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Original article

An enigmatic mortality event in the only population of the Critically Endangered Cameroonian frog *Xenopus longipes*

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Abstract.—Contemporary global declines and mortality events in amphibian populations have been often attributed to infectious disease and climate change, separately and in combination. We report on an enigmatic mortality event in the only known population of the Critically Endangered frog species *Xenopus longipes*. This aquatic and biologically distinctive species is restricted to Lake Oku, a high-elevation crater lake on Mt. Oku in Cameroon. Neither a quantitative PCR-based screen nor histopathological analysis revealed the presence of the chytrid fungus *Batrachochytrium dendrobatidis*, which is believed to be responsible for many declines and mortality events in amphibian populations around the world. Histopathology revealed widespread epidermal hyperplasia and multifocal saprolegniasis suggesting that the animals have been exposed to a source of skin irritation. These sources might include acidified surface waters, perhaps derived from inorganic fertilisers or other human-related pollutants, or to local geological processes distinctive of the Cameroonian Volcanic Line. Currently, the causes underlying this mortality event remain obscure.

Key words.—Africa, amphibian declines, *Batrachochytrium dendrobatidis*, Cameroon Volcanic Line, histopathology, PCR

Contemporary amphibian population declines, global in scope and multi-faceted in nature, are perhaps most alarming where habitats remain relatively intact (Stuart *et al*. 2004). Recent research on enigmatic declines of amphibians has focused on infectious disease and climate change (Berger *et al*. 1998; Pounds *et al*. 2006; Lips *et al*. 2008; Rohr *et al.* 2008). However, it is clear that multiple causal factors, sometimes acting in concert, underlie many declines (e.g. Kiesecker *et al.* 2001; Wake & Vredenburg 2008; Rovito *et al*. 2009). There has been surprisingly little documentation of mortality events or declines in amphibian populations from many biodiverse regions, including sub-Saharan Africa. For example, the fauna of Cameroon and neighbouring countries is considered one of the world's biodiversity hotspots (Myers *et al*. 2000), yet there have been few published reports of amphibian declines in this area (IUCN 2009). The amphibian fauna of Cameroon is one of the most diverse in all of Africa (Stuart *et al*. 2004) and much of this diversity has been described only during the past

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forty years. The principal threat to Cameroonian amphibians is habitat loss and degradation, which is widespread throughout the many mountains in which Cameroonian endemics occur (Gartshore 1986; Gonwouo *et al*. 2006; IUCN 2009). During research in Cameroon in August of 2006, one of us (DCB) observed a mortality event on a scale not previously reported for the frog species *Xenopus longipes* (family Pipidae) that is restricted to Lake Oku, a high-elevation crater lake on Mt. Oku. Due in part to its very small range ($< 10\,km^2$), *Xenopus longipes* is Critically Endangered (IUCN 2009). This species is among the most morphologically distinctive species of *Xenopus* (Kobel *et al.* 1996) and is one of only two described vertebrate species known to be dodecaploid (the other is a closely related *Xenopus* species; Evans 2007). During previous surveys of this lake in 1984 (Gartshore 1986), 1990 (Loumont & Kobel 1991), 2003 (BJE), and September 2004 (DCB), similar mortality events were not observed. Although Doherty-Bone *et al*. (2008) did not comment on the mortality event, it was also observed by these authors in 2006 (T. Doherty-Bone, pers. comm.).

Chytridiomycosis, an infectious disease of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), is present on every continent where amphibians occur and is implicated in the declines of many populations (Berger *et al*. 1998; Skerratt *et al*. 2007). *Bd* has been identified in at least seven countries in sub-Saharan Africa, though nearly all records are restricted to southern and eastern Africa (Table 1). Thus far, *Bd* has been identified as a possible culprit in declines of at least one species of African amphibian (*Nectophrynoides asperginis*; Weldon & du Preez 2004; Channing *et al*. 2006). However, not all amphibian population declines and mortality events are attributable, or at least not entirely, to *Bd* (e.g. Vredenburg 2004; Lampo & Señaris 2006). Mass mortality events, in particular, can have a variety of infectious and non-infectious bases, one of the most reported of which is infection with ranaviruses (e.g. Cunningham *et al*. 1996; Bollinger *et al*. 1999; Green *et al.* 2002). We report on a mortality event in a Critically Endangered frog for which two independent lines of inquiry (histopathology and PCR-based screen) reveal no evidence of *Bd* and histology reveals no pathologies typical of lethal ranavirus infection. Our work underscores that amphibian declines and mortality events may be multi-faceted and not always clearly attributable to the spread of either infectious disease or climate change.

MATERIAL AND METHODS

Study Area and Sampling

Lake Oku is a crater lake located at 2 227 m elevation on western Mt. Oku. With a total area of $2.43\,km^2$, it is the largest montane Cameroonian lake, yet it is relatively shallow (maximum depth, 52 m) in comparison to other nearby montane lakes (Kling 1988). Because of its high elevation and colder temperatures, this lake is only weakly stratified (Kling 1988), containing a microstructure with many narrow temperature bands.

During previous work in 2003 (BJE) and 2004 (DCB), adult *Xenopus longipes* were found to be moderately abundant at a single accessible site (6° 12′ N, 10° 27′ E) at the margin of Lake Oku and no records were made of dead or dying specimens. In 2004 (September 22–23) and 2006 (August 18), specimens of *X. longipes* were collected from the same point at the northwest edge of Lake Oku; sick or dead

Table 1. Countries and frog taxa from sub-Saharan Africa with reports of infection by *Batrachochytrium dendrobatidis*.

Country	Frog taxon infected	Reference
Botswana	*Xenopus*	Aanensen & Fisher 2010
Cameroon	*Xenopus*	Soto-Azat *et al.* 2010
Dem. Rep. Congo	*Hyperolius*	Greenbaum *et al.* 2008
Ghana	*Silurana*, *Xenopus*	Carey *et al*. 2003 Morehouse *et al*. 2003 Aanensen & Fisher 2010
Kenya	*Afrixalus*, *Amietia*, *Amietophrynus*, *Hyperolius*, *Kassina*, *Phrynobatrachus*, *Ptychadena*, *Xenopus*	Speare & Berger 2000 Kielgast *et al*. 2009 Aanensen & Fisher 2010
Lesotho	*Amietia*	Aanensen & Fisher 2010
Malawi	*Xenopus*	Soto-Azat *et al.* 2010
Nigeria	*Chiromantis*	Imasuen *et al*. 2009
South Africa	*Amietia*, *Cacosternum*, *Heleophryne*, *Kassina*, *Strongylopus*, *Tomopterna*, *Vandijkophrynus*, *Xenopus*	Hopkins & Channing 2003 Lane *et al*. 2003 Weldon *et al*. 2004 Aanensen & Fisher 2010 Soto-Azat *et al.* 2010
Swaziland	*Xenopus*	Weldon *et al.* 2004
Tanzania	*Afrixalus*, *Hyperolius*, *Kassina*, *Nectophrynoides*, *Petropedetes*, *Phrynomantis*, *Ptychadena*,	Weldon & du Preez 2004 Channing *et al*. 2006 Aanensen & Fisher 2010
Uganda	*Amietophrynus*, *Leptopelis*, *Ptychadena*, *Xenopus*	Goldberg *et al*. 2007 Soto-Azat *et al.* 2010
Zambia	*Xenopus*	Aanensen & Fisher 2010

animals were only observed in 2006. At midday in 2004, an ad hoc visual encounter survey (counting individuals anywhere in the water column) revealed densities at this site, in clear water approximately 0.3–0.8 m deep, to be approximately 20 individuals per m^2. In the late evening (ca. 1900 h), densities were at least twice this. Mortality events were not observed for any frog species at nearby sites or sympatric with *X. longipes*. During the 2006 mortality event, dozens of dead and decomposing individuals were found floating near the bottom or at the surface along the lake margin (Fig. 1A). Many listless, and apparently dying, individuals were found, some with grossly visible mats of soft white-tan material on the limbs and especially the feet. Animals (both living and dead) were collected by hand or dip-net and stored overnight in water in plastic bags; the following day, live animals were euthanised in an aqueous solution of chloretone. Following removal of liver tissue samples for genetic analysis, specimens were fixed overnight in 4% neutral buffered formalin and then stored in 70% ethanol. Approximately 30 live and apparently healthy *X. longipes*

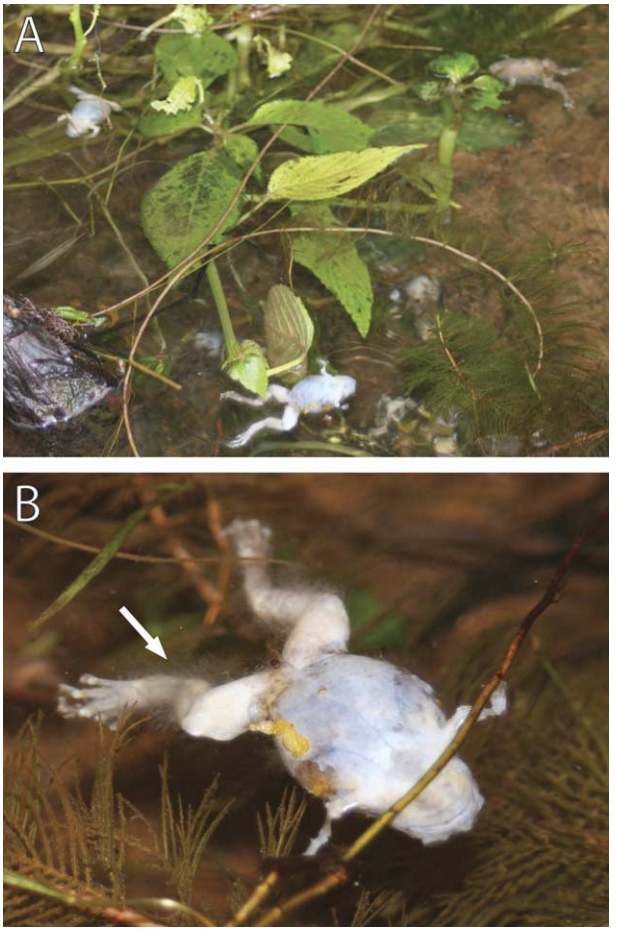

Figure 1. Dead and decomposing *Xenopus longipes* floating at margin of Lake Oku during 2006 mortality event (A). A close-up image (B) of dead *X. longipes* shows extensive coverage by oomycete hyphae (arrow).

collected during the 2006 mortality event were exported from Cameroon and subsequently housed in the aquaculture facilities of Columbia University (D. Kelley Lab) and then transported to the lab of BJE. During transport from Cameroon, all individuals were housed in a single container. All male individuals died during transport from Cameroon to New York in 2006, as also occurred during a previous attempt to export a breeding population by BJE in 2003. As of the time of publication, four female individuals remain alive in captivity. Sex-specific mortality during transport of *X. longipes* is one of many challenges faced by captive breeding programs and indicates that more information is needed on appropriate breeding conditions.

PCR-based Screen for *Batrachochytrium dendrobatidis*

To determine infection status, 16 of the live captive frogs (temporarily housed at Columbia University) were swabbed with a synthetic-cotton swab using a standardised

swabbing protocol (30 strokes, five between the toes on each hindfoot, five on each thigh, and five on each side of the ventral abdomen). Samples were analysed using a standard Real Time PCR assay specific for *Bd* (Boyle *et al.* 2004; Hyatt *et al*. 2007). We also analysed tissues collected from 10 dead animals at the field site. Either a toe or a small amount of skin tissue (approx. 10×10 mm) was removed from the dorsal side of the cadaver and stored in 95% EtOH. The same assay was used on these samples; a bead-beater was used to break up tissue during the extraction process. All samples were run in triplicate to ensure best detection of *Bd*.

Histopathological Analysis

Histopathology was carried out in the Wildlife Disease Laboratories of San Diego Zoo's Institute for Conservation Research. Four 'sick' specimens collected from the 2006 mortality event (XL-1 through XL-4) and two 'normal' specimens collected from the same locality in 2004 (MCZ A-136857, A-136871) were selected for histologic processing. All specimens were adult females except XL-2, which is an adult male. Immediately prior to histologic processing, specimens were post-fixed in 10% neutral buffered formalin and demineralised in hydrochloric acid (RDO Rapid Decalcifier, Apex Engineering Corp.). Following demineralisation, specimens were automatically processed using a Tissue-Tek VIP vacuum infiltration processor (Sakura Finetek USA Inc.), embedded in paraffin, and then serially sectioned transversally. Resulting histologic sections were stained with haematoxylin and eosin; selected sections were stained with Gomori's methenamine silver to detect the presence of water moulds or other fungi. Parasites were identified in section and via standard references (e.g. Gardiner *et al.* 1988; Gardiner & Poynton 1999). Histological sections of both sick animals from 2006 and normal specimens from 2004 are deposited in the Museum of Comparative Zoology at Harvard University.

Results

Molecular Diagnostics for *Batrachochytrium dendrobatidis*

The Real Time PCR assays for *Bd* revealed no positives despite the proper functioning of positive controls in each analysis.

Histopathology

On gross examination and in comparison to normal specimens from 2004, each of the sick specimens from the 2006 mortality event appeared emaciated with generalised muscle wasting. Two of the specimens selected for histopathology (XL-1 and XL-4) exhibited grossly visible tufts of soft white-tan material covering one of the hindfeet (e.g. Fig. 1B).

Histopathologic findings unique to and present in all of the sick specimens of 2006 were limited to the skin and associated soft tissues and to the gastrointestinal tract. The skin and underlying muscles of the feet (XL-1, XL-3, and XL-4) and legs (XL-2) had focally-extensive areas of necrotising dermatitis and myositis associated with large numbers of thin-walled, aseptate, non-branching hyphae that are morphologically

suggestive of oomycete-type water moulds ('saprolegniasis'). These areas of dermatitis corresponded to the tufts of material observed grossly on the feet of XL-1 and XL-4. In addition, all of the sick animals from 2006 had mild to moderate epidermal hyperplasia as determined by varying degrees of thickening and/or disorganisation of the epidermis (Fig. 2). Epidermal hyperplasia was present diffusely and not solely in the areas infected by the presumptive water moulds. Healthy animals from 2004 had distended stomachs filled with both arthropods and plant material, whereas the stomachs of sick animals from 2006 were mostly empty.

Histopathologic findings observed only in some, but not all, sick animals include small granulomas on the serosal surface of the stomach with a central nematode parasite in XL-1 and XL-2, and focal lymphocytic pancreatitis, mild granulocytic and histiocytic salpingitis and mild multifocal single cell necrosis in the gastric and intestinal mucosa of XL-1. None were considered to be of a magnitude or nature likely to contribute to death.

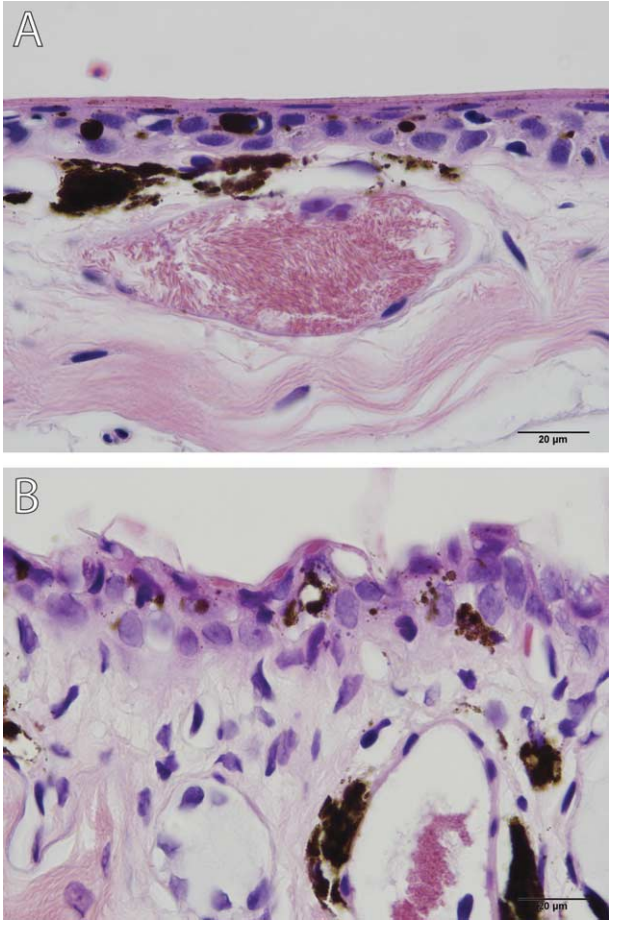

Figure 2. Normal skin from a healthy *Xenopus longipes* from 2004 (A) compared to a section of skin (B) from a sick *X. longipes* (XL-4) from 2006 showing relative epidermal cell disorganisation (epidermal hyperplasia).

Histopathologic findings that were common to both sick animals and normal animals included a wide variety of endoparasites and evidence of adipose tissue atrophy. These included an unidentified trematode parasite free in the coelomic cavity and/or pericardial sac that was associated with mild granulocytic inflammation (XL-1, XL-2, and MCZ A-136871). A different unidentified trematode parasite was found in small numbers encysted in the submucosa of the oral cavity and cloaca of all animals examined. This trematode was associated with moderate granulomatous inflammation in only one sick frog (XL-3). A third morphologically distinct trematode was found within the lumen of the small intestine in the two normal frogs. Other notable parasites included a spirurid-type nematode in the lumen of the stomach (XL-1, XL-2, XL-3 and MCZ A-136857); a myxozoan parasite (possible *Myxidium* sp.) in the lumen of the gallbladder (XL-1, XL-3 and MCZ A-136871) and a cestode parasite in the lumen of the small intestine (MCZ A-136857). Finally, in both the sick and normal specimens examined, we found evidence of either bone marrow (all specimens) or gonadal fat body (XL-1, XL-4, MCZ A-136871) adipose tissue atrophy. In all pathological and normal specimens, there are mild cases of accumulation of tubular cytoplasmic brown-yellow pigment within the kidneys and melanomacrophage hyperplasia in the liver. In addition, two specimens (XL-2 and XL-4) also exhibit hepatocellular atrophy.

Other tissues that were examined but appear to be normal histologically are as follows (relevant specimens listed in parentheses): thyroid gland (XL-1, MCZ A-136871); pituitary gland (XL-3, MCZ A-136871); brain (all specimens); spinal cord (all specimens); eye (all specimens); inner ear (all specimens); heart (all specimens); lung (XL-4, MCZ A-136871); urinary bladder (XL-1, MCZ A-136871); gallbladder (MCZ A-136857); subcutaneous lymph sac (all specimens); ovary (all female specimens); oviduct (XL-3, XL-4, MCZ A-136857, A-136871); testis (XL-2; i.e. the only male specimen); gonadal fat body (XL-3, MCZ A-136857); spleen (XL-2, XL-3, MCZ A-136871); small intestine (XL-1 through XL-4); large intestine (XL-1 through XL-4); pancreas (XL-2, XL-3, XL-4, MCZ A-136857, A-136871); bone (all specimens); skeletal muscle (all specimens); bone marrow hematopoietic tissue (all specimens); liver, sub-capsular hematopoietic tissue (all specimens); skin (MCZ A-136857, A-136871).

Discussion

There is no evidence from either the PCR-based screen or histopathology to suggest that the mortality event observed in August 2006 is associated with *Bd* infection. This result is consistent with a previous study searching for *Bd* in various frog species from around Mt. Oku (Doherty-Bone *et al*. 2008). We therefore reject the hypothesis that *Bd* is responsible for the mortality event of *Xenopus longipes* observed in Lake Oku in 2006. It is also notable that similar mortality events were not observed for any other amphibian species at Lake Oku or elsewhere on Mt. Oku or in nearby watersheds (Doherty-Bone *et al*. 2008). Surveys in and since 2006 suggest that levels of morbidity are relatively stable through time in this population (T. Doherty-Bone, pers. comm.).

Our histopathological study could not determine a specific cause underlying the mortality event, although some findings, while non-specific, suggest potential contributing factors. All of the sick frogs examined, including additional specimens

that were collected but not sectioned, exhibited skin infections with presumptive water moulds (i.e. saprolegniasis; Pessier 2002). In captive amphibians, water mould infections are often secondary infections to a variety of skin injuries (Pessier 2002), which can include skin trauma and poor water quality. In post-metamorphic wild amphibians, water mould infections are not commonly reported except in association with mortality of egg masses or larvae (e.g. Romansic *et al*. 2006, 2009; Ruthig 2009). Our observations of skin changes (epidermal hyperplasia) away from areas of water mould colonisation suggest that skin injury or irritation may have predisposed these animals to saprolegniasis. Based on our knowledge of contributing factors in captive amphibians, we speculate that several factors could predispose animals to saprolegniasis in wild populations. These include trauma from conspecifics, predators, or changes in substrates, and skin irritation or injury caused by changes in water quality. Because we did not perform a molecular screen for ranaviruses, we cannot definitively rule out that the skin lesions might have a primary viral etiology similar to the ranaviral "cutaneous ulcerative disease" observed in *Rana temporaria* (Cunningham *et al*. 1996). Yet, the lack of other histopathologic lesions that could suggest systemic ranaviral disease, such as necrosis in liver, kidney, or hematopoietic tissue, multicentric haemorrhage, or the observation of viral inclusion bodies makes lethal ranavirus infection unlikely.

All of the specimens from the 2006 mortality event appeared significantly emaciated in comparison to the 'normal' specimens collected in 2004. However, using criteria of body fat stores such as bone marrow adipose tissue and gonadal fat bodies, both groups exhibited signs of adipose depletion. All animals had atrophy of bone marrow fat stores and one of the normal specimens exhibited atrophy of the gonadal fat bodies. Based on these observations, we speculate that it may be that the emaciation of the specimens from the 2006 mortality event is due more to muscle wasting than depletion of body fat stores. Another potential explanation for the emaciated appearance of these specimens is the substantial difference in the quantity of stomach contents in comparison to the normal animals collected in 2004. The latter specimens had distended stomachs with abundant arthropods and plant debris whereas these were rarely present in the stomachs of the abnormal animals (data not shown). This relative lack of recent ingesta may be a non-specific finding relating to systemic illness in the abnormal group or might reflect a recent lack of prey items resulting in nutritional debilitation. Yet, this may not be unusual for *X. longipes* as Loumont & Kobel (1991, p. 735) reported that specimens of *X. longipes* collected from Lake Oku in 1990 "looked rather emaciated and starving." Thus, the possibility remains that our characterisation of both normal and sick *X. longipes* as exhibiting adipose depletion may somehow be characteristic of this unusual species.

The species of trematode observed in the coelomic cavity and pericardial sac of several specimens could not be determined from the sections examined, but it may be of interest to recover intact parasites from other preserved animals for parasitological examination. Previous reports of trematodes in other *Xenopus* species include *Clinostomum* sp. from the body cavity of *X. laevis* (Kuperman *et al*. 2004), *Diplostomulum* sp. from the pericardial sac of *X. laevis* (Nigrelli & Maraventano 1944), and gorgoderid trematodes, which are typically found in the urogenital system and are observed occasionally in the coelomic cavity of other anurans (Li & Lipman 1995). A different encysted trematode was commonly observed in the cloacal and oral cavity region of both normal and abnormal specimens, but was

associated with inflammation in only one animal (XL-3). Three animals had asymptomatic gallbladder infections with a myxozoan parasite, which have been previously documented only from frogs in southern Africa (Delvinquier *et al.* 1992); it is likely that this is a new species of *Myxidium* (e.g. Jirků *et al*. 2006). The stomach nematode observed in most of the normal and abnormal animals has features consistent with the Spirurida, including coelomyarian musculature, a large intestine with uninucleate cells, uterine larvae, and a sclerotised buccal capsule.

Among anthropogenic factors that might underlie the mortality event observed in Lake Oku, the most likely is exposure to agricultural run-off from nearby farms. Instances of epidermal hyperplasia have been documented in fish as a result of exposure to inorganic fertilisers (Paul & Banerjee 1996). Run-off of floodwaters from nearby fields conceivably could introduce such fertilisers into the surface waters of Lake Oku. Similar effects might be also expected from contamination of lake waters via purposeful dumping of toxic substances, though there is no evidence that this is the case.

In addition to potential anthropogenic factors, this mortality event might be explained by geological processes distinctive of the Cameroon Volcanic Line. Catastrophic and powerful CO_2 limnic eruptions ('degassing') from within crater lakes have been documented only twice (Lake Monoun, 1984; Lake Nyos, 1986) and both occurred at other Cameroonian lakes less than 75 km from Lake Oku (e.g. Zhang 1996; Zhang & Kling 2006). Release of CO_2 from bottom water in stratified lakes could lead to changes in water chemistry (e.g. dissolved O_2, pH) that might lead to epidermal hyperplasia, necrosis, and even sloughing (Daye & Garside 1976; Callinan *et al.* 2005). Yet, Kling (1988) found Lake Oku to be both weakly stratified and shallow in comparison to other crater lakes. Without strong stratification, it would be difficult for large quantities of CO_2 to build up in bottom waters. In addition, there are no reports from this area in 2006 of seismic activity, which may generate the release of a pocket of gas, and there is no prior evidence of a leak of CO_2 or of salt build-up, which would be expected as the gas-charged soda springs in Cameroon are rich in salts (G. Kling, pers. comm.). Taken together, this evidence suggests that Lake Oku does not contain a large amount of CO_2 in its bottom waters and that a release of CO_2-saturated water is an improbable cause of the observed mortality event in *X. longipes* (G. Kling, pers. comm.).

As a closed hydrological system, the likelihood of introduction of aquatic-borne diseases to the lake is lower than that of an open system, such as a lake with out-flowing streams. However, it also means that populations within the lake may not be readily and quickly replenished in the event of a mortality event, regardless of its cause. As such, special attention should be given to populations of aquatic organisms restricted to lakes throughout the Cameroon Volcanic Line. These have generally received little attention from biologists and pose a unique and significant challenge for conservation. Though the cause of this mortality event in *Xenopus longipes* remains unknown, our documentation of this event has been significant by leading to the formulation of a conservation action plan for this biologically unique and Critically Endangered species (Browne *et al.* 2009; R. Browne, pers. comm.).

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