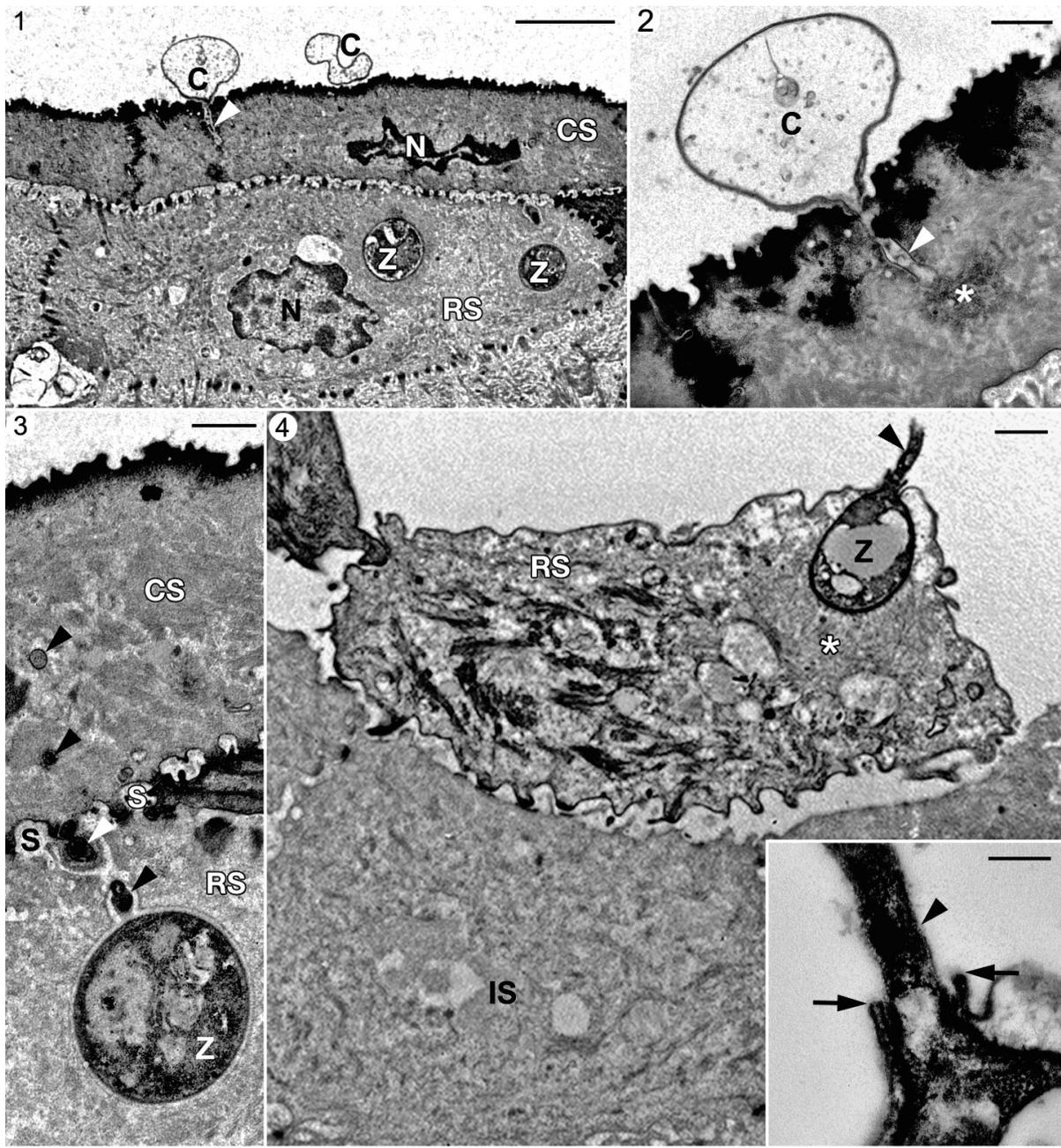
## DISCUSSION

Transmission electron microscopy (TEM) is a powerful descriptive and diagnostic tool in *Bd* research.

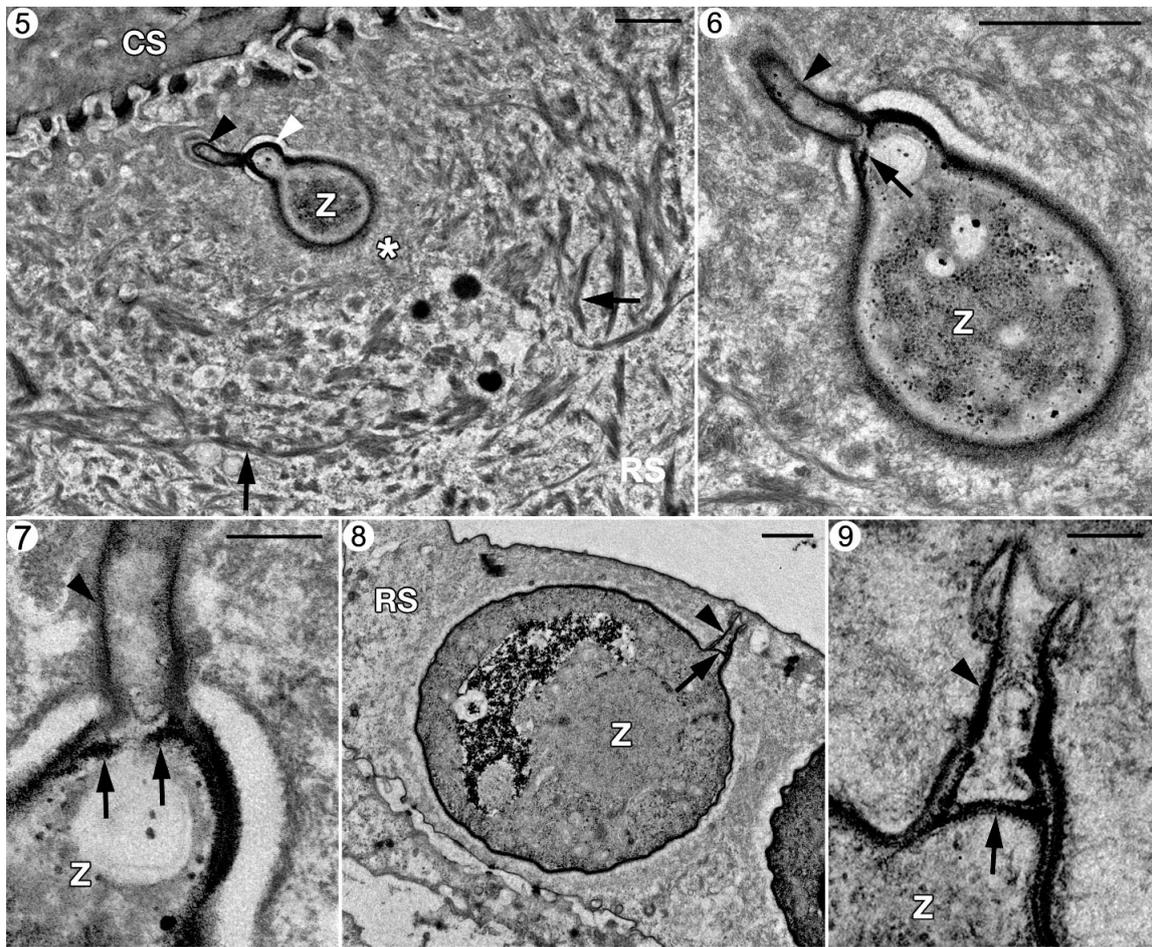
We build on existing reports of *Bd* thallus ultrastructure (Berger et al. 1998, 2005, Pessier et al. 1999, Davidson et al. 2003, Ouellet et al. 2005, Simoncelli et al. 2005, Une et al. 2008) by providing TEM micrographs of *Bd* in bullfrogs and wood frogs and, to our knowledge, the first account of the host invasion process in any host species. Host invasion by *Bd* parallels that in other exogenous chytrid fungi and appears morphologically similar in experimentally infected bullfrogs and wood frogs, species that display disparate levels of mortality risk for chytridiomycosis. At least for these species, how *Bd* enters host cells seems unrelated to the degree of pathogenicity. Given our modest sample size, however, it is possible that we did not fully capture morphological variation in host invasion. Also, possible interspecies responses to enzymatic activity in the colonization process were not addressed in this study (Rosenblum et al. 2008). Our experimental infection protocol seemed to mimic natural infection processes and our tissue fixation protocol effectively preserved frog and fungal tissue and limited artifacts (organelles appeared well preserved and cytoplasm lacked perforations and compression). These protocols may be adapted for use with other host species and *Bd* strains.

The morphological infection process in both species, and probably in other hosts, is as follows. Within as few as 12 h after exposure to a frog host, a zoospore encysts on the skin surface of the host and produces a germination tube that penetrates the surface host cell membrane and usually extends through more than 1 cell layer. Cyst contents migrate into host tissue through the tube, and a single or colonial zoosporangium arises from a portion of the tube within which cyst contents have accumulated. A septum forms that segregates the zoosporangium from the tube. Fig. 15 presents a schematic view of the life cycle of *Bd* in skin based on this work and on the literature (Berger et al. 2005). Zoospore cysts and germination tubes were somewhat smaller in *Bd* than in some other exogenous chytrid species; however, sizes were reasonably similar and, unlike in wet-mounted specimens, plane of section could have influenced measurement of *Bd* features. Infrequent observation of rhizoids in skin corroborates ultrastructure studies of the dainty green tree frog *Litoria gracilentia* (Berger et al. 2005) and White's tree frog *L. caerulea* (Pessier et al. 1999).

Infected cells of bullfrogs and wood frogs bore a close resemblance to those of dainty green tree frogs with regard to displacement of host organelles and clear zones around zoosporangia (Berger et al. 2005). Altered cytoplasm may indicate enzymatic activity



Figs. 1 to 4. *Batrachochytrium dendrobatidis* (*Bd*) infecting *Lithobates* spp. Transmission electron micrographs. C: zoospore cyst; CS: corneal stratum (stratum corneum); IS: intermediate stratum (stratum spinosum); N: nucleus; RS: replacement stratum (stratum granulosum); S: intercellular space; Z: zoosporangium. **Fig. 1.** *L. catesbeianus* at 12 h post-inoculation. Two empty zoospore cysts rest on the skin surface. On the left, a germination tube (arrowhead) produced by a cyst has penetrated the host cell membrane and extends into the CS cell cytoplasm and potentially deeper. Two zoosporangia are in the RS; whether they originated from the cysts shown is unknown. Scale bar = 5  $\mu$ m. **Fig. 2.** Higher magnification of zoospore cyst and germination tube (arrowhead) shown in Fig. 1. Black blotches are stain residue. Host cytoplasm appears condensed where the germination tube exits the plane of section (\*). Scale bar = 1  $\mu$ m. **Fig. 3.** *L. catesbeianus* at 12 h post-inoculation. Germination tube (arrowheads) extends through CS and into RS. Zoosporangium has formed from portion of tube. Germination tubes of *Bd* are curvilinear (in micrographs, they often exit and re-enter plane of section), making it difficult to image the entire tube. Scale bar = 1  $\mu$ m. **Fig. 4.** *L. sylvaticus* at 60 h post-inoculation. CS (not shown) has separated from RS cell, leaving the germination tube (arrowhead) behind. RS cell contents have been displaced to the left side of the cell, leaving a clear zone (\*) around the zoosporangium. Inset shows part of the same thallus at higher magnification; germination tube (arrowhead) has penetrated CS (not shown) and RS cell membrane (arrows). Scale bar = 1  $\mu$ m. Scale bar in inset = 0.25  $\mu$ m

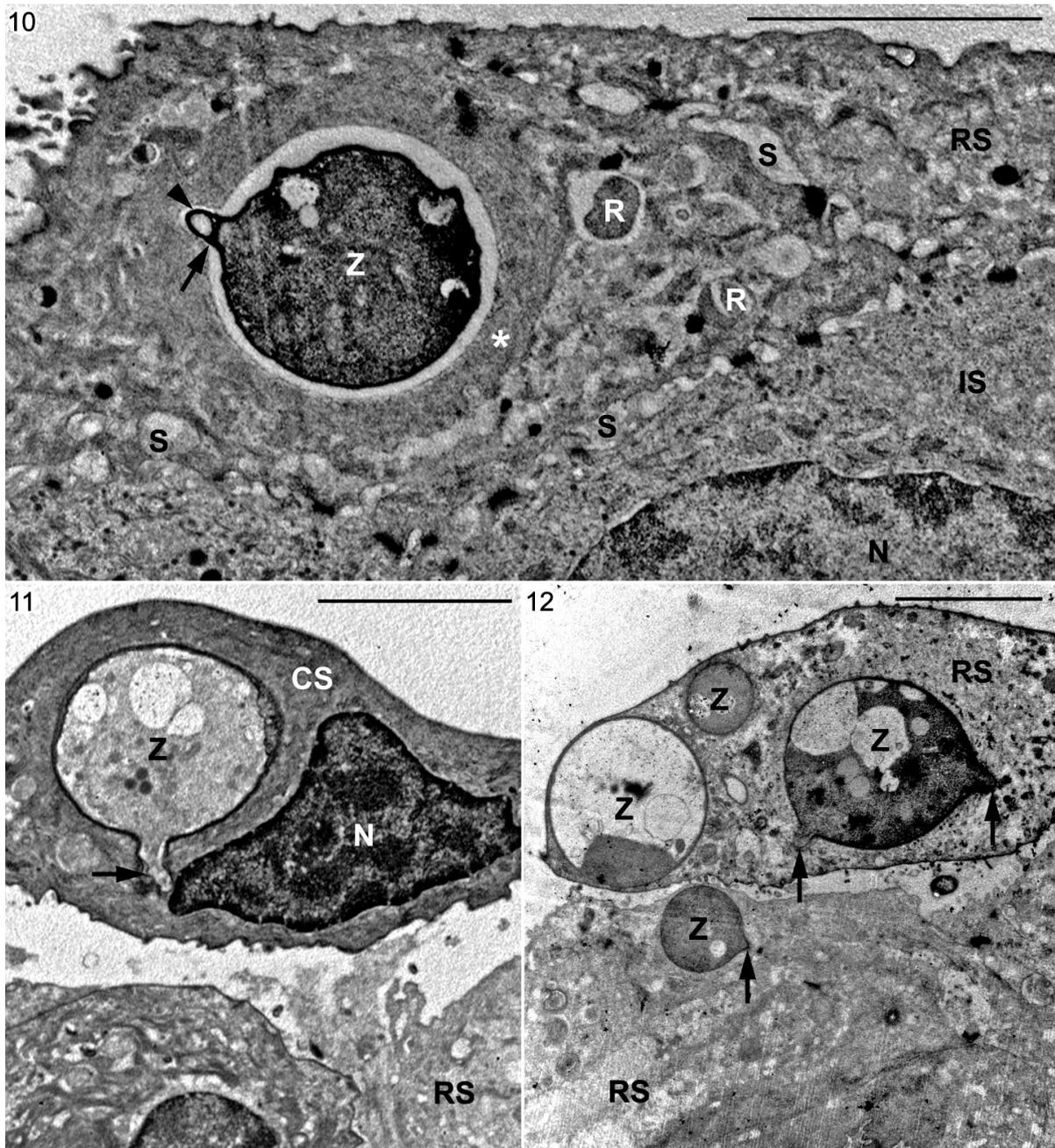


Figs. 5 to 9. *Batrachochytrium dendrobatidis* infecting *Lithobates* spp. Transmission electron micrographs. CS: Corneal stratum (stratum corneum); RS: replacement stratum (stratum granulosum); Z: zoosporangium. Fig. 5. *L. catesbeianus* at 12 h post-inoculation. Zoosporangium attached to germination tube (black arrowhead) bulges (white arrowhead) near tube–zoosporangium junction. Keratin filaments in RS cell cytoplasm appear reorganized (arrows), leaving a clear zone (\*) surrounding the zoosporangium. Scale bar = 1  $\mu$ m. Fig. 6. Higher magnification of thallus shown in Fig. 5. Septum (arrow) is forming between zoosporangium and germination tube (arrowhead). Scale bar = 1  $\mu$ m. Fig. 7. Higher magnification of germination tube (arrowhead) and incomplete septum (arrows) shown in Figs. 5 & 6. Scale bar = 0.25  $\mu$ m. Fig. 8. *L. sylvaticus* at 132 h post-inoculation. Septum (arrow) separates zoosporangium from germination tube (arrowhead). CS is absent. Scale bar = 1  $\mu$ m. Fig. 9. Higher magnification of germination tube (arrowhead) and complete septum (arrow) shown in Fig. 8. Scale bar = 0.25  $\mu$ m

Table 2. Diameter of zoospore cysts and germination tubes of chytrid fungi from 3 orders. We measured parts of thalli in micrographs with ImageJ software. For JEL423 (*Batrachochytrium dendrobatidis*), we measured diameters of zoospore cysts (n = 2) and germination tubes (n = 16) in transmission electron micrographs. For other isolates, we measured diameters of zoospore cysts (n = 1) and germination tubes (n = 1) in light micrographs of live, wet-mounted specimens (Longcore 1995, J. E. Longcore unpubl. data)

Order (isolate)	— Zoospore cyst ( $\mu$ m) —		Germination tube ( $\mu$ m)
	Short axis	Long axis	
Chytridiales (JEL129)	4.9	5.0	1.0
Cladochytriales (JEL036)	4.3	4.4	1.3
Cladochytriales (JEL044)	5.5	6.3	1.9
Cladochytriales (JEL075)	3.8	7.9	2.2
Rhizophydiales (JEL326)	3.3	4.5	0.7
Rhizophydiales (JEL423)	2.7–3.1	3.2–3.8	0.2–0.6

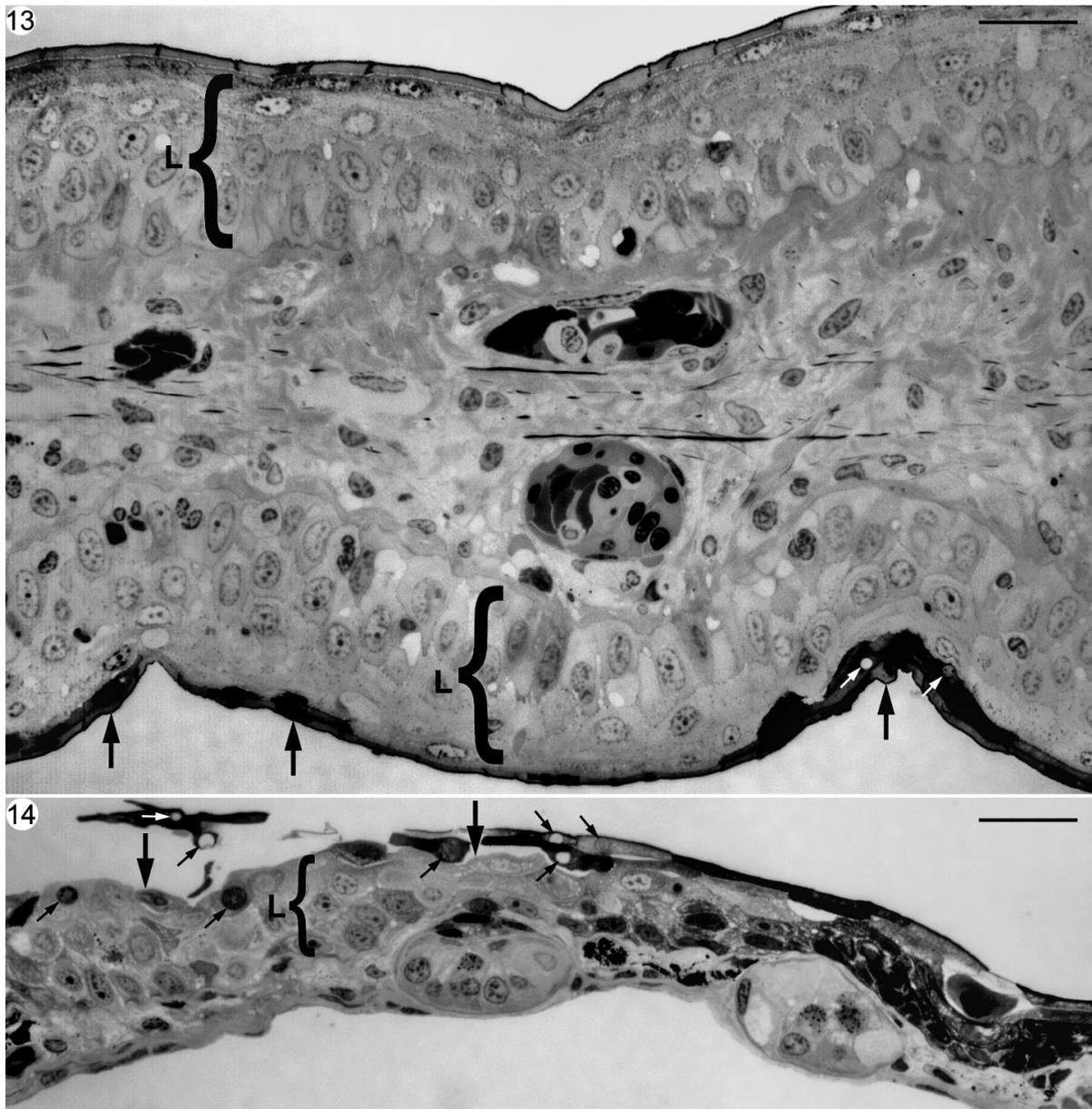
by *Bd*, the release of other compounds by *Bd*, or host immune response, but more research is needed to fully interpret these changes. This research direction may further our understanding of the effects of *Bd* on host tissue outside of localized infection sites (Berger et al. 2005). We observed anecdotally that bullfrog epidermis, usually with more numerous replacement (stratum granulosum) and intermediate (stratum spinosum) cell layers, was consistently thicker than wood frog epidermis regardless of infection status (Figs. 13 & 14), but



Figs. 10 to 12. *Batrachochytrium dendrobatidis* infecting *Lithobates sylvaticus*. Transmission electron micrographs. CS: corneal stratum (stratum corneum); IS: intermediate stratum (stratum spinosum); N: nucleus; R: remnants of organelles; RS: replacement stratum (stratum granulosum); S: intercellular space; Z: zoosporangium. Fig. 10. At 60 h post-inoculation. Host cytoplasm is clear (\*) around zoosporangium and remnants of organelles appear displaced to the right side of the cell. Septum (arrow) separates zoosporangium from germination tube (arrowhead). Scale bar = 5  $\mu$ m. Fig. 11. At 60 h post-inoculation. Zoosporangium with rhizoid (arrow) in CS cell. Rhizoid extends toward RS and lacks septum, distinguishing it from germination tube. Scale bar = 5  $\mu$ m. Fig. 12. At 132 h post-inoculation. Zoosporangia with rhizoids (arrows) in RS cells. CS is absent. Rhizoids extend toward deeper epidermal strata and septa are absent. Black speckles are stain residue. Scale bar = 5  $\mu$ m

our study design precluded robust statistical comparisons. Our finding that disruption of the epidermal cell maturation cycle was mild and localized in bullfrogs, a low-mortality-risk species for chytridiomycosis,

and more severe in wood frogs, a high-mortality-risk species for chytridiomycosis (at least at some life stages under laboratory conditions), supports previous work (Daszak et al. 2004, Berger et al. 2005).



Figs. 13 & 14. *Batrachochytrium dendrobatidis* infecting *Lithobates* spp. Light micrographs. Fig. 13. *L. catesbeianus* at 24 h post-inoculation. Premature keratinization (2 or 3 layers of darkened cells) at localized infection sites (large arrows). Small arrows point to zoosporangia. Fig. 14. *L. sylvaticus* at 60 h post-inoculation. Heavy infection. Some outermost cells have separated from epidermis, exposing incompletely keratinized epidermal surface (large arrows). Small arrows point to zoosporangia. Note that epidermis is thinner than bullfrog epidermis in Fig. 13; live epidermal strata (L; all strata except corneal stratum [stratum corneum]) are demarcated for reference. Scale bars = 25  $\mu$ m

Abnormal molting is a clinical sign of chytridiomycosis that could be beneficial, by ridding animals of infected skin layers (Davidson et al. 2003, Berger et al. 2004), or detrimental, by impeding water and electrolyte transport (Voyles et al. 2011). Bullfrogs may slough rapidly during experimental inoculations with *Bd* (Greenspan et al. 2012). A relatively large supply of intermediate (stratum granulosum) and

replacement (stratum spinosum) cell layers could confer molting plasticity to the bullfrog, allowing this species to derive benefits from abnormal molting without damage to cutaneous function. We encourage further research on skin structure and function (e.g. supply of epidermal layers, rate of epidermal cell turnover) as potential determinants of mortality risk for chytridiomycosis.

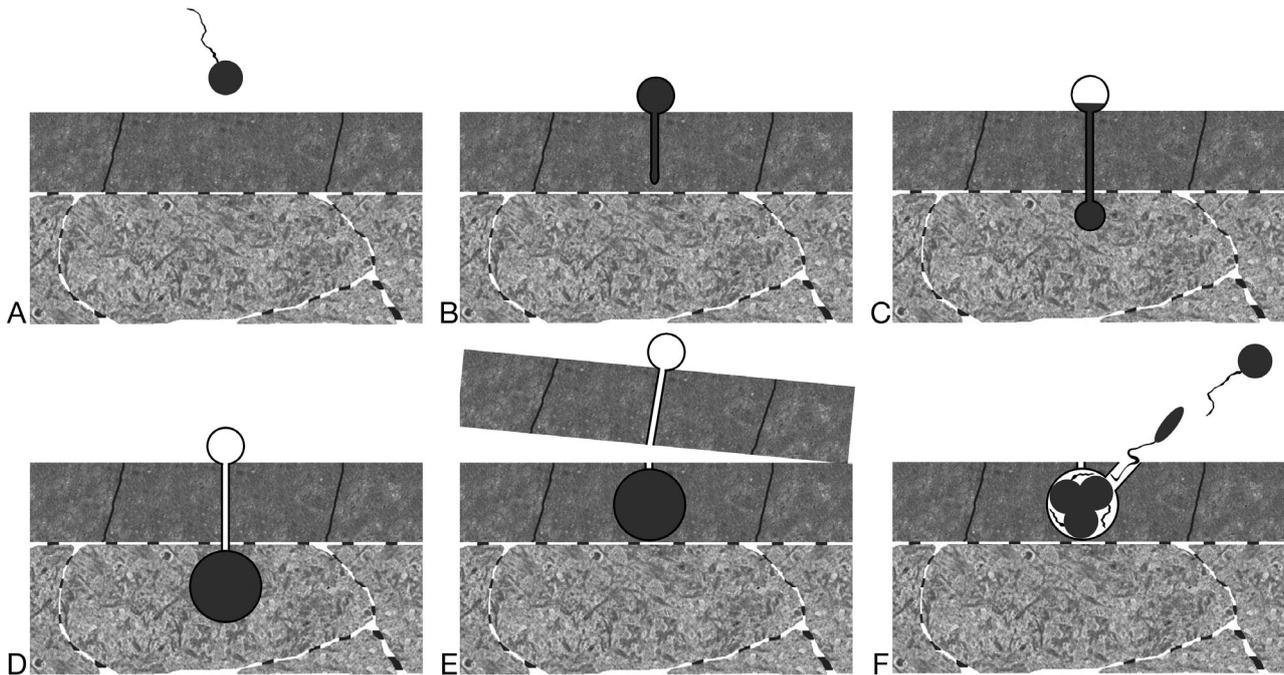


Fig. 15. *Batrachochytrium dendrobatidis*. Schematic of life cycle in frog skin. (A) After a motile period, usually lasting  $\leq 24$  h, a zoospore attaches to the skin surface, retracts the flagellum, forms a wall of chitin around the spore body, and (B) produces a germination tube that penetrates the surface cell membrane of the host and usually extends through the corneal stratum (stratum corneum) and (C) into deeper cell layers. Cyst contents migrate into host tissue through the tube, and a single or colonial (not shown) zoosporangium arises from a portion of the tube within which cyst contents have accumulated. (D) A septum forms to segregate the zoosporangium from the tube. (E) Host cells undergo maturation and outward movement as the zoosporangium develops (Berger et al. 2005). (F) Maturation of the zoosporangium culminates in cleaving of zoosporangial contents into zoospores, formation of at least 1 zoospore-discharge papilla, and release of zoospores (Berger et al. 2005)

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