

Diagnostic Histological Findings in Yosemite Toads (*Bufo canorus*) from a Die-off in the 1970s

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ABSTRACT.—Twelve adult and 25 larval Yosemite toad (*Bufo canorus*) specimens from the eastern Sierra Nevada of California were examined histologically for evidence of infectious, toxicological, and degenerative diseases. The preserved toads were selected from 21 that had been salvaged or collected during a die-off in 1976–1979 that immediately preceded a population decline. Causes of death of four toads were determined histologically; clinical signs and field observations suggested causes of death of three more. Four toads died of infectious diseases, including chytridiomycosis of the skin ($N = 1$), bacillary septicemia ($N = 2$), and combined chytridiomycosis and bacterial septicemia ($N = 1$). Infections by a funguslike organism (*Dermosporidium penneri*), renal myxozoa (*Leptotheca ohlmacheri*), larval *Rhabdias*, various gastrointestinal nematodes, urinary bladder flukes, and lung flukes were detected in five specimens. No evidence of degenerative diseases, virus infections, or intoxications was found. The variety of lethal diseases and our inability to determine the causes of death of five specimens suggests that one or more histologically undetectable diseases or intoxications may have also contributed to the deaths and population decline.

Amphibian populations have declined throughout the world (Heyer et al., 1988; Bradford, 1991; Carey, 1993; Pounds and Crump, 1994; Stebbins and Cohen, 1995; Laurance et al., 1996). Many declines can be traced to anthropogenic factors, but some have occurred in relatively protected habitats with minimal human impacts (e.g., national parks or remote mountain regions: Crump et al., 1992; Drost and Fellers, 1996; Lips, 1998, 1999). These declines have been of two general types: (1) abrupt die-offs in which many sick and dead animals are found, followed by population crashes 3–24 months later (Kagarise Sherman and Morton, 1993; Laurance et al., 1996; Lips, 1998, 1999); and (2) declines without documented die-offs or detected field casualties (Fellers and Drost, 1993; Pounds and Crump, 1994; Drost and Fellers, 1996; Fisher and Shaffer, 1996).

The significance of field casualties is twofold. First, the observation of dead and dying toads (or other small vertebrates) suggests that actual mortality numbers are much higher because the removal of carcasses by predators and scavengers has been overwhelmed by the number of deaths (Wobeser and Wobeser, 1992). Second, the sudden occurrence of casualties has epidemiologic features characteristic of an introduced infectious disease or intoxication.

In California, population declines have been

documented or suggested for *Ambystoma californiense*, *Bufo boreas*, *Bufo canorus*, *Bufo microscaphus californicus*, *Rana aurora*, *Rana boylei*, *Rana cascadae*, *Rana muscosa*, *Scaphiopus hammondi*, *Scaphiopus intermontanus*, and *Taricha* spp. (Bradford, 1991; Fellers and Drost, 1993; Kagarise Sherman and Morton, 1993; Stebbins and Cohen, 1995; Drost and Fellers, 1996; Fisher and Shaffer, 1996). A documented die-off in one *B. canorus* population near Yosemite National Park preceded widespread population declines (Kagarise Sherman and Morton, 1993; Drost and Fellers, 1996); this die-off coincided with observed mass mortality, rapid population declines, and local extinctions in the mountain yellow-legged frog (*R. muscosa*) in nearby Kings Canyon National Park (Bradford, 1991).

Recent studies involving mass casualties of entire amphibian assemblages in Costa Rica, Panama, and Australia (Berger et al., 1998; Lips, 1998, 1999) as well as more localized and species-specific mass mortality of eggs, larvae, neotenes, and postmetamorphic adults (Worthylake and Hovingh, 1989; Bradford, 1991; Blaustein et al., 1994a; Jancovich et al., 1997) have implicated bacteria, fungi, and viruses as etiologies. The mass mortality among Yosemite toads at Tioga Pass was similar in its epidemiology and symptomatology to amphibian casualties reported in Central America and Australia. To determine whether infectious disease was a factor in the Sierra Nevada die-offs, histologic examinations were performed on adult toads preserved in 1976–1979, normal-appearing tadpoles collected

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in 1977 and 1978, and skeletal and skin remains preserved in 1990.

MATERIALS AND METHODS

Study Species.—*Bufo canorus* is a montane species that historically inhabited an area of 240 km × 60 km in the central Sierra Nevada (Karlstrom, 1962). The species is diurnal (Mullally, 1953; Mullally and Cunningham, 1956) and sexually dichromatic (males are olive-green, females are mottled black and grayish-white). Individuals are active above ground for only about four months each year between May and September, and hibernate beneath deep snow for the remainder of the year (Karlstrom, 1962; Kagarise Sherman, 1980; Morton, 1981; Kagarise Sherman and Morton, 1993).

From 1971 to 1982, a population of *B. canorus* adjacent to Yosemite National Park at Tioga Pass, California, was censused yearly (Morton and Sokolski, 1978; Kagarise Sherman, 1980; Morton, 1981, 1982; Kagarise Sherman and Morton, 1993). Beginning in the late spring of 1976, field casualties were observed for three years, and by 1982, the population had declined by 90% (Kagarise Sherman and Morton, 1993). The majority of observed field casualties (19 of 36) had no gross abnormalities or apparent causes of death; six toads died while crossing a large snowfield near the pools, and two females were found dead after being amplexed by several males. However, nine (25%) casualties had evidence of infectious disease, characterized as reddened skin of the ventrum and toes ($N = 8$), or ventral skin nodules ($N = 1$). An additional 21 toads were found alive but with similarly reddened ventral skin and toes (Kagarise Sherman and Morton, 1993).

Collection of Specimens.—Twenty-one dead or dying adult *B. canorus* were collected at two locations: at or near breeding sites in Tioga Pass Meadow (TPM) at the southern end of Tioga Lake, Mono County (119°E, 38°N, elevation 3030 m) in 1976–1979 ($N = 19$); and at a breeding area at the northwest end of Saddlebag Lake, Mono County (119°E, 38°N, 3072 m, 6.8 km from Tioga Pass) in 1977 ($N = 2$). In addition, the remains of 22 *B. canorus* (skins, partial skeletons, and ovaries and oviducts of females) were salvaged in 1990 from Mildred Lake, Mono County (119°E, 37°N, elevation 2975 m, 53.1 km from Tioga Pass). Twenty-five Yosemite toad tadpoles, alive and normal-appearing, were collected in 1977 and 1978 from the main breeding area in TPM where sick and dead adults were found. All collection sites were east of the crest of the Sierra Nevada.

The specimens from Tioga Pass Meadow and Saddlebag Lake were fixed in neutral buffered 10% formalin and deposited in the Museum of

Zoology, University of Michigan at Ann Arbor, where they were stored in ethanol in the herpetological collection. Partial carcasses from Mildred Lake and the tadpoles were fixed and stored in isopropyl alcohol and 10% formalin, respectively, and kept in the personal collection of CKS.

From 1971 to 1975, and from 1979 to 1982, toads were captured by searching throughout TPM and around Saddlebag Lake. In addition, during 1976–1978, toads entering the main breeding area at TPM were caught by a drift fence with pitfall traps. Adult toads were permanently marked by clipping a unique combination of toes (Kagarise Sherman, 1980; Kagarise Sherman and Morton, 1993).

Pathology and Histology.—Adult specimens were selected to span the range of collection dates, sites, and causes of death as assessed at the time of collection. Toad carcasses were examined, weighed, and portions of organs and tissues were removed. Skin from the pelvic patch and one whole hind-limb digit (usually digit IV, but in three toads another digit because of ante mortem toe clipping) were examined from 12 toads collected from 1976–1979 (11 at TPM, one from Saddlebag Lake). Digits were decalcified in saturated EDTA solution for 24–48 h. The livers, spleens, mesonephroi, lungs, urinary bladders, stomachs, and cloacae of eight toads were examined histologically. At least four pieces of skin were selected for histological evaluation from each of 14 carcasses from Mildred Lake; wherever possible, yellowish ventral skin was included. Tadpoles' oral discs (toothrows and jaw sheaths) were examined under a dissecting microscope. Tadpoles were Gosner's stages 27–36, with one exception (stage 43–44). Tissues and whole tadpoles were processed routinely through ethanol and xylene, and embedded in paraffin. Six micron-thick sections were stained with hematoxylin and eosin (H&E) and Giemsa stains.

RESULTS

Three toads that had been accidentally killed in May 1976 were chosen to serve as pre-die-off specimens (Table 1). Gross and histological examinations of these specimens revealed no significant infectious diseases (Table 2).

Ages of 10 toads, determined by counting growth rings in phalangeal bones, ranged from four to nine years (Table 2). The minimum ages of six toads, calculated from first capture dates and likely age at sexual maturity, ranged from five to eight years (Table 1). In five toads, age was determined by both methods. In four cases, the toad's age was estimated within 1–2 yr by the two methods. One toad was nine years by

TABLE 1. Field data for *Bufo canorus* preserved from 1976–1979. Abbreviations: TPM, Tioga Pass Meadow breeding areas; TPM*, nonbreeding areas; SBNW, breeding areas at northwest end of Saddlebag Lake; M, male; F, female; †, sex determined by external characteristics only; Minimum age calculated from first capture date in breeding area, assuming that males were 3 years old and females 4 years old when first captured in breeding area (Kagarise Sherman, 1980); ND, not determined; Condition, status when captured or collected for preservation: P, pre-die-off specimen.

Catalog no.	Collection date	Toe clip date	Location	Sex	Minimum age	Condition	Presumed cause of death
144321	16May76	12June71	TPM	M†	8 yr	Dead	Probably trampled, P
144322	21May76	10June74	TPM*	F	6 yr	Dead	Probably trampled, P
144323	27May76	none	TPM	F	ND	Alive	Died after chalk injection, P
156674	5June77	4June77	TPM	F†	ND	Dead	Amplexed by 6 males
156678	15June77	none	TPM	M	ND	Dead	Unknown
156685	16June77	24June76	SBNW	F	5 yr	Dead	Unknown
156681	24June77	12June77	TPM	M	ND	Dead	Dead in shallow burrow at fence
156677	4July77	19June74	TPM	M	6 yr	Alive	Accidental hyperthermia in plastic bag
156714	6July78	27May76	TPM	F	6 yr	Dead	On snow bank after breeding
156717	14July78	29June78	TPM	F	ND	Dead	Swollen red toes
156718	14July78	30June78	TPM	F	ND	Sick	Swollen red toes
156716	8June79	25May76	TPM	M	6 yr	Dead	Unknown, possibly trampled

growth rings and was known to be at least six years old based on its first capture date.

Beginning in June 1976, Yosemite toads at Tioga Pass Meadow began to show signs of illness (CKS, pers. obs.). One male was bloated and weak when captured and was found dead five days later; two emaciated individuals were found in late summer, and several other males were found dead but with no external abnormalities. During the next three years, more dead and moribund toads were found throughout the population at TPM (Table 3). The number and percentage of dead and moribund males varied from 1976 to 1982. Female deaths peaked in 1978, and only one dead female was found after 1978. One dead and one dying *B. canorus* were also found during a survey of the Saddlebag Lake (northwest) population on 16 June 1977.

Between 34.9% and 68.4% of the adult males that had been toe clipped at or near the main breeding area in TPM were recaptured in the same area one year after being marked (Fig. 1). These one-year recapture rates were significantly lower for males marked from 1976–1978 compared to those marked from 1971–1974 and in 1979 and 1981 (unpaired *t*-Test, $t = 3.23$, $df = 7$, $P = 0.015$, percentages transformed using square-root of arcsine values). Males marked in 1978 had the lowest one-year recapture rates (Fig. 1). In addition, only 13.3% (2/15) of the males toe clipped in 1978 that were recaptured one year later were known to be alive two years after marking. Comparable recapture percentages two years after marking for males toe clipped in the other study years were: 1971, 91.7% (33/36); 1972, 94.4% (17/18); 1973, 68.4% (80/117); 1974, 87.9% (80/91); 1976, 74.2% (89/

120); 1977, 46.3% (25/54); and 1979, 50.0% (2/4).

Serious infectious diseases were detected in histological examinations of four of 12 toads (Table 2). These diseases were identified as epidermal chytridiomycosis ($N = 1$; Figs. 2–3), bacillary bacterial septicemia ($N = 2$; Fig. 4), and a combination of both diseases ($N = 1$). Infectious diseases of uncertain significance were detected in an additional five specimens (Table 2): a systemic fungal infection by *Dermosporidium* sp. ($N = 1$; Fig. 5), myxozoan infection (*Leptotheca ohlmacheri*) of the mesonephros ($N = 1$; Fig. 6), and systemic larval *Rhabdias* sp. infections ($N = 3$; Fig. 7). A variety of helminths were found in the gastrointestinal tracts, lungs, and urinary bladders of five toads (Table 2). The trematodes in the urinary bladders ($N = 3$) were consistent with *Gorgodera* sp., *Gorgoderina* sp., or *Megalodiscus* sp. (Flynn, 1973) and were considered innocuous. The trematodes in the lungs ($N = 2$) caused very mild inflammatory reaction and probably were *Haematoloechus* sp.

The skins of 14 toads that probably were killed by ravens (*Corvus corax*, Kagarise Sherman and Morton, 1993) at Mildred Lake in 1990 were free of chytridial and dermosporidial fungi. Autolysis (decomposition) hindered examinations of about half of the skins; postmortem bacterial and water mold invasion of the skin was evident in seven of the toads.

Because of autolysis of internal organs, histologic examinations of the 25 tadpoles were limited to skin, oral discs, and gills. Grossly, there was no evidence of deformed, malformed, or supernumerary limbs, nor depigmentation or ulceration of the keratinized segments of the

TABLE 2. Results of pathological examinations of *Bufo carinatus* preserved from 1976-1979. Abbreviations: \wedge , missing organ(s), thus mass is greater than shown weight; SVL, snout-vent length; SUL, snout-urostyle length; age estimated by counting growth rings in hind-limb phalangeal bones; ND, not determined; De, *Dermosporidium* infection; L, lung flukes; M, Myxozoan (*Leptotheca oltmachi*) infection; Ub, flukes in urinary bladder; 0, lesion not present; +, minimal lesion; ++, mild lesion; ++++, moderate lesion; +++++, severe lesion.

Catalog No.	Mass, g	SVL/SUL, mm	Age	Gross lesions	Histologic lesions					
					Bacterial septicemia	Chytrid fungi	Rhabdias larvae	Flukes	Other	
144321	13.60	52.5/48.5	>9 yr	(Unopened body cavity)	0	0	0	0	0	
144322	12.60 \wedge	61.5/57.0	5 yr	Dysedcysis of fingers & chin; red fingertips; ovaries & part of liver absent	0	0	0	0	0	
144323	15.33 \wedge	62.0/58.5	ND	Ovaries & spleen absent	0	0	0	0	0	
156674	9.65 \wedge	56.5/51.5	5 yr	5 ulcers on chin, chest & belly; 3 nodules with ulcers next to urostyle; liver, spleen & gonads absent	0	0	0	0	De	
156678	13.05	56.0/49.0	4 yr	None	0	0	0	Ub	M	
156685	14.83	61.5/57.0	ND	Atrophied fat bodies; nodules in urinary bladder	+	0	++	Ub	0	
156681	8.18	45.0/44.5	7 yr	Emaciated	++++	++	0	0	0	
156677	11.13	52.0/47.5	5 yr	None	0	++	0	Ub	0	
156714	14.69	58.0/54.0	8 yr	None	0	0	++	0	0	
156717	11.76	56.5/50.5	7 yr	Worms in stomach & lungs (3)	+++++	0	0	L	0	
156718	10.09	51.0/48.5	6 yr	Splenomegaly; 1 lungworm	+++++	0	0	L	0	
156716	11.54	54.0/50.0	9 yr	None	0	0	++	0	0	

TABLE 3. Total number of dead and moribund adult male and female *Bufo canorus* found in Tioga Pass Meadow from 1976–1979 and in 1981 and 1982, and the percentage of each year (data from Kagarise Sherman and Morton, 1993) that were found dead or moribund.

	Males		Females	
	Number dead	% (sample size)	Number dead	% (sample size)
1976	6	1.8% (342)	3	5.5% (55)
1977	13	8.0% (162)	3	9.5% (63)
1978	8	3.7% (216)	13	13.0% (100)
1979	4	5.3% (75)	0	0% (46)
1981	1	3.9% (33)	1	1.6% (63)
1982	3	10.7% (28)	0	0% (81)

oral discs. Histologically, there was no evidence of viral, bacterial, fungal, or helminthic infections in the skin, oral discs, or gills. No dead or sick tadpoles were observed in TPM except when pools dried up before the tadpoles completed metamorphosis.

DISCUSSION

To determine the cause of the die-off at TPM, we must account for the following observations. First, the onset of mortalities began abruptly in 1976 and continued for at least six years, as indicated by finding dead toads in the field. Second, no single known infectious disease, such as chytridiomycosis or bacillary septicemia, was found in more than 25% of the dead toads, in marked contrast to reports of infection rates of

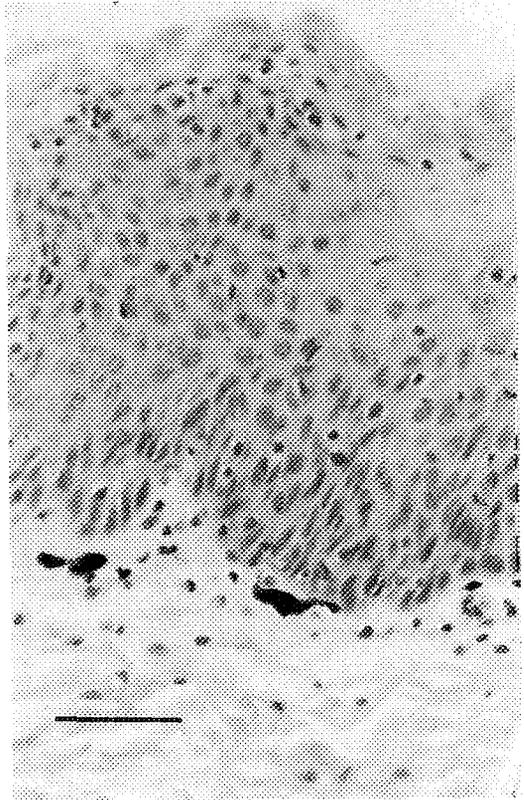


FIG. 2. Toad number 156677, skin of pelvic patch. Chytrid fungi are present in the superficial (stratum corneum) layer of epidermis with marked proliferation (acanthosis) of the middle layers of epidermis (stratum spinosum and stratum granulosum) and thickening of the stratum corneum (hyperkeratosis). Note paucity of inflammatory cell response. H&E stain; bar, 50 microns.

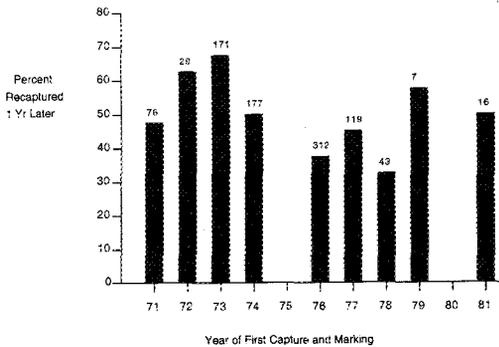


FIG. 1. Percentage of male *Bufo canorus* that were first captured and toe clipped in a given year and recaptured one year later (or known to be alive because they were recaptured two or more years later). Only males marked at or within 200 m of the main breeding area at Tioga Pass Meadow are included. Numbers above bar are total number toe clipped each year; data from 1975 are not included because of partially missing marking records; no marking was done in 1980.

more than 90% associated with amphibian die-offs caused by chytrids or bacilli (Worthyale and Hovingh, 1989; Berger et al., 1998). Third, a variety of serious or potentially serious diseases were found in the toads (chytridiomycosis, bacillary septicemia, systemic dermosporidiosis, and systemic rhabdiasis). Fourth, the absence of lesions, observed illness, and death among tadpoles at TPM, except that caused by pool desiccation and insect predation (Kagarise Sherman, 1980), indicates that adult Yosemite toads were more susceptible. Fifth, males declined before females at TPM (Kagarise Sherman and Morton, 1993). Sixth, several toads had no detectable histological abnormalities that could explain their deaths. Seventh, the Yosemite toad population at TPM has continued to decline and has not recovered (Kagarise Sherman and Morton, 1993; CKS, pers. obs., 1999). Populations of *B. canorus* elsewhere in the species' range have

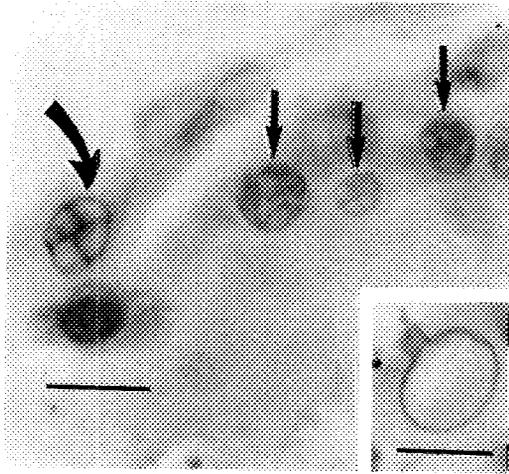


FIG. 3. Toad number 156677, skin of pelvic patch, Giemsa stain. Zoosporangia of *Batrachochytrium dendrobatidis* (arrows) in various stages of development within keratinized epidermal cells. One zoosporangium has multiple septae of uncertain function (curved arrow). The observed characteristic morphology of chytrids (Berger et al., 1998) were a roughly spherical zoosporangium of 3–16 micron diameter (arrows; $N = 100$; median: 6.1 microns; mean: 6.5 microns; range: 2.8–16.0 microns), strictly intracellular location, a discharge tube (inset), and one to more than 15, 1–2 micron diameter intrasporangial zoospores (not shown). Bar, 10 microns. Inset: one empty zoosporangium with discharge tube to the skin surface; all zoospores have been discharged. Bar, 10 microns.

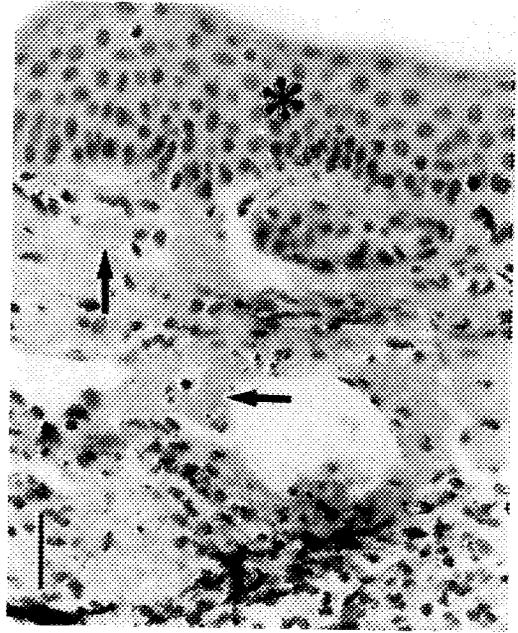


FIG. 4. Toad number 156718, skin and lymphatic sac of toe. The normal-appearing epidermis (asterisk) overlies dermis and lymphatic sac that have been damaged by bacillus bacteria, are severely edematous, filled with fibrin (arrows), and contain many necrotic cells. The presence of fibrin, necrotic cells, and intracellular bacteria within phagocytic cells and endothelial cells of many organs (e.g., livers, spleens, and bone marrow) of this toad and two others are diagnostic of ante mortem bacterial septicemia. Bacilli were small ($< \frac{1}{2}$ micron long), but morphology alone is insufficient to identify the bacteria, even to a family. Compare the thickness of the normal epidermis of this chytrid-free toad to the epidermis in Figure 2; also compare the dermal inflammation in this figure to the normal dermis in Figure 2. H&E stain; bar, 30 microns.

declined or disappeared completely (Kagarise Sherman and Morton, 1993; Stebbins and Cohen, 1995; Drost and Fellers, 1996).

Two intriguing epidemiological observations are that males died and suffered population declines 1–2 yr before females, and tadpole mortality was not found. The most significant behavioral difference between male and female Yosemite toads is that adult males usually return to pools to breed each year and spend more time in the breeding pools than females; adult females may return to breed only every second to fourth year (Kagarise Sherman, 1980; Kagarise Sherman and Morton, 1993). This suggests that some agent(s) in the water is associated with the die-off and persistent population decline. However, the absence of morbidity and mortality in tadpoles suggests either that tadpoles are resistant to the agent(s), the agent(s) is more prevalent at the peak of spring breeding than in the summer, the agent(s) requires more than one year to accumulate in toad tissues, or the agent(s) is associated with terrestrial prey of adults rather than aquatic vegetation consumed by tadpoles.

Chytrid Fungus Infections.—The mortality

events and rapid population declines of amphibian genera in Panama and Australia have been attributed to a single new disease, chytridiomycosis (Berger et al., 1998), in which more than 90% of all sick and dead anurans had widespread chytrid skin infections. By contrast, only two Yosemite toads in this study had chytrid infections. Consequently, epizootic chytridiomycosis is not considered the cause of the mortality event in the 1970s. The absence of chytrid fungi in the keratinized oral discs of Yosemite toad tadpoles is additional evidence that chytridiomycosis was not widespread at TPM. However, this study establishes the susceptibility of adult Yosemite toads to chytrid infection and confirms the presence of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (Longcore et al., 1999), in the United States at least 18 yr before the massive epizootic in western Panama (Berger et al., 1998; Lips, 1999).

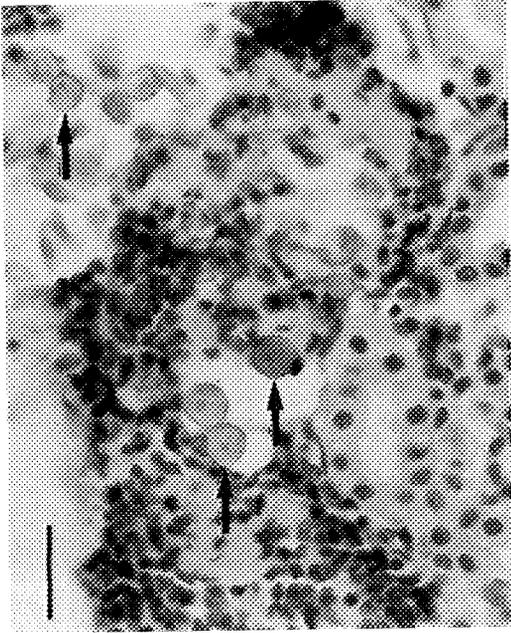


FIG. 5. Toad number 156674, mesonephros. Multiple spores of *Dermosporidium penneri* (arrows) in intertubular capillaries have elicited a granulomatous inflammatory cell response. The spores probably entered the mesonephric capillaries from the lymphatic sacs where they were carried to the pelvic lymph hearts and were pumped in the lymph to the renal portal circulation. Dermal and renal spores were spherical, extracellular, 10 microns in diameter ($N = 20$; median: 10.15 microns; range: 7.2–11.7 microns). Each spore contained 19 to more than 24, 1–4 micron diameter inclusions and a central nucleus. Embolic spores were also found in the liver, renal glomeruli, and fat bodies. Grossly, eight centrally ulcerated nodules were found in the ventral skin and around the urostyle. H&E stain; bar, 20 microns.

The three principal sites of fatal chytridial infections in tropical bufonids (*B. conifer*, *B. haematiticus*, and *B. marinus*) were the pelvic patch, ventral abdominal skin, and hind-limb digits (Berger et al., 1998). The chytrid infections of adult Yosemite toads were atypical because the digital skin was not affected. However, only one hind-limb digit from each toad was examined histologically. Unfortunately, no data indicate the number of digits that are infected and unaffected in lethal chytridiomycosis.

The absence of fungal, bacterial, and helminthic pathogens in the skins of toads probably killed by predators at Mildred Lake suggests that the toads were not selected as prey items because of underlying infections. The absence of chytrids and dermosporidia in this population, approximately 12 yr after the die-off at TPM and Saddlebag Lake, supports our conclusions that these fungal pathogens have a low preva-

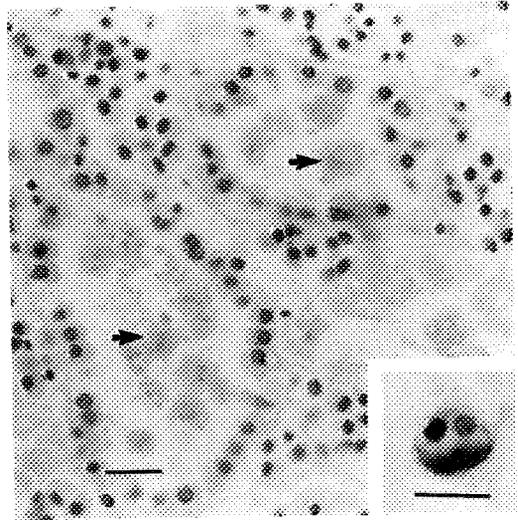


FIG. 6. Toad number 156678, mesonephros. Dilated renal tubules contain weakly stained intraluminal myxozoan trophozoites (arrows) and spores of *Leptotheca ohlmacheri*. The size of trophozoites could not be determined because of their irregular shape and weak staining by H&E and Giemsa. H&E stain; bar, 20 microns. Inset: One mature spore of *L. ohlmacheri*. Note the ovoid shape, bilateral symmetry, and pair of nearly spherical, bluish-black polar capsules. The median size of spores was 10.1×8.4 microns ($N = 12$; range 7.9–12.5 \times 6.1–9.9 microns; mean: 10.1×8.3 microns). Polar capsules had a median size of 3.25×3.0 microns ($N = 20$; range 2.7–3.5 \times 2.4–3.3 microns; mean: 3.2×2.9 microns). Giemsa stain; bar, 10 microns.

lence in Yosemite toads and are an unlikely cause of the die-offs that began in 1976.

Bacteremias.—Acute, overwhelming bacterial septicemia resulting from small bacilli, also called "red leg" disease, was lethal in three toads. The presence of these bacteria in the endothelial cells of many blood vessels and within phagocytic cells indicates that the bacteria were present in the circulation before the toads died and were not decompositional bacteria.

Of major concern to us was the possibility that wounds resulting from toe clipping became infected by bacteria and progressed to septicemia. All three *B. canorus* with bacterial septicemia had been captured and clipped 12 to 14 days prior to death; thus, the probable cause of septicemias in these three toads was toe clipping. However, several findings suggest that some additional factor may have been present beginning in 1976 that promoted bacterial infections of toe wounds or weakened the toads' resistance to bacteria. First, toe clipping was done on this population for five years before field mortalities were first observed in 1976. In addition, Kagarise Sherman (1980) found no significant correlation between the percent of



FIG. 7. Toad number 156685, lung. Two larval *Rhabdias* (Nematoda; arrows) are present in the airway and a capillary; larval and mature *Rhabdias* were numerous in the lungs of this toad. A maximum of three *Rhabdias* larvae were detected histologically in partial mesonephroi of two other toads. Larvae were 11.0–12.3 microns in diameter ($N = 5$), had indistinct platymyarian muscle cells, a smooth cuticle, and an intestine composed of uninucleate, low cuboidal epithelium; these are characteristics of *Rhabdias* sp. (Chitwood and Lichtenfels, 1972). H&E stain; bar, 30 microns.

males recaptured and the number of toes removed from *B. canorus* caught during the breeding season in 1974 and 1976 and recaptured in 1976 and 1978, respectively. Finally, the population has not recovered (Kagarise Sherman and Morton, 1993; CKS, pers. obs., 1999) despite the cessation of toe clipping in 1982.

Although toe clipping is a standard, widely used technique for marking amphibians (Donnelly et al., 1994), its effects on subsequent mortality are not well studied. Clarke (1972) found a negative association between recapture probability and number of toes removed in Fowler's toads (*B. woodhousei fowleri*), probably because of

the absence of toes rather than the presence of open wounds because wounds healed rapidly without signs of swelling. Studies of *R. pretiosa* (Reaser and Dexter, 1996) and *Crinia signifera* (Lemckert, 1996) found signs of infection in less than 1.0% and 1.2%, respectively, of the toe clipped frogs when they were recaptured. However, 18.2% of 66 *B. calamita* that were toe clipped developed infections ranging from infection of the toe stump to necrosis of the entire foot (Golay and Durrer, 1994).

Dermosporidium Infection.—The morphology of the skin nodules and spores is consistent with *Dermosporidium penneri* (Jay and Pohley, 1981), although the occurrence of this infection in the western United States, in this host, and spores in visceral organs have not been reported previously. The presence of fibrin and inflammatory cells around some spores in the mesonephros (Fig. 5) is evidence that spores became embolic and may have produced infarctions in multiple vital organs. Infarctions affecting the brain, spinal cord, ear labyrinths, or eyes could be immediately fatal, or cause slow lingering death resulting from paralysis, torticollis, or blindness. Because vital organs of the central and sensory nervous systems were not examined histologically, it is uncertain whether this disseminated infection contributed to the death of the infected toad, or whether amplexus by six males was the sole cause of this female toad's death.

Rhabdias and Myxozoan Infections.—Nematodes of the genus *Rhabdias* are widely regarded as common innocuous lungworms of many amphibians (Brannian, 1984), but in large numbers, *Rhabdias* larvae are fatal to juvenile *B. marinus* (Williams, 1960). During invasion of the host, larvae may be found in the skin, eyes, and body cavity (Williams, 1960; Flynn, 1973). It is not clear whether the number of larval nematodes in non-lung sites in the *B. canorus* specimens were equivalent in intensity to the fatal infections described by Williams (1960). In the experience of DEG, detection of more than one larval *Rhabdias* outside of the lungs and gastrointestinal tract is rare. The myxozoan morphology and tissue tropism are diagnostic of *Leptotheca ohlmacheri* (Kudo, 1922). Although *L. ohlmacheri* is widespread in North American anurans (DEG, pers. obs.), their life cycle, host morbidity and mortality rates, and effect on renal function are unknown. The mild infection in one Yosemite toad was considered innocuous.

Was Immunosuppression a Cause of the Die-off?—The absence of one predominant disease in a majority of toads and the variety of infectious diseases suggests two possible explanations. First, we were simply unable to detect the primary etiological agent(s). Our diagnostic tests

on the formalin-fixed toads were limited to gross and histological examinations. To preserve the museum specimens intact, we did not examine histologically the special, sensory and central nervous systems, hearts, and some tissues of the immune system (thymus, lymphomyeloid organs, etc.) and would not have detected any infectious or toxic agents that had their principal effects on these systems. Although histological examinations can detect infections caused by iridoviruses and most bacteria, fungi, protozoa, and helminths, often there are no observable abnormalities with some virus infections and most intoxications (Haschek and Rousseaux, 1998).

Second, the variety of infections in these Yosemite toads suggests that their immune systems were suppressed. Virus infections and intoxications by pesticides and other contaminants may be acutely lethal, impair the immune system, or cause other nonlethal injuries (e.g., malformations, disruption of hormones, impairment of other organs; Carey and Bryant, 1995; Carey et al., 1999). Two studies have documented immune impairment and lethal secondary bacterial infections following virus infections in frogs (Cunningham et al., 1996) and pesticide exposure in toads (Taylor et al., 1999). Cunningham et al. (1996) found that iridovirus-infected adult *R. temporaria* were prone to secondary bacterial infections, and Taylor et al. (1999) found that adult *B. woodhousei* had much higher mortality rates with combined exposure to a pesticide (malathion) and a Gram-negative bacteria (*Aeromonas hydrophila*) than when exposed to only one of the two agents. Such immune suppression is not selective for bacterial infections but allows opportunistic infections by a variety of bacterial, fungal, protozoan, and helminthic organisms (Griffin, 1997).

Evidence of virus infections was not found in these Yosemite toads. Most viruses isolated from amphibians have been ranaviruses in the family, Iridoviridae (Tweedell and Granoff, 1968; Wolf et al., 1968; Smith et al., 1986; Speare et al., 1991; Speare and Smith, 1992; Bennati et al., 1994; Cullen et al., 1995; Cunningham et al., 1996; Jancovich et al., 1997; Mao et al., 1997, 1999). Adult and larval *B. canorus* lacked the histological abnormalities associated with acute amphibian ranavirus infections. However, the abnormalities associated with chronic ranavirus infections have not been described, and information on amphibian virus diversity and pathology is inadequate. Thus, the Yosemite toads might have had an unrecognized virus infection that could have been lethal to some individuals and caused immune suppression in others.

A variety of chemicals are known to produce immunosuppression in vertebrates, including

halogenated hydrocarbons, (e.g., dioxin, hexachlorobenzene), polycyclic (nonhalogenated) hydrocarbons, insecticides, organotin, heavy metals, and oxidizing air pollutants (Haschek and Rousseaux, 1998; Taylor et al., 1999; Voccia et al., 1999). Many agricultural and pollutants are known to be lethal to amphibians (Kaplan and Overpeck, 1964; Kaplan et al., 1964, 1967; Matsui and Hayashi, 1992; Berrill et al., 1993; Schneeweiss and Schneeweiss, 1997; Marco et al., 1999). Direct application of pesticides (J. Van Wagtenonk, pers. com.), toxic chemical spills, industrial effluents, and agricultural run-offs have not been reported at TPM. However, atmospheric drift of pesticides from the heavily agriculturalized Central Valley is well documented in the Sierra Nevada (Cory et al., 1970; Zabik and Seiber, 1993; Astor and Seiber, 1997; McConnell et al., 1998), and concentrations of some pesticides in mountain surface waters are sufficient to be lethal to amphibians but not amphibians (LeNoir et al., 1999). Bioaccumulation of pesticides into tissues of Sierra Nevada amphibians has been reported (Cory et al., 1970; Datta et al., 1998). However, the synergistic effects of pollutants and concentrations necessary to cause immunosuppression or other sublethal effects are unknown. Histological changes associated with intoxication by most xenochemicals, even at acutely lethal doses, are slight and nonspecific (Haschek and Rousseaux, 1998). Toad specimens that predate the use of agricultural chemicals in the Central Valley were not available for comparison, and our histological examinations did not detect changes associated with chronic intoxication(s). Nevertheless, we conclude that the seven epidemiological and pathological features of this die-off of Yosemite toads are consistent with immunosuppression by a virus infection or chronic intoxication by one or more chemicals.

Other Possible Causes of Amphibian Die-offs.—Several hypothesized causes of amphibian die-offs and population declines were ruled out as factors in the die-off at TPM. Acute lethal intoxications, as occur in a direct application of pesticides or accidental chemical spill (Kirk, 1988; Matsui and Hayashi, 1992), were unlikely for four reasons: (1) there are no records of pesticide applications at Tioga Pass (e.g., for the control of lodgepole needle miners, *Coleotechnites milleri*; Struble, 1972); (2) the deaths spanned several years rather than being limited to a few days; (3) tadpoles were not affected; and (4) no other taxa suffered concurrent mortalities. The habitat at TPM is protected and has not been degraded by farming, ranching, or logging (Kagarise Sherman and Morton, 1993), and there has been no introduction of potential predators or competitors such as bullfrogs (*R. catesbeiana*)

at TPM (CKS, pers. obs.). Although nonnative trout have been stocked in lakes throughout the Sierra Nevada, Yosemite toads breed in shallow, ephemeral pools that are not inhabited by fish.

Physical and environmental stressors, such as handling, toe clipping, unusual temperatures or weather, and ultraviolet radiation, may cause deaths and immune suppression in amphibians (Carey, 1993; Carey et al., 1999). At TPM, Yosemite toads were captured, handled, and toe clipped for five years before casualties occurred (Kagarise Sherman, 1980; Morton, 1981, 1982; Kagarise Sherman and Morton, 1993). Toad populations declined at Saddlebag Lake, Mildred Lake, and elsewhere in the eastern Sierra Nevada (Kagarise Sherman and Morton, 1993; Drost and Fellers, 1996) where human visitors were infrequent, and handling and toe clipping were rare. Weather fluctuations (Pounds and Crump, 1994), or atmospheric warming (Pounds et al., 1999), may be causally linked to some amphibian declines. Although temperature and weather at TPM in 1977 and 1978 may have temporarily stressed adult *B. canorus* (Kagarise Sherman, 1980; Kagarise Sherman and Morton, 1993), such variations, which probably have occurred repeatedly for many millennia, cannot explain the persistence of the population decline for two decades. Ultraviolet radiation cannot be ruled out completely as a factor in the die-offs and persistent population declines; however it should be noted that casualties associated with rising levels of UV-B radiation have been reported only in some amphibian eggs, embryos, and larvae (Blaustein et al., 1994a; 1994b; Kiesecker and Blaustein, 1995; Blaustein et al., 1998; Hatch and Burton, 1998; Cummins et al., 1999) but not in adult amphibians.

This study demonstrates the potential value of museum specimens for investigating amphibian mortality events and population declines. We encourage salvaging and preservation of field casualties for museum specimens or prompt diagnostic examination. We also recommend the collection of apparently healthy individuals from declining amphibian populations and casualty sites for thorough diagnostic examinations and placement in museums. Not only can museum collections be used to assess changes in biodiversity (Shaffer et al., 1998), but preserved specimens may yield critical information to help understand the causes of historic amphibian population declines.

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