Journal of Herpetology, Vol. 35, No. 2, pp. 330–335, 2001
Copyright 2001 Society for the Study of Amphibians and Reptiles

Mucocutaneous Fungal Disease in Tadpoles of *Bufo marinus* in Australia

LEE BERGER,[1,2,3] RICK SPEARE,[2] ANNETTE THOMAS,[4] AND ALEX HYATT[1]

[1]*CSIRO Australian Animal Health Laboratory, Geelong, Victoria 3220, Australia*
[2]*School of Public Health and Tropical Medicine, James Cook University, Townsville, Queensland, 4811, Australia*
[4]*Oonoonba Veterinary Laboratory, Department of Primary Industries, Oonoonba, Townsville, Queensland 4811, Australia*

Infectious diseases in wild tadpoles have been reported rarely. *Saprolegnia* was reported to kill tadpoles of *Spea bombifrons* (Bragg and Bragg, 1958), *Rana berlandieri* and *Pseudacris streckeri* in pools in Oklahoma (Bragg, 1962). In West Virginia, tadpole edema virus killed tadpoles of *Rana catesbiana* (Wolf et al., 1968). The bacterium, *Aeromonas hydrophila*, was reported to be the cause of mass mortality of tadpoles of *Rana sylvatica* in a pond on Rhode Island (Nyman, 1986) and tadpoles of *Alytes obstetricans* in the Spanish Pyrenean Mountains (Marquez et al., 1995). *Prototheca richardsi*, an alga isolated from the feces of tadpoles in Britain, was found to cause growth inhibition (Wong and Beebee, 1994). Sporangia of *Batrachochytrium*, a nonhyphal chytrid fungus causing mortality in adult anurans, occur in the epidermis of the mouthparts of apparently healthy tadpoles in Australia (Berger et al., 1999).

Fungi of the family Saprolegniaceae (Class: Oomycetes) are common fish and crustacean pathogens (Willoughby, 1994). *Saprolegnia* has been observed in captive amphibians (Raphael, 1993) and on amphibian eggs (Blaustein et al., 1994), but *Aphanomyces* infections have not been previously reported in amphibians. We report fungal infection in tadpoles of the introduced giant toad, *Bufo marinus*, in two separate outbreaks in ponds in north Queensland, Australia. Hyphal fungi grew in tufts attached to the nostrils, mouth parts, skin of the head, and also occasionally on the hind legs and tail. Several fungi were isolated, with *Aphanomyces* a likely pathogen.

The first outbreak occurred in 1989 near Mt. Margaret Station north of Townsville, Australia. This was in a large dam with muddy, opaque water to which cattle had access. Nine diseased tadpoles were collected and fixed in 10% neutral buffered formalin ($N = 5$) or submitted fresh for mycological culture ($N = 4$).

In September 1995, a second outbreak was observed in an ephemeral pond in Major's Creek, near Woodstock about 45 km west of Townsville, Australia. The tadpoles were collected from a pond behind a sand dam in the creek. This pond was approximately 2.5×3.5 m wide, reaching a depth of about 15 cm. The creek ran along the border of two properties where mangoes, avocadoes, grapes, and potatoes were grown. Although there was a possibility of fertilizer and pesticide run off, the water appeared clear. Two groups of tadpoles were collected. Group A was a random sample of 100 tadpoles including affected and unaffected tadpoles from a pond containing thousands of tadpoles. They were immediately formalin-fixed and later examined under a dissecting microscope for the presence of hyphae, stage of limb development and body condition. Group B contained 40 severely infected tadpoles selected individually on the basis of visible abnormalities from this pond. These were examined, placed in individual containers, transported to the laboratory and subsequently fixed in 10% formalin for histology, 2.5% glutaraldehyde for electron microscopy, frozen at −80°C for virus isolation, or dissected when fresh for mycology.

For histological examination, formalin fixed tadpoles were dehydrated and embedded whole on their sides in paraffin wax. Histological sections were cut at 6 μm and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or Grocott's methenamine silver (Luna, 1968).

Mycological identification for the first outbreak was conducted on samples submitted on DMEM (Dulbecco's Modification of Eagle's Medium) plus antibiotics or Potato Dextrose Agar. Cultures that grew after incubation at 25°C for 4–14 days were examined.

Mycological culture for the second outbreak was attempted by dissecting the visible mycelia from the heads of tadpoles. Hyphae from each tadpole were transferred to three petri dishes containing Sabour-

[3] Corresponding Author. E-mail: lee.berger@li.csiro.au

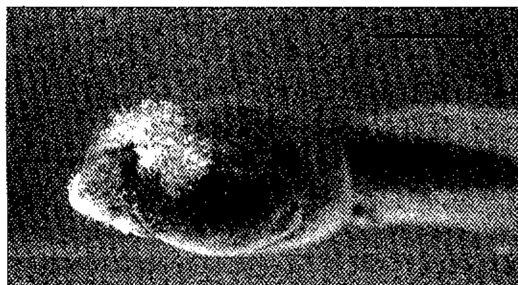

FIG. 1. Glutaraldehyde-fixed tadpole of *Bufo marinus* photographed underwater with a mycelium of fungus consistent with *Aphanomyces* growing on and between the nostrils, as well as on the mouth. Bar = 3.5 mm.

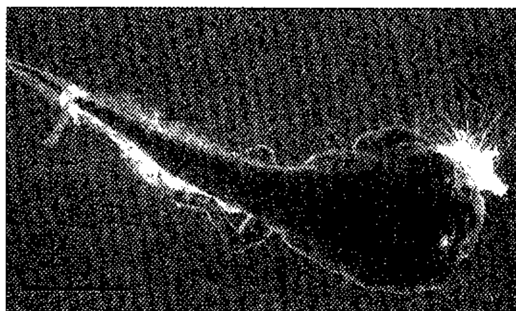

FIG. 2. Tadpole of *Bufo marinus* with a tuft of fungal hyphae attached to the left nostril. The dense mycelial matt near the skin is consistent with *Aphanomyces;* there is also an unidentified fungus with thicker hyphae radiating out (arrow). Note poor body condition. Bar = 4 mm.

aud's Dextrose Agar (SDA), Glucose Yeast Extract Agar (GYA) or 20 mL of tap water plus penicillin and streptomycin. The culture media were incubated at 25°C and checked daily for growth for 14 days.

In both outbreaks, identification of isolates belonging to the zygomycetes and the fungi imperfecti involved subculture onto SDA. Any fungal growth typical of the oomycetes were transferred to GYA and reincubated at 25°C until pure cultures were obtained. To identify the oomycetes, 5 × 5 mm blocks of fungal growth were taken from the periphery of the growing colonies (3–4 days of growth) and transferred to glucose-yeast extract broth and incubated at 25°C (or room temperature) for a further 3-4 days. The blocks of growing hyphae were then washed three times in autoclaved pond water before being placed in a petri dish containing 20 mL pond water. After overnight growth at room temperature, the fungal growths were examined microscopically for the presence of encysted or actively motile zoospores and the pattern of spore discharge, for differentiation of the oomycete genera (Fuller and Jaworski, 1987).

For virological testing, 18 frozen tadpoles from the second outbreak were homogenized with glass beads in Eagle's Modified Essential Medium (1:20 w/v) and centrifuged at 4000 rpm for 2 min. The supernatant was passed through a 0.45 μm filter and 200 μl added to a monolayer of African clawed toad kidney (A6; ATCC CCL 102), bullfrog tongue (FT; ATCC CCL 41), and chinook salmon embryo (CHSE; ATCC CCL 1681) cells for 1 h in 24 well plates. Two milliliters of the appropriate media containing 10% fetal calf serum, penicillin and streptomycin were then added to the wells that were incubated between 22°C and 24°C. Cells were observed for cytopathic effects during three passages of at least seven days each.

For scanning electron microscopy, the heads of one formalin-fixed tadpole from the first outbreak and three glutaraldehyde-fixed tadpoles from the second outbreak were washed in 0.2 M cacodylate buffer, postfixed in 1% osmium tetroxide, slowly dehydrated through graded alcohol, critical point dried, sputter coated with gold, and examined using a JEOL JSM 840 scanning electron microscope at 5 KV.

Tadpoles collected in the first outbreak were active but thin with pale, cotton wool-like fungal mycelium growing from their heads and obstructing the mouths. The histological appearance was similar to outbreak 2 (below) with masses of branched, aseptate, basophilic fungi attaching to a swollen dermis with focal ulceration of the epidermis and a mild inflammatory cell response. Fungi stained with methenamine silver but not with PAS. The intestines were empty. Scanning electron microscopy of one tadpole revealed a fungus with fine hyphae that branched almost at right angles, that covered the mouth, grew radially toward the nostrils and extended outward in a large tuft. Achlyoid-type clusters of round zoospores were present throughout the mycelium, consistent with an *Aphanomyces* sp. Significant fungi were cultured from only one tadpole that grew a *Mucor* sp.

The second outbreak occurred about four weeks after rain in the headwaters resulted in pond formation in the previously dry creek and breeding of *B. marinus.* At the time of collection, the pond was evaporating rapidly. Healthy metamorphs of *B. marinus* were emerging, indicating a fairly normal rate of development. Hundreds of tadpoles had a superficial fungal infection of the head, with a predilection for the mouth and the nostrils. The fungal mass was pale grey, projecting from the mouth or nostrils in a cotton wool-like mycelium (Figs. 1–2). In some tadpoles, the fungal mycelium was extensive and covered the mouth, nostrils, and rarely one or both eyes. Occasional tadpoles had foci of fungi on other areas of the body, particularly the limbs and the tail, in addition to heavy infection of the head. In the random sample of tadpoles (Group A), 37 of 100 (37%) were infected with fungi. Of the affected tadpoles, 10 (27%) had obvious tufts of fungus, and 27 (73%) only had a few strands of hyphae. The infected tadpoles were relatively immature and in poor body condition, with shrunken abdomens, muscle wasting and prominent cranial bones, compared to uninfected tadpoles (Table 1). Of 40 severely affected tadpoles examined in detail (Group B), all had fungi on the head with 30 (75%) involving the mouth and at least one nostril. Of the remainder, five (13%) had invasion of the mouth only, and five (13%) had invasion of nostrils only. Other lesions occurred on 25% of Group B tadpoles: four (10%) had lesions on both body and limbs; four (10%) had lesions on limbs only; and two (5%) had lesions

TABLE 1. Stage of development and body condition of infected and uninfected tadpoles from the second outbreak of fungal disease.

	Group A (random scoop)		Group B
	Uninfected (N = 63)	Infected (N = 37)	Severely infected (N = 40)
Limb buds or immature hind limbs	6 (10%)	11 (30%)	18 (45%)
Mature hind limbs only	41 (65%)	26 (70%)	18 (45%)
Forelimbs	16 (25%)	0 (0%)	4 (10%)
Thin	1 (2%)	4 (11%)	37 (93%)

on body only. Almost all were thin (Table 1). After collection, 43% of tadpoles in Group B died during 15 h in transit to the laboratory.

Histological examination revealed fungi attached to the dermis on the head or lower jaw, and focal ulceration occurred at sites of fungal attachment. Masses of branching, aseptate hyphae overlaid areas of necrotic dermis, thickened by mononuclear cell infiltration and distension (Fig. 3). Fungi did not invade through the dermis. Chains of bacterial rods, some cocci, occasional yeasts and debris such as vegetable matter were present in the mycelium. Mycelia filled the oral cavity of some tadpoles and occasionally hyphae were seen attaching within the mouth to a mildly inflamed submucosa with focal or extensive ulceration of the mucosal epithelium (Fig. 4). Hyphae stained negative with PAS and positive with methenamine silver. Significant lesions were not observed in the gills or internal organs. Intestinal ciliates were observed in 8/10 infected and 4/10 healthy tadpoles.

On scanning electron microscopy of three tadpoles there was a fungus with a similar appearance to that seen in the first outbreak (Fig. 5). Focal thickening of the skin occurred where the fungal mass attached, and this is consistent with the swelling seen histologically (Fig. 6). Tadpoles had matts of branching, fine hyphae adhering over the skin surface. These hyphae had rounded tips and branched almost at right angles to the main axis. Achlyoid-type clusters of encysted primary zoospores were attached by a stem (lateral evacuation tube; Fig. 7). These features are consistent with an *Aphanomyces* sp. (Lilley et al. 1998). One of these tadpoles was also infected with a morphologically different fungal species with longer, thicker, branching hyphae of varying width radiating out from the head; the identity of this fungus is unknown.

Of the samples from five other tadpoles from which we attempted mycological culture, *Aphanomyces* sp. grew in two, a *Fusarium* sp. and a *Leptolegnia* sp. grew in one, a *Rhizopus* sp. and *Achlya* sp. grew in another,

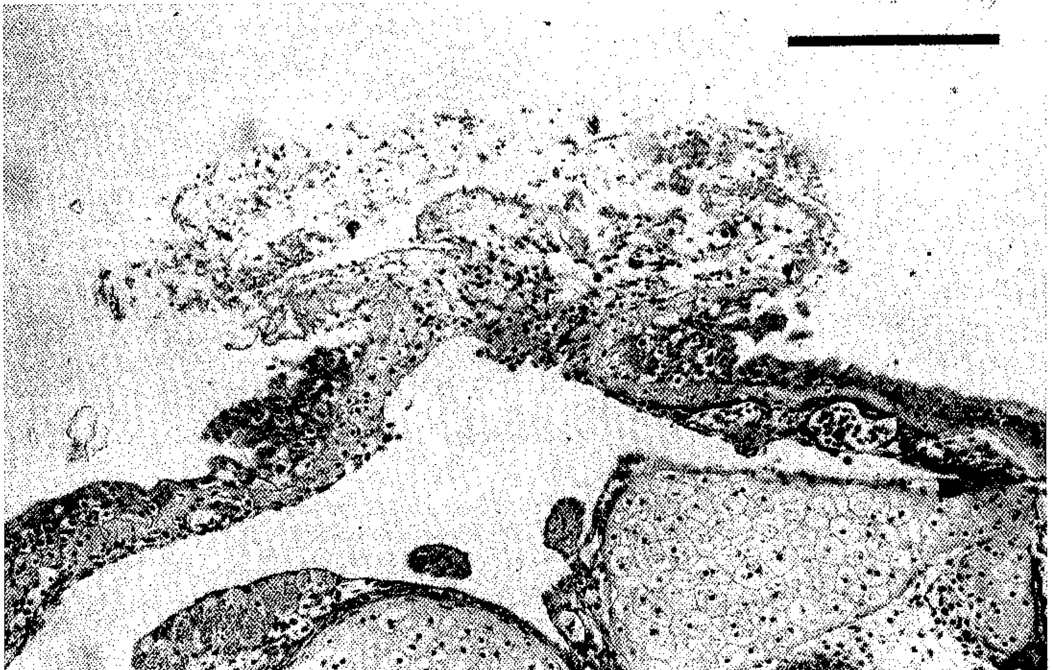

FIG. 3. Histological section of an of infection foci on lower jaw, with ulceration of the epidermis, and inflammation and distension in the dermis. Hyphae do not invade through the dermis. (H&E) Bar = 200 μm.

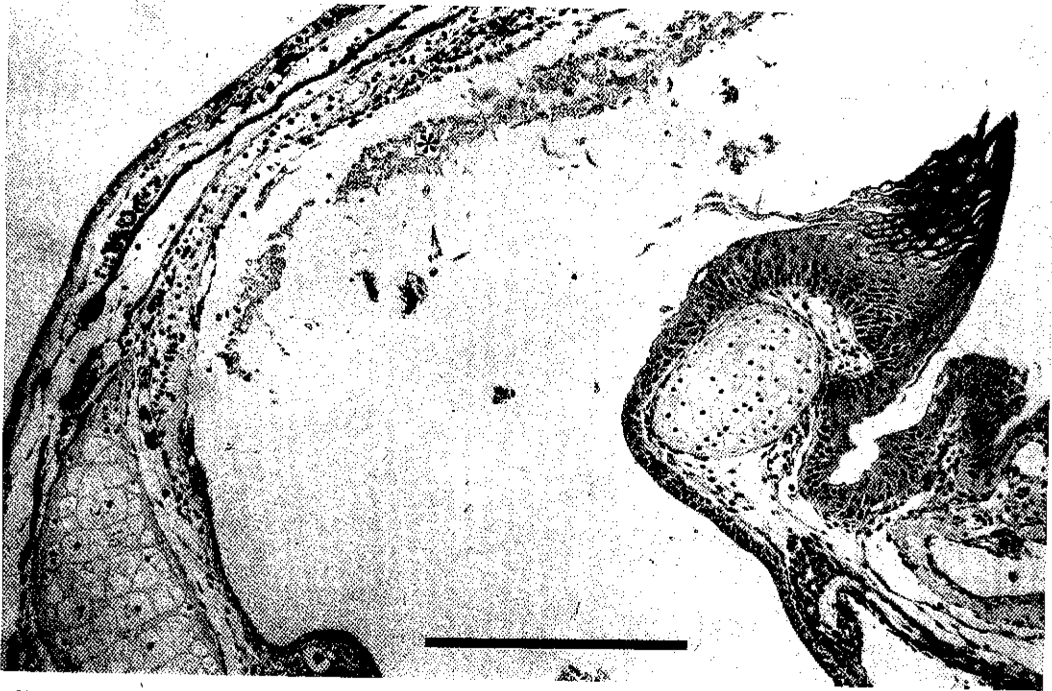

FIG. 4. Histological section of the mouth showing fungal hyphae (asterisk) adhering to the palate with extensive ulceration of the mucosal epithelium. (H&E) Bar = 280 μm.

and no fungi were observed in the remaining sample. No viruses were isolated on cell culture, although some toxic effects were observed in cells in the initial inoculation these were not transmitted with passaging.

Starvation was likely because the fungal infection primarily attacked the mouth and could have prevented the tadpoles eating. Obstruction of the eyes and nostrils would have major effects on sensory functions. Fungus-infected tadpoles were less mature than healthy ones, suggesting that this was a disease of young tadpoles or the disease may have slowed their development. Although dead tadpoles were not found in the wild, we suspect that severe infections were terminal because of the extensive lesions and the high mortality rate observed after collection. Dead and moribund tadpoles may have been removed rapidly by predation from tadpoles or other animals.

Identification of the primary species involved in cutaneous fungal diseases in fish is typically complicated by the presence of a range of opportunists (Lilley et al., 1998). The predominant fungus observed by scanning electron microscopy in the second outbreak was consistent with the *Aphanomyces* sp. identified by culture from two other tadpoles. The presence of the

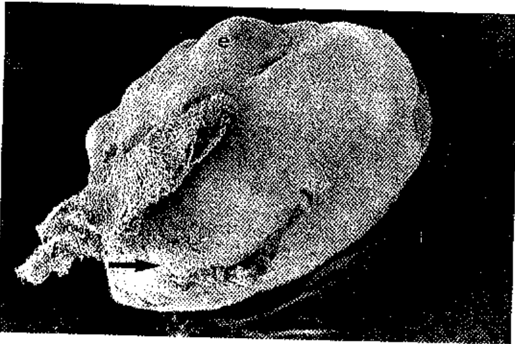

FIG. 5. Scanning electron micrograph of the tadpole in Figure 1. with mycelium growing between the nostrils. Note two smaller patches of fungus above the mouth (arrow). e = eye. Bar = 1 mm.

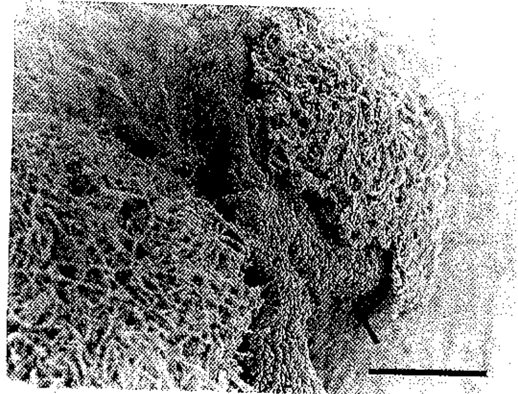

FIG. 6. Scanning electron micrograph of the patches of fungal infection above the mouth surrounded by a rim of swollen skin (arrow). Bar = 100 μm.

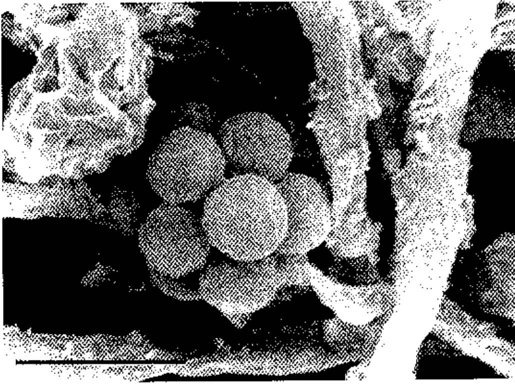

FIG. 7. Scanning electron micrograph of a cluster of primary zoospores on a lateral evacuation tube. Bar = 10 μm.

faster growing *Achlya* and *Fusarium* spp. isolated from the other tadpoles from which culture was attempted could have restricted growth of any *Aphanomyces* that may have been present. Culture was not informative in the first outbreak, but scanning electron microscopy on one tadpole revealed a fungus consistent with *Aphanomyces*.

Aphanomyces spp. are aseptate, zoosporic fungi in the family Saprolegniaceae. Members of this family may grow saprophytically and some species are common external pathogens of fish and shell fish (Willoughby, 1994). In outbreaks caused by Saprolegniaceae in fish, particular sites, such as a single fin, may be affected. This might be a result of variations in the epithelium or mucous layer making some areas more susceptible. Atlantic salmon fry have been observed dying from starvation with hyphal masses of *Saprolegnia diclina* blocking the pharynx, similar to these tadpoles.

Aphanomyces sp. infecting fish invade the muscle invoking granuloma formation, and sporulation is not seen except on culture (Lilley et al., 1998). In contrast, the infection of these tadpoles was superficial and extended away from the host, with growth and sporulation occurring as in culture. The cotton wool-like tufts appear similar to lesions of *Saprolegnia* infections in fish, but the morphology of the fungus is clearly consistent with *Aphanomyces*. The noninvasive lesions suggest it is not a highly pathogenic strain.

Aphanomyces has been described as a primary pathogen but also may need some stress to allow it to invade. In fish, *A. invadans* causes the fatal epizootic ulcerative syndrome (EUS; Lilley et al., 1998). A diverse group of biotic and abiotic agents that are thought to initiate skin lesions have been associated with outbreaks of EUS. For example, increased acidity of the water (as may occur after rain drains off peat soils), low dissolved oxygen, bacteria, and rhabdoviruses are suspected to precipitate epidemics by causing damage to the epithelium which then attracts fungal spores (Lilley et al., 1998). Lower temperatures are thought to contribute to outbreaks of disease by decreasing the immune response. However, it is unlikely that any specific factor is essential to cause an outbreak, and the only common agent in all epidemics was the presence of *A. invadans*. An experimental increase in water siltation led to an increased susceptibility to *Saprolegnia parasitica* infection in *Ambystoma tigrinum* (Lefcort et al., 1997). A synergistic effect between UV-B radiation and *Saprolegnia ferax* was experimentally demonstrated to increase mortality in amphibian eggs (Kiesecker and Blaustein, 1995). We do not know what environmental factors may have precipitated this fungal disease of giant toad tadpoles in agricultural areas, and concurrent diseases were not detected.

The *Saprolegnia* infection of wild tadpoles reported by Bragg and Bragg (1958) and Bragg (1962) is described as a fuzz on the surface of dead tadpoles, with no mention of infection on live tadpoles. Therefore, it is likely they were observing postmortem invasion.

During mass mortalities associated with amphibian population declines in Australia, tadpoles appeared to be unaffected (Laurance et al., 1996). The marine toad was introduced to Australia in 1935, and its distribution has since been increasing (Freeland, 1985). Studies of amphibian disease are relevant to conservation because lack of knowledge in this subject hinders investigations and management of the health of endangered species.

Acknowledgments.—We thank R. Alford, L. Speare, and J. Pritchard for collection of specimens; A. Gaeth and G. Russell for histology; F. Filippi for photography; L. Schneider for help with electron microscopy; and P. Hooper and M. Williamson for commenting on the manuscript. L. Berger was supported by a grant from Environment Australia.

LITERATURE CITED

BERGER, L., R. SPEARE, AND A. HYATT. 1999. Chytrid fungi and amphibian declines: overview, implications and future directions. *In* A. Campbell (ed.), Declines and Disappearances of Australian Frogs, pp. 23–33. Environment Australia, Canberra, Australian Capital Territory, Australia.

BLAUSTEIN, A. R., D. G. HOKIT, R. K. O'HARA, AND R. A. HOLT. 1994. Pathogenic fungus contributes to amphibian losses in the Pacific northwest. Biol. Conserv. 67:251–254.

BRAGG, A. N. 1962. *Saprolegnia* on tadpoles again in Oklahoma. Southwest. Nat. 7:79–80.

BRAGG, A. N., AND W. N. BRAGG. 1958. Parasitism of spadefoot toads by *Saprolegnia*. Herpetologica 14: 34.

FREELAND, W. J. 1985. The need to control cane toads. Search 16:211–215.

FULLER, M. S., AND A. JAWORSKI. 1987. Zoosporic fungi in teaching and research. South-eastern Publishing Corporation, Athens, GA.

KIESECKER, J. M., AND A. R. BLAUSTEIN. 1995. Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. Proc. Natl. Acad. Sci. USA 92:11049–11052.

LAURANCE, W. F., K. R. MCDONALD, AND R. SPEARE. 1996. Epidemic disease and catastrophic decline of Australian rain forest frogs. Conserv. Biol. 10:1–9.

LEFCORT, H., K. A. HANCOCK, K. M. MAUR, AND D. C. ROSTAL. 1997. The effects of used motor oil, silt and the water mold *Saprolegnia parasitica* on the growth and survival of mole salamanders (genus

Ambystoma). Arch. Environ. Contam. Toxicol. 32: 383–388.

LILLEY, J. H., R. B. CALLINAN, S. CHINABUT, S. KANCHANAKHAN, I. H. MACRAE, AND M. J. PHILLIPS. 1998. Epizootic Ulcerative Syndrome (EUS) Technical Handbook. The Aquatic Animal Health Research Institute. Bangkok, Thailand.

LUNA, L. 1968. Manual of histological staining methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw-Hill Book Company, New York.

MARQUEZ, R., J. L. OLMO, AND J. BOSCH. 1995. Recurrent mass mortality of larval midwife toads *Alytes obstetricans* in a lake in the Pyrenean mountains. Herpetol. J. 5:287–289.

NYMAN, S. 1986. Mass mortality in larval *Rana sylvatica* attributable to the bacterium, *Aeromonas hydrophila*. J. Herpetol. 20:196–201.

RAPHAEL, B. L. 1993. Amphibians. Vet. Clin. North Am. Small Anim. Pract. 23:1271–1285.

WILLOUGHBY, L.G. 1994. Fungi and Fish Diseases. Pisces Press, Stirling, U.K.

WOLF, K., G. L. BULLOCK, C. E. DUNBAR, AND M. C. QUIMBY. 1968. Tadpole edema virus: a viscerotropic pathogen for anuran amphibians. J. Inf. Dis. 118:253–262.

WONG, A., AND T. BEEBEE. 1994. Identification of a unicellular, non-pigmented alga that mediates growth inhibition in anuran tadpoles: a new species of the genus *Prototheca* (Chlorophyceae: Chlorococcales). Hydrobiologica. 277:85–96.

Accepted: 27 June 2000.