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ABSTRACT: Wyoming toads (*Bufo baxteri*) that died from January 1989 to June 1996 were submitted to the Wyoming State Veterinary Laboratory (Laramie, Wyoming, USA) for postmortem evaluation. These consisted of 108 free-ranging toads and 170 animals from six captive populations. Ninety seven (90%) of 108 free-ranging toad carcasses were submitted during September and October. From 1989 to 1992, 27 (77%) of 35 mortalities in the captive populations occurred in October, November, and December. From 1993 to 1996, mortality in captive toads occurred without a seasonal pattern and coincided with changes in hibernation protocols that no longer mimicked natural cycles. Cause of mortality was determined in 147 (53%) of the 278 cases. Mycotic dermatitis with secondary bacterial septicemia was the most frequent diagnosis in 104 (71%) of 147 toads. *Basidiobolus ranarum* was found by microscopic examination of skin sections in 100 (96%) of 104 of these mortalities. This fungus was isolated from 30 (56%) of 54 free-ranging and 24 (48%) of 50 captive toads. This research documents the causes of mortality for both free-ranging and captive endangered Wyoming toads over a 7 yr period.

Key words: Amphibian, *Basidiobolus ranarum, Bufo baxteri,* mortality, mycotic dermatitis, redleg, Wyoming toad.

INTRODUCTION

Amphibian populations are declining worldwide (Pechmann et al., 1991). These animals are considered environmental health indicator species due to their sensitivity to changes in their habitat (Beiswenger, 1988). Habitat changes related to pesticide spraying, changes in agricultural practices, increased predation, disease, increased UV radiation, decreased habitat, and climatic changes have all been suggested as possible contributing causes to these declines (Carey, 1993; Scherman and Morton, 1993; Blaustein et al., 1994). While numerous theories have been proposed to account for the declining amphibian populations, little research has been done to determine specific causes of mortality (Rabb, 1990; Bishop and Pettit, 1992; Wake, 1992).

The Wyoming toad (*Bufo baxteri*) and its drastic decline in numbers is an example of what appears to be occurring worldwide to amphibians. This toad, believed to be a glacial relic subspecies of the Canadian toad (*B. hemiophrys*), has historically only been known to inhabit the Laramie Basin of Albany County (Wyoming, USA) (Withers, 1992). They emerge from hibernation in mid-May and breed through mid-June; the young metamorphose in July, and hibernation begins in September and October (Withers, 1992). In the mid-1970's, the Wyoming toad population rapidly declined (Lewis et al., 1985) and the U.S. Fish and Wildlife Service listed the toad as an endangered species in February of 1984 (U.S. Fish and Wildlife Service, 1991). This decline coincided with the widespread aerial application of the pesticide fenthion for mosquito control in the area however, mortality investigations and toxicology were not done at that time (Baxter et al., 1982). We examined carcasses of 278 Wyoming toads that died from January 1989 to June 1996 and were submitted to the Wyoming State Veterinary Laboratory (Laramie, Wyoming,) for postmortem evaluation.

METHODS AND MATERIALS

Toad carcasses were from the free-ranging population ($n = 108$) and the six captive populations ($n = 170$). Tadpoles were not studied. Free-ranging toad carcasses were collected opportunistically during field surveys from the two areas inhabited in Albany County (Wyoming, USA; 41°20′N, 105°35′W). Surveys for free-ranging toads were conducted on a daily basis during June, July, and August and sporadically in May, September, and October during 1991 and 1992.

The entire captive population of toads is derived from one lake in Albany County. Toads being used for the captive breeding programs were housed at: (1) Wyoming Game and Fish Department Sybille Wildlife Research and Conservation Education Unit (Wheatland, Wyoming, USA); (2) Henry Doorly Zoo (Omaha, Nebraska, USA); (3) Houston Zoo (Houston, Texas, USA); (4) Toledo Zoological Park (Toledo, Ohio, USA); (5) Cheyenne Mountain Zoo (Colorado Springs, Colorado, USA); and (6) Cincinnati Zoo (Cincinnati, Ohio, USA). Specific husbandry protocols varied between the facilities.

Dead Wyoming toads were usually placed in sterile plastic bags, placed on ice, and delivered or shipped via overnight mail to the Wyoming State Veterinary Laboratory. The toad carcasses varied in degree of postmortem decomposition. Carcasses were weighed, sexed, and age determined as juveniles (metamorphosed to 2-yr-old) or adults (over 2-yr-old). Postmortem evaluation included gross examinations and subjective evaluation of body condition based on the size of intestinal fat bodies and pericardial fat.

Sections of ventral abdominal skin, digits, tongue, gluteus muscle, lung, heart, liver, kidney, stomach, intestine, fat bodies, reproductive tract, and abnormal tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 to 7 μm, and stained with hematoxylin and eosin. Brain and dorsal skin sections were occasionally collected for histology. Periodic-acid Schiff (PAS), Gomori methenamine silver (GMS), and Gram's stains were utilized as necessary (Chandler et al., 1980; Rippon, 1982). Tissues were evaluated by light microscopy.

Swabs of abdominal skin, subcutaneous fluid, liver, lung, kidney, and intestine were collected and placed in modified Stuart's bacterial transport medium (S/P Brand Culturette Systems, Baxter Diagnostics, Deerfield, Illinois, USA). These were plated onto Columbia agar with 5% sheep blood (Acumedia Manufacturing Inc., Baltimore, Maryland, USA). The plates were incubated at 35 C in atmospheric air for 96 hr.

Each day plates were examined for bacterial growth. Bacterial isolates were identified using one of two commercial aerobic bacterial identification systems (PASCO Panels, Difco Laboratories, Detroit, Michigan, USA; and/or Biolog Panels, Biolog, Inc., Hayward, California, USA). Due to the large number of isolates obtained at certain times, some were frozen prior to identification. Prior to summer 1993, only the predominant bacteria were isolated and identified to genus. Beginning in the summer of 1993, all bacterial isolates from a tissue or sample were identified to genus and species.

Fungal cultures were conducted by placing sections of abdominal skin and digits on Sabouraud's dextrose agar slants and incubating them at room temperature (22 C) for 1 mo (Rippon, 1982). Resulting cultures were identified by colony morphology and microscopic examination. Dissected culture elements from isolates were stained with lactophenol cotton blue and identified microscopically by morphology (Rippon, 1982). Impression smears of ventral skin were air dried, heat fixed, stained by PAS and examined by light microscopy.

A subset of Wyoming toads were examined by electron microscopy. Liver and intestine with contents were examined by negative stain electron microscopy for viruses (Nunamaker and Williams, 1986).

RESULTS

Complete information and data could not be collected from all toads due to autolysis or incomplete reporting from the submitting institution. Of the 278 Wyoming toads that died from January 1989 to June 1996, 108 (39%) were from the free-ranging population and 170 (61%) were from the six captive populations. Ninety seven (90%) of 108 free-ranging toad carcasses were submitted during September and October. From 1989 to 1992, 27 (77%) of 35 mortalities occurred in the captive populations during October, November and December. From 1993 to 1996 mortality in the captive populations occurred throughout the year without a seasonal pattern.

Of 244 carcasses for which age could be determined, there were 112 (46%) adults and 132 (54%) juveniles: From the wild population, 90 carcasses were aged and this equaled 58 (64%) adults and 32 (36%) juveniles. The carcasses of captive toads

TABLE 1.   Results of postmortem examination of captive and free-ranging Wyoming toads submitted to the Wyoming State Veterinary Laboratory for postmortem evaluation, January 1989 to June 1996.

Diagnosis	Number of free-ranging toads	Number of captive toads	Total toads
Mycotic dermatitis			
Basidiobolus ranarum	54	46	100
Mucor sp.		4	4
Septicemia			
Edema syndrome	1	23	24
Generalized	1		1
Hepatitis		1	1
Peritonitis		1	1
Muscle			
Degeneration	2	2	4
Dysplasia		1	1
Gastrointestinal			
Rupture		2	2
Obstruction		3	3
Hibernation related			
Dehydration		3	3
Emaciation		2	2
Toxic: Chlorhexidine diacetate		1	1
Undetermined	15	37	52
Unsuitable for evaluation	35	44	79
Total	108	170	278

that were aged totaled 154, with 54 (35%) adults and 100 (65%) juveniles. Overall, there were 206 toads that could be sexed with 110 (53%) females and 96 (47%) males. Within the free-ranging population 45 (42%) of 108 carcasses were female while 39 (36%) were males. Within the captive population 65 (37%) of 178 carcasses were females while 57 (32%) were males. Carcasses in which both age and sex could be determined in both the free-ranging and captive populations equaled 198 carcasses consisting of 65 (33%) adult females, 45 (23%) juvenile females, 42 (21%) adult males, and 46 (23%) juvenile males. The 78 carcasses of free-ranging toads were categorized as 34 (43%) female adults, 11 (14%) female juvenile, 20 (26%) male adults, and 13 (17%) male juvenile. Of the 120 captive toads submitted, there were 31 (26%) female adults, 34 (28%) female juveniles, 22 (18%) male adults, and 33 (28%) male juveniles.

Primary causes of mortality were determined on 147 (53%) of the 278 carcasses (Table 1). Of the toads suitable for evaluation and where a primary mortality diagnosis could be determined, mycotic dermatitis with secondary septicemia occurred in 104 (71%) of 147 toads. This included 54 (93%) of 58 free-ranging and 50 (56%) of 89 captive toads. *Basidiobolus ranarum* was involved in 100% of the free-ranging cases and 92% of the captive cases. There were four cases of mycotic dermatitis within the captive population caused by *Mucor* sp.

Upon gross examination, toads affected with *B. ranarum* mycotic dermatitis displayed hyperemia and sloughing of the epidermis on the ventral abdomen. Small erosions or ulcers were often found on the ventrum of the toes which were also hyperemic. Histologically, the sections of skin were characterized by thickening and presence of numerous fungal spherules

TABLE 2.   Mycotic isolates from captive and free-ranging Wyoming toad carcasses submitted to the Wyoming State Veterinary Laboratory, January 1989 to June 1996.

Mortality diagnosis	Asper-gillus spp.	Basidio-bolus ranarum	Blasto-myces sp.	Clado-sporium sp.	Fusarium spp.	Geotri-chum sp.	Mucor sp.	Penicil lium sp.	Tricho-sporon sp.
Free-ranging toads									
Dermatitis: Mycotic	6	30	NI	1	12	1	17	8	NI
Septicemia									
Edema syndrome	1	NI	NI	NI	NI	1	1	1	1
Generalized	1	NI	NI	NI	1	1	1	1	NI
Captive toads									
Dermatitis: Mycotic	12	24	2	NI	14	6	14	9	2
Septicemia	NI[a]	NI	NI	NI	NI	NI	NI	NI	NI
Edema syndrome	2	NI	NI	1	4	NI	3	1	10
Peritonitis	1	NI	NI	NI	1	1	1	NI	NI
Gastrointestinal	NI	NI	NI	NI	NI	NI	NI	NI	NI
Rupture	1	NI	NI	NI	2	NI	1	1	NI
Obstruction	1	NI	NI	1	2	NI	1	NI	NI
Hibernation									
Dehydration	3	NI	NI	NI	1	NI	1	1	NI
Emaciation	1	NI	NI	1	NI	NI	NI	NI	NI

[a] NI = Not isolated.

and occasional hyphae in the superficial layers of the epidermis without significant inflammatory reaction. These lesions were morphologically identical to those caused by *B. ranarum* in Canadian toads described by Taylor et al. (1999a). The erosions in the skin of the toes contained fungi but these did not invade into the underlying dermis. Periodic-acid Schiff and GMS stained sections demonstrated the walls of the spherical fungi in the skin. These toads usually had livers that were enlarged, mottled, and grey. Microscopically, hepatocytes were swollen and pigment-bearing macrophages was common.

Fungal isolates are listed in Table 2. Of the toads that died of mycotic dermatitis, *B. ranarum* was cultured from 30 (56%) of the 54 free-ranging toad carcasses and 24 (48%) of the 50 affected captive toads. *Aeromonas hydrophila* was isolated from 63% of the free-ranging toads, but in only 38% of the captive toads. *Pseudomonas aeruginosa* was isolated from 35% of the free-ranging and 32% of the captive toads. In addition, *Acinetobacter lwoffi, Aero-monas veronii, Alcaligenes faecalis, Bacillus sphericus, Bacillus* sp. *Bordetella bronchiseptica, Chromobacter* sp., *Citrobacter freundii, Edwardsiella tarda, Eikenella corrodens, Enterbacter cloacae, Escherichia coli, Flavobacterium indolgenes, Halfenia* sp. *Klebsiella ozaenae, Morganella morganii, Pasturella multocida, Pleisomonas shigelloides, Proteus mirbilis, Proteus vulgaris, Pseudomonas fluorescens, Pseudomonas mallei, Pseudomonas maltophila, Salmonella arizonae, Serratia liquefaciens, Staphylococcus aureus, Staphylococcus epidermis, Streptococcus* spp., *Vibrio mettchnikovii,* and *Yersinia enterocolitica* were cultured in various combinations from ≤14 toads each.

Subcutaneous and coelomic edema was observed in one toad from the free-ranging population, while it was the second most common mortality diagnosis in the Wyoming toad captive populations, associated with 23 deaths. Externally, these toads displayed only mild ventral hyperemia. However, there was marked excess of clear to yellow fluid in the subcutis and

celomic cavity. Histology revealed scattered foci of ballooning degeneration occurred in the epidermis of some cases. There was hepatocellular degeneration in many toads and the livers often contained pigment-bearing macrophages. Renal tubular degeneration and glomerulopathy also was present. Bacteria were not observed microscopically. The predominant bacterial isolates were *Proteus* spp (10 toads), *A. hydrophila* (9 toads) and *P. aeruginosa* (8 toads).

Peritonitis with secondary septicemia was diagnosed in one juvenile male captive toad. The animal was in poor body condition and had increased fluid in the abdomen and hemorrhage and fibrin clots over the intestine. Hepatitis occurred in one juvenile captive toad. Microscopically, the liver contained aggregates of granulocytes and some mononuclear cells in the sinusoids. A generalized septicemia of most organ systems was noted in one free-ranging toad. Bacterial isolates revealed *P. shigelloides*. Two captive adult toads were diagnosed with intestinal obstruction and rupture leading to acute peritonitis. The causes of the ruptures were not apparent. Two captive toads had intestinal obstructions in which we were able to locate a blockage at the pelvic canal. These blockages appeared to be the result of old pelvic fractures that had healed with a lot of bone callus that in turn was restricting the normal function of the intestine. One toad had an intestinal obstruction of undetermined cause.

One emaciated captive male toad weighed 10 g; the predominant lesion was muscle dysplasia. Skeletal muscle sections were characterized by variation in size and staining properties of fibers. Nuclei were unusually abundant and the fibers appeared disorganized. Muscle degeneration was the primary lesion noted in two free-ranging toads and two captive toads. Sections of the skeletal muscle from these toads showed acute degeneration characterized by eosinophilia, swelling, and frag-

mentation without inflammatory cell response.

Additionally, three captive toads died of dehydration and two died of emaciation either during hibernation or within 2 wk pre or post hibernation. Presumed toxicity occurred when one captive male juvenile toad escaped and was found in a foot bath containing chlorhexidine diacetate disinfectant (Novasan, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA). The toad was found alive but the skin displayed a bluish tint. The toad died later that day. The only histologic lesion noted was that sections of the liver were characterized by marked swelling of hepatocytes with vacuolated cytoplasm.

Several abnominalies were noted in the captive population. Three adult toads had died that had been born with ocular defects. In one toad an eye protruded from the socket and was covered by skin. Histologically there was no apparent cornea and the retina was folded and atrophic in areas. The other two toads grossly had only one eye which was located in a normal position. The tissues were unsuitable for further evaluation. One male toad had polydactyly. The right rear foot had three extra toes on the medial aspect joined by skin. All of these toads were in good body condition when they died from mycotic dermatitis.

Of the tissues examined by electron microscopy from 17 free-ranging toads only one (6%) had virus-like particles. These were observed in the intestine/feces of an adult male toad and measured 24 to 29 nm in diameter. They were not further identified. The toad died of mycotic dermatitis. Virus-like particles were not observed in any of the 14 captive toads examined.

DISCUSSION

Mycotic dermatitis with secondary septicemia was the most frequently diagnosed cause of mortality in both the free-ranging and captive populations. Mycotic dermatitis was first observed in the free-ranging population in September and October of

1990 and it occurred again the following fall. In 1991, *B. ranarum* was identified histologically and by culture as the predominant pathogen causing skin lesions. This condition appeared to be 100% fatal once clinical signs were observed. The skin lesions may have provided an avenue for secondary bacterial invasion.

Basidiobolus ranarum, a saprophytic fungus, is a member of the family Entrophthoraceae (Coremans-Pelseneer, 1973). It was first isolated from healthy amphibian intestines by Eidam in 1886 (Hutchinson and Nickerson, 1970). The only description of *B. ranarum* causing disease in amphibians as a primary pathogen was in a captive colony of dwarf African clawed frogs (*Hymenochirus curtipes*) (Groff et al., 1991). This outbreak of mycotic dermatitis resulted in a morbidity and mortality rate of almost 100%. The clinical course of the disease appeared to be similar to that which occurred in Wyoming toads. *Basidiobolus* sp. is known to have chitinolytic activity (Gugnani and Okafor, 1980). This may explain why it has an affinity for insect cuticle and has been recovered frequently from insects. Thus, the toad's food base may actually be a source of infection.

Wyoming toads with mycotic dermatitis were treated with various pharmaceutical regimes. In 1994, we began using itraconazole (Janssen Pharmaceutica, Titusville, New Jersey, USA) with success, administered orally at a rate of 1 micro bead from a 100 mg capsule for 9 days. Treatment was started at the first sign of ventral skin or toe hyperemia. During the course of treatment some toads displayed dorsal skin darkening, abdominal edema, and a hunched posture. However, within 48 hr of treatment completion the toads appeared to have returned to normal. Due to the small size and endangered status of this species, no attempt was made to determine blood or tissue levels of the itraconazole. From 1994 on, toads were treated at the first detectable sign of hyperemia. We were unable to confirm with diagnostic evaluation that all treated toads had mycotic dermatitis.

Ninety percent of free-ranging and 77% of captive toad carcasses were submitted during the months of September and October. Causes of mortality were more readily defined in the captive populations because carcasses were usually in fresher postmortem condition. From 1993 to 1996 the captive population mortality trends changed and mortality occurred without a seasonal pattern. This trend coincided with changes in the hibernation protocols that no longer mimicked natural cycles. Some facilities did not hibernate all toads and some elected to hibernate toads for periods as short as 4 wk. There is some evidence to support seasonal variation in the immune system of amphibians (Zapata et al., 1992). In frogs, maximal development of the thymus occurs in the summer with marked regression as winter approaches. Even when they were maintained in an active state in a thermoregulated environment and not hibernated, the thymi regressed. Toads have lower humoral responses to heterologous erythrocytes in the summer and autumn as compared to the spring and summer (Zapata et al., 1992). Thus, an increased mortality in the fall may have occurred due to this seasonal variation in the toad's immune system.

Of the other fungal species identified from the Wyoming toads, several have been reported to be pathogenic to amphibians. However, most fungal infections are believed to occur opportunistically in amphibians, as they do in other species. *Geotrichum* sp. has infected *Bufo granulosus* and *Trichosporon* sp. has caused white piedra in *B. granulosus* and *Bufo marinus* (Mok et al., 1982). *Cladosporium* sp. has caused one case of mycotic dermatitis in a *B. marinus* (Bube et al., 1992). *Mucor* sp., *Aspergillus* spp., *Penicillium* sp., and *Fusarium* sp. are frequently considered nonpathogenic contaminants in fungal cultures (Rippon, 1982; Koneman and Roberts, 1985). The species of *Mucor* we isolated could not be identified (Taylor

et al., 1999b). This isolate was morphologically different then the *Mucor amphibiorum,* reported to cause systemic infection in cane toads (*B. marinus*) (Speare et al., 1997). The significance of *Blastomyces* sp. in amphibians is unknown.

Normal bacterial flora has not been characterized for most amphibian species, and therefore it has been difficult to assess which bacteria may be pathogenic. In toads that died of mycotic dermatitis with secondary septicemia the most common bacteria isolated was *A. hydrophila* followed by *Pseudomonas* spp. *Aeromonas* spp. have frequently been reported to cause "red leg" in captive amphibians (Gibbs, 1963; Fowler, 1986; Nyman, 1986). Reports of naturally occurring "red leg" epizootics occurring in free-ranging populations are rare. In 1948 a mortality event was reported to occur in *Bufo americanus* in West Virginia (USA) (Dusli, 1948). Within 48 hr of onset the majority of the population estimated at 300 individuals was dead. The causative organism *A. hydrophila* was isolated from heart blood. *Aeromonas* spp. are common in aquatic environments (Hazen et al., 1978; Palumbo, 1993). In experimental studies to determine the pathogenicity of *A. hydrophila* in *Rana pipiens,* extremely high numbers of bacteria (1.5×10^9) injected intraperintoneally were required to induce mortality (Rigney et al., 1978). This information supports the findings of a survey for *A. hydrophila* in free-ranging *R. pipiens* conducted in Minnesota (USA) (Hird et al., 1981). The bacteria was isolated from 94 (32%) of 294 clinically healthy animals thus demonstrating that it is not always pathogenic.

Of the other species of bacteria isolated from the Wyoming toad several are known to be pathogenic to amphibians. *Pseudomonas* spp. has been experimentally shown to induce mortality at suboptimal environmental conditions in *R. pipiens* (Brodkin et al., 1992). In addition, splenic mass was higher in the *Pseudomonas* spp. group than in the saline controls. *Staphylococcus*

sp. and *Citrobacter* sp. have also been associated with "red leg" in *R. pipiens,* however, *A. hydrophila* has always been present in combination with these other bacteria (Gibbs, 1963). *Flavobacterium* sp. has been reported to cause septicemia with subcutaneous edema in *R. pipiens* (Olson et al., 1992). *Salmonella* sp. has been reported in amphibians located in third world countries (Anver and Pond, 1984). The isolation of this bacteria is thought to reflect sewage contamination of their ponds. *Acinetobacter* sp., *Pleisomonas* sp., *Bacillus* sp., *Enterobacter* sp., *Escherichia* sp., *Klebsiella* sp., *Proteus* spp., *Serratia* sp., *Staphylococcus,* spp. and *Streptococcus* sp. have all been isolated from the intestine of *Rana catesbeiana* (Carr et al., 1976). However, the role of these bacteria in amphibian disease is not clear.

Muscle lesions occurred in a few toads. This could indicate a handling or substrate problem with muscle exertion, a dietary deficiency such as vitamin or mineral (vitamin E, or selenium) exists, or possibly, a developmental defect.

Three adult Wyoming toads were noted to have congenital ocular defects. Few diseases affecting the amphibian eye have been reported and none were found describing developmental ocular defects. However, ocular congenital abnormalities such as microphthalmia, anophthalmia, and cyclopia have been reported in many species of mammals and reptiles and the cause has usually been undetermined (Jones and Hunt, 1983; Millichamp, 1990).

In terms of health of the population, it is interesting that Wyoming toads, whether free-ranging or captive, appear to be highly susceptible to infection from a fungus that is common in their environment. Research is needed in evaluating potential predisposing factors that might cause immunosuppression and increased disease susceptibility in this species. This effort should focus on studying factors that have changed in the toad's environment and that it will face upon reintroduction to the

wild, such as the evaluation of the effects of pesticide application on the toad's disease susceptibility.

ACKNOWLEDGMENTS

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