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Histopathological Diagnosis of Diseases Affecting Amphibians Inhabiting Kihansi Gorge, Tanzania

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Abstract

Kihansi gorge is a natural habitat to Kihansi spray toad (KST), *Nectophrynoides asperginis*. The toad is extinct in its natural habitat partly due to diseases. Efforts to successfully re-introduce it are primed by awareness and mitigation of prevalent diseases at the gorge. This study aimed at exploring the prevalent amphibian diseases at and around Kihansi gorge. A total of 182 individuals from *Petropedetes yakusini* (n = 60), *Nectophrynoides tornieri* (n = 60) and *Leptopelis uluguruensis* (n = 62) species were collected, humanely sacrificed, fixed in formalin, routinely processed, sectioned, stained with haematoxylin and eosin and analysed for diseases by histopathology. Disease prevalence (percentage) in *P. Yakusini*, *N. Tornieri* and *L. uluguruensis* were strongyloidiasis 23.3, 31.7 and 29%; chytridiomycosis 8.3, 38.3 and 11.3%; inflammatory reactions 13.3, 6.7 and 14.5%; viral inclusion bodies 15, 11.7 and 9.7%; and granuloma 0, 0 and 1.6%, respectively. It is concluded that there are potentially fatal disease conditions at Kihansi gorge and its surroundings that can threaten the life of the re-introduced KSTs.

Keywords: Kihansi gorge, amphibians, Kihansi spray toad (KST), diseases, prevalence

Introduction

In 1993, the government of Tanzania approved construction of the lower Kihansi hydropower plant (LKHP). The plant was intended to use water from the Kihansi River. The river had a total water flow of 16 m^3/s at the completion of LKHP. Ninety percent of the water flow was diverted leaving only 1.6–1.8 m^3/s to the Kihansi gorge wetlands. The gorge wetlands were natural habitats to various fauna and flora, including the Kihansi spray toad, KST. The toad was discovered in 1996 during implementation of the environmental management plan in the course of establishing the LKHP. At the time of discovery, the number of KSTs was estimated to be around 22,000. A few years later, the numbers alarmingly declined and threatened the existence of the toad as a species. Five hundred toads were translocated to six zoos in the United States (USA) in 2001 to rescue against the presaged possibility of extinction. The KSTs in the gorge continued to die and the population finally crashed in 2003. Factors considered to account for the crash include reduced water flow to the gorge wetlands, collapse of sprinkler system that mimicked the natural water spray, infection with chytrid fungus, flushing of sediments from the dam, and human activities upstream the dam that used pesticides. The KST was last seen in its natural habitat in 2004 (Channing et al. 2009). In 2009, the International Union for Conservation of Nature (IUCN) declared that the KST was extinct in the wild.

Emergency of diseases due to changed microenvironments following the dam construction could have played roles to the extinction of the KST in the natural habitat. Indeed, chytrid infection has been proposed to account for the extinction of amphibians (Daszak et al. 1999). There could be other diseases in the gorge that might have

contributed to the population decline. The prevalence of these diseases poses a great survival challenge to the KST upon re-introduction. It is therefore worthy to establish the prevalent diseases at and around the gorge in order to mitigate any impending threats to the re-introduced KSTs.

Amphibians can be affected by viruses, bacteria, fungi, helminths and ectoparasites. Reported viral diseases in free-living amphibians include Ranaviral infection, Lucke frog herpesvirus (kidney cancer), Frog erythrocytic virus, and West Nile virus. The most common is Ranavirus. It causes emaciation, erythema associated with petechial or paintbrush haemorrhage around the mouth or base of the hind limbs; raised, vesicular, or erosive skin lesions; focal to generalized swelling due to effusions in the lymphatic sacs and body cavity; oedema, enlargement, and haemorrhage or discolouration of the spleen, liver, kidney, and gastrointestinal tract; focal to diffuse necrosis throughout the skin and internal organs, particularly the liver and hematopoietic tissues; basophilic intracytoplasmic inclusions in the liver (Bollinger et al. 1999, Docherty et al. 2003, Johnson and Wellehan 2005). Infection with Lucke herpesvirus is associated with renal carcinoma with intranuclear inclusions (Lucke 1934, Lunger et al. 1965). Currently, the disease is only diagnosed through histopathology. Other isolated viruses like frog adenovirus 1, Crotalus calicivirus type 1, and retroviruses appear to be non-pathogenic (Smith et al. 1986, Granoff 1989, Johnson and Wellehan 2005).

Bacteria diseases include bacterial dermatosepticemia also called Red leg disease caused by *Aeromonas hydrophila*. It appears as a reddening of the skin, particularly on the belly and underside of the thighs. The reddening is due to vasodilation, congestion, and petechial, paintbrush, or ecchymotic haemorrhages. Other changes include coelomic effusions, and epidermal erosions, ulcers, sloughing, or necrosis (Mauel et al. 2002). Flavobacteriosis or oedema syndrome caused by *Flavobacterium* is another bacterial disease characterised by effusions in the lymphatic sacs, hydrocoelom, lingual or corneal oedema, panopthalmitis, petechiation, and visceral congestion (Taylor et al. 2001). A less common mycobacteriosis, is a chronic, slowly progressive disease causing granulomatous inflammation that may appear grossly as solitary or multifocal nodules on the skin, liver, spleen, intestines, and mesonephros (kidney) (Green 2001).

Amphibian fungal infections include chytridiomycosis, zygomycosis, chromomycosis, *Basidiobolus* fungi, dermosporidiosis, ichthyophoniasis, *Dermocystidium* and *Dermomycoides*. Generally, they show up as areas of red inflammation based on soft white tissues, and sometimes any noticeable abnormal skin colour. Currently, chytridiomycosis is the most significant and well-described fungal disease of amphibians caused by *Batrachochytrium dendrobatidis* (Bd). It is a global threat to a broad host range of wild amphibian populations (Berger et al. 1998) and can cause severe population declines, extirpations of populations, and extinctions (Daszak et al. 1999). Lesions include thickening of the skin (hyperkeratosis) associated with dysecdysis, massive numbers of intracytoplasmic thalli in multiple retained epidermis layers, increased number of cell layers in the epidermis from a normal number (3–5) to a much larger number (about 8–15), and acanthosis (Daszak et al. 1999).

Protozoan parasites include amoeba, ciliates, flagellates, and sporozoans. The numbers of parasites encountered, the presence or absence of lesions, and the general condition of the host are important to assess the significance of infection. Amoebae are most often found in the gastrointestinal tract, liver, or kidney (Wright 2006). Ciliates, opalinids, and flagellates are commonly found in the gastrointestinal tract and on the skin of amphibians, and the majority of these organisms are commensal and nonproblematic

(Densmore and Green 2007). Sporozoan parasites can be found in amphibian blood, the gastrointestinal tract, and other organs or tissues with varying pathogenicity. They include coccidia (*Eimeria* and *Isospora*) (Poynton and Whitaker 2001) and microsporidians that have a tropism for muscle, connective tissues of various organs, and oocytes (Schuetz et al. 1978).

Common metazoan parasites include myxozoans, helminths (particularly trematodes and nematodes) and arthropods. Trematodes (flukes) cause diseases in amphibians only when associated with high numbers of trematodes encysting in, attaching to, or migrating through host tissues. Nematodes infect amphibians from egg to adult life stages and affect a variety of organs and tissues. Nematodes of the genus *Rhabdias* are lungworms that are problematic among captive amphibians (Williams 1960) whereas *Strongyloides* may cause protein-losing enteropathy and death (Patterson-Kane et al. 2001). Other helminths reported to infect amphibians and occasionally produce disease include filarid nematodes and cestodes (Wright 2006). Arthropods are less problematic to free living amphibians. Amphibians have a rich diversity of helminthic parasites (Poynton and Whitaker 2001). In general, most cestodes, trematodes and nematodes of amphibians are innocuous and not linked to specific clinical signs or mortalities.

Being a descendant of about 70 individuals and having stayed in captivity for more than a decade, the current KST population is thought to have acquired a new steady state away from the original state in the wild. Hypothetically therefore, the KST to be re-introduced to its natural habitat will face survival challenges before they acquire new steady state population numbers in the wild. The challenges include tolerance as well as survival against endemic amphibian diseases in the wetlands. These diseases have the potential to be fatal prior to immunity acquisition and adaptation of the toads to the wild environment. Thus, establishing endemic amphibian diseases that pose a potential impending threat to the survival of KST in the wild is pertinent to instituting interventions geared at successful re-introduction of the toad. Unfortunately, the endemic amphibian diseases at Kihansi gorge are not currently known. This study was therefore aimed at exploring the endemic amphibian diseases affecting amphibians in and around Kihansi gorge.

Materials and Methods

Study area

The study was conducted at and around Kihansi gorge, Tanzania.

The animals

A total of 182 frogs were collected from Kihansi gorge wetlands and the area around the gorge from February 2009 to February 2010. The collection was done by placing a net over the frog and gentle wrap of the hand around the frog's back and legs. The frogs were then placed in aerated containers and transported to the laboratory within 30 minutes. The animals were examined for any gross lesions prior to euthanization under chloroform and an autopsy examination. Faecal samples were taken and smears were prepared and examined for parasites.

Tissue processing

Following autopsy, tissue samples from all organs were taken and fixed in 10% neutral buffered formalin for at least 24 hours. Thereafter, they were trimmed and put in cassettes for routine tissue processing whereby the tissues were dehydrated in graded alcohol (70%, 90%, 95%, absolute alcohol), cleared in chloroform, impregnated, embedded and molded in molten (56 °C) paraffin wax. They were then sectioned with a rotary microtome at 4 μm, deparaffinized and stained with haematoxylin and eosin. The stained tissue sections were observed under a light microscope (BX41, Olympus, Japan) as done before (Malago and Nondoli 2008) and

digital photomicrographs were taken by a DP21 camera (Olympus, Japan) mounted on the microscope.

Statistical analysis

Numeric data were expressed as percentage prevalence, whereas pathologic changes were presented as photomicrographs.

Results

Figure 1 shows the prevalence of diseases at and around Kihansi gorge. According to the figure, fungal infection and strongyloidiasis were highest in *N. tornieri* and *P. yakusini* (38.3% and 31.6%, respectively). Strongyloidiasis was the most prevalent disease condition encountered while granulomatous lesion was the least and occurred only in *L. uluguruensis* at 1.6%. The least diseased animal species was the *P. yakusini* where 40% of the animals were normal, while *N. tornieri* was the most affected animal with only 11.6% of the animals being normal.

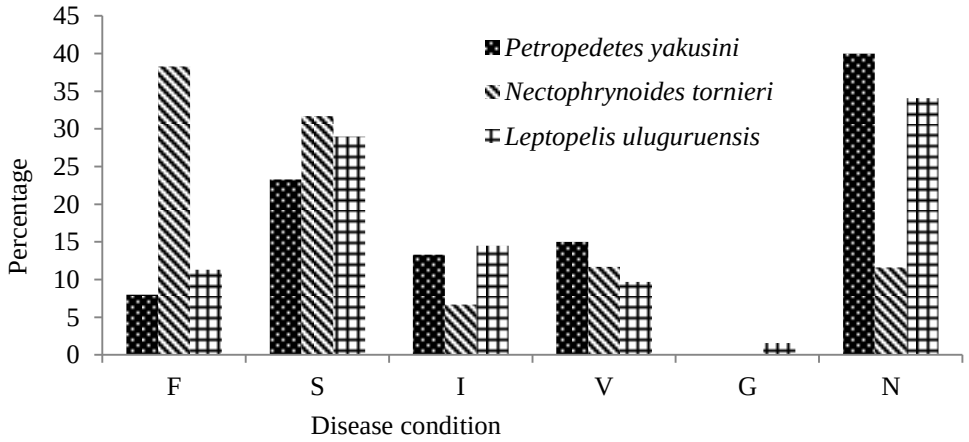


Figure 1: Prevalence of amphibian diseases at and around Kihansi gorge. F = fungal infections; S = strongyloidiasis; I = inflammatory reactions; V = viral infections; G = granulomatous lesions; and N = normal animals.

Figure 2 depicts mixed infections encountered at and around the Kihansi gorge. The figure shows that *N. tornieri* had the highest (15%) fungal and strongloides mixed infections followed by *P. yakusini* (5%) for the same mixed infections and *L. uluguruensis* (4.8%) for strongyloidiasis and inflammatory reactions. *P. yakusini* did not have inflammatory reactions during fungal infection and only *L. uluguruensis* had strongyloidiasis with viral or granulomatous lesions (Figure 2) prevalent at 1.6%.

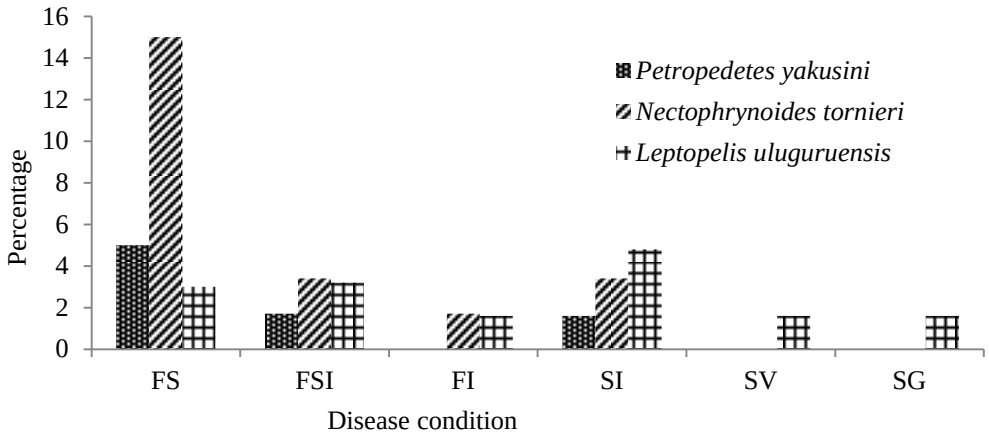


Figure 2: Prevalence of mixed amphibian infections at and around Kihansi gorge. FS, FSI, FI, SI, SV, and SG denote mixed conditions of F, fungal infections; S, strongyloidiasis; I, inflammatory reactions; V, viral infections; G, granulomatous lesions.

Among the diseases observed in amphibians at and around Kihansi gorge was chytridiomycosis. It is clear from the section that Bd proliferates in epidermal cells and causes thickening of the epidermis (hyperkeratosis), parakeratosis and sloughing of the outer skin layer (Figure 3).

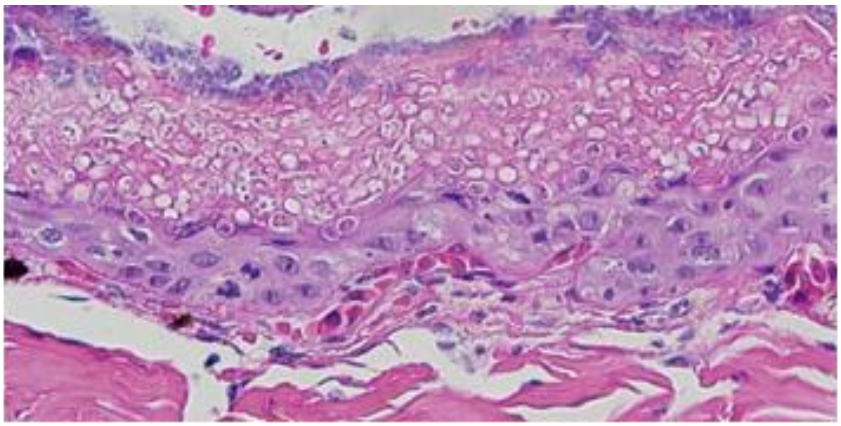


Figure 3: Section of skin from *L. uluguruensis* showing chytrid fungus infection. Note the hyperkeratosis, chytrid fungus appearing as clear circular spaces in the epidermis, homogenous immature stage, zoosporangium with discharging papillae containing zoospores, and empty zoosporangium formed after zoospores have discharged in the sloughing epithelium.

Nematodal infections, particularly strongyloides were also observed in the intestines of several amphibians. Some of them caused intestinal disturbances (Figure 4).

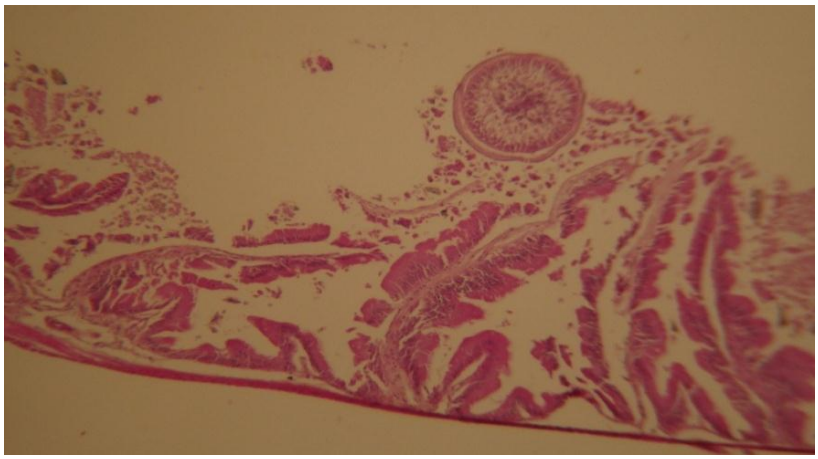
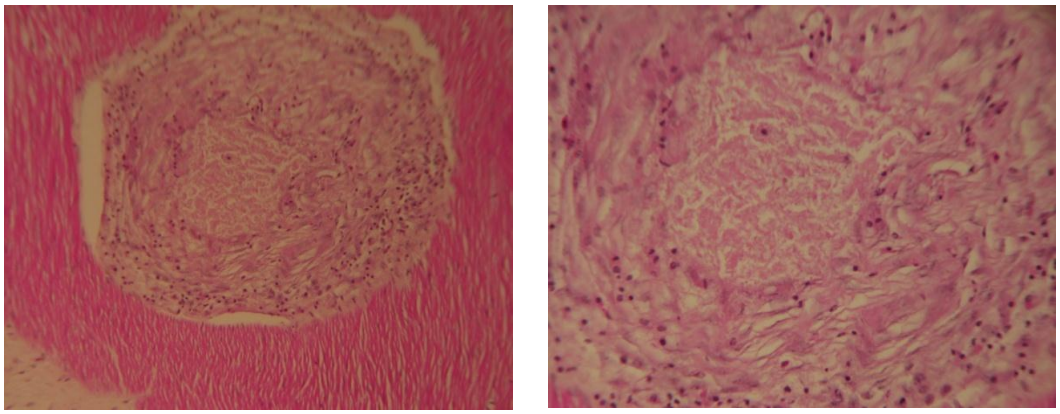


Figure 4: Cross section of a nematode parasite and associated disturbances to the intestine.

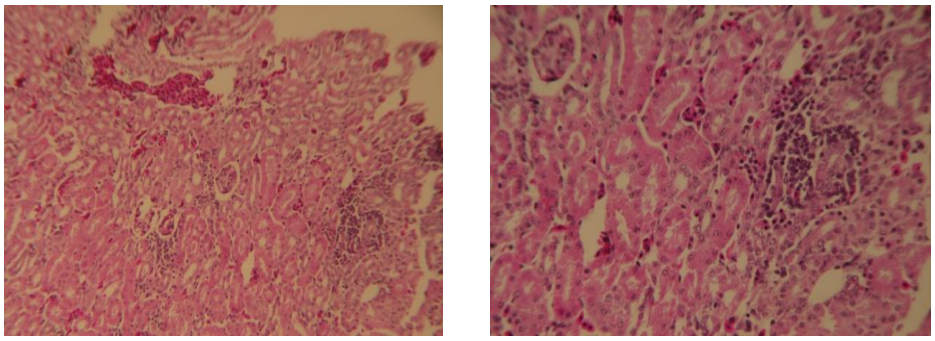
There were frogs observed to have well circumscribed lesions in the muscles (Figure 5A). The lesions had typical features of a hypersensitivity reaction granuloma with central area of necrosis, mononuclear cellular infiltration with epitheloid macrophages and giant cells and fibroplasias when viewed under higher magnification (Figure 5B).



(A) (B)

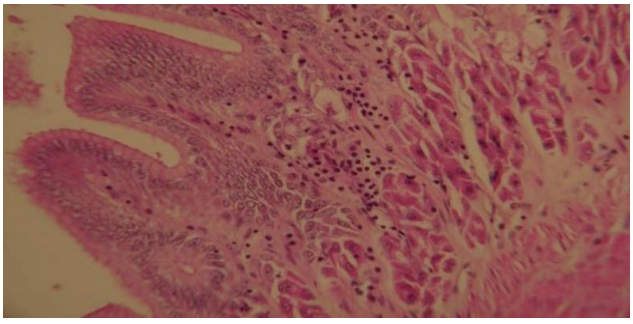
Figure 5: Granulomatous lesion in cardiac muscle at (A) low magnification and (B) high magnification. Note the typical features of the lesion: central area of necrosis, mononuclear cellular infiltration with typical epitheloid macrophages and giant cells and ongoing fibroplasias.

There were non-specific infiltration of inflammatory cells to various organs including the kidney, stomach and liver. The cells were mainly lymphocytes and some macrophages (Figure 6).



A B

Figure 6: Section of a kidney of toad under low magnification (A) and high magnification (B). Interstitial nephritis with monocytic infiltration in the interstitium (macrophages and lymphocytes), necrosis of tubular epithelium, haemorrhages, presence of eosinophilic homogeneous materials (proteins) in the tubular lumen.



C

Figure 6C: Section of stomach of *L. uluguruensis*. Lymphoplasmacytic infiltration into the epithelial mucosa. Note also the high number of gastric glands, especially the chief (peptic) cells.

Discussion

Various disease conditions occur in amphibians at and around Kihansi gorge. According to the current study, amphibian diseases prevalent at Kihansi include chytridiomycosis, helminthosis and non-specific conditions most probably attributed to bacterial and viral infections.

Toads can be infected by chytrid fungus (Lettoof et al. 2013). The presence of chytrid fungus around Kihansi has been established (Makange et al. 2014). Although chytrid is known to wipe up some amphibian populations, its infections do not always lead to fatal diseases. It appears that some factors like species differences, environmental stress and the immune status of the host determine the outcomes of the infections (Rollins-Smith et al. 2002, Ramsey et al. 2010). Chytrid kills frogs by causing massive skin disruptions (Rosenblum et al. 2012). In this study, chytrid infections were observed in healthy sacrificed *N. tornieri*, *P. yakusini* and *L. uluguruensis* at prevalence of 38.3%, 8.3% and 11.3%, respectively (Figure 1). This indicates that some amphibians at and around Kihansi gorge harbour the fatal chytrid fungus without developing the disease. This is in harmony with earlier findings by Ramsey et al. (2010) who reported infection of South African clawed frog, *Xenopus laevis* with chytrid fungus without fatal illness.

Parasites are ubiquitous members of biotic communities. A study by Men et al. (2016) on investigating the infective status of helminth parasites in 90 frogs (*Pelophylax nigromaculatus*), found that 86.67% of the frogs were infected with 1 to 367 internal parasites. The parasites included trematodes, nematodes, cestodes, and acanthocephalas. Majority (60.04%) of parasites were parasitic in the intestines, followed by urinary bladder (24.8%) and lungs (7.38%). Based on morphological features, these researchers found 13 different helminth species parasitizing the frogs. Toads can also contain gastric-encysting nematodes (Lettoof et al. 2013). Other researchers observed a parasite spectrum comprising monogeneans, trematodes (both adults and metacercaria stages), cestodes (both adult and larval stages), nematodes and acanthocephalan taxa (Imkongwapang et al. 2014). Consistently, all studied amphibian species in this very study (*N. tornieri*, *P. yakusini* and *L. uluguruensis*) were parasitized by nematodes at 31.7%, 23.3% and 29%, respectively.

The findings in this study that nematodes parasitize the intestines and cause morphological and possible functional disturbances are in harmony with earlier observations (Lettoof et al. 2013, Men et al. 2016). These disturbances may or may not be significant to the health of the amphibians. It depends on the available nutrients. A recent study exploring the effects of diet on frog resistance and tolerance against skin-penetrating, gut nematode *Aplectana* spp. infection has shown that a high resource diet enhances frog resistance to worm penetration and tolerance as worms travel to the gut. In contrast, a low resource diet increases resistance to establishment of the infections (Knutie et al. 2017). Furthermore, the study showed that following infection and parasitic access of food resources in the gut, a high resource diet enhanced host tolerance of worms. In high resource diets, frogs infected with nematodes consume more food than non-parasitized frogs. Under food restriction, the mass of parasitized frogs decreases, while that of non-parasitized does not change (Knutie et al. 2017). Another study (De Donato et al. 2017) found a strong positive correlation between nematode prevalence and parasite numbers in frogs with trace elements like Mn, Co, Ni, As, Se and Cd. These observations strongly concluded that the outcome of parasitic infection is guided largely by the nutritional status of the animal. The free living amphibians at and around Kihansi have a wide range of resources that partly could explain the lack of clinical nematodiasis.

Granulomatous lesions result from body immune reactions against organisms that are hard to be killed by the body such as *Mycobacteria* or *Brucella*. It is a hypersensitivity reaction primed by cellular immunity and characterised by mononuclear cell infiltration (Malago 2015). In amphibians, granulomatous lesions have been reported in some frogs (Sanchez-Morgado et al. 2009, Sailasuta et al. 2011). It is a chronic granulomatous non-lethal disease but with low mortality rates. It is caused by various organisms including *Mycobacterium* and *Hepatozoon* species (Cosma et al. 2006, Sanchez-Morgado et al. 2009, Sailasuta et al. 2011). To the best available knowledge, the observed granulomatous lesion in this study observed in *L. uluguruensis* is a first report on occurrence of granulomatous lesions in amphibians at and around Kihansi gorge.

Conclusion and recommendations

This study has highlighted some of the conditions affecting amphibians inhabiting Kihansi gorge and the surroundings. The knowledge is pertinent to the re-introduction of the KST to its natural habitats at Kihansi wetlands. Although most of the encountered conditions are not fatal to the native amphibians, they may be mortal to the KST that has spent more than a decade in captivity with limited resource diversity. As indicated above, disease development and outcomes depend partly on nutritional and environmental factors as well as host immune

status. It is therefore important to consider these factors during re-introduction of KSTs that are moved from a biosecured captive facility to wide taxa of parasites in the wild. Future research focusing on parasite pathogenicity under limited resources portraying life in captivity are encouraged so as to generate information pertinent to mitigating any fatality caused by endemic conditions at and around Kihansi gorge following re-introduction of KSTs.

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