Mycobacterial arthritis and synovitis in Painted reed frogs (*Hyperolius marmoratus*)

Michelle Barrows¹, Katja Koeppel², Anita Michel², Emily Mitchell³

¹Bristol Zoological Society, Clifton, Bristol BS8 3HA.
²Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.
³National Zoological Gardens of South Africa, Pretoria, South Africa

Correspondence: Michelle Barrows, Bristol Zoo Gardens, Clifton, Bristol BS8 3HA.
+44 (0)117 9747339  mbarrows@bristolzoo.org.uk

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**Summary:**

Several species of atypical mycobacteria have been isolated from wild and captive amphibians. In captive anurans cutaneous and visceral mycobacteriosis are common and can result in significant mortality, particularly when animals are immunocompromised. Mycobacterial arthritis and synovitis have rarely been reported in amphibians. We describe 20 cases in painted reed frogs, *Hyperolius marmoratus* which presented with cachexia, limb paresis or paralysis or ‘spindly leg syndrome’. Histopathology revealed multifocal histiocytic to granulomatous synovitis affecting appendicular, rib or spinal intervertebral joints. Periarticular granulomata, granulomatous cellulitis and skeletal muscle atrophy, necrosis and degeneration were also present. In one case granulomatous spinal osteomyelitis was recorded. Ziehl-Neelson stains showed large numbers of acid-fast bacteria in macrophages and histiocytes. The mycobacterial isolates obtained from culture were identified as members of the *Mycobacterium chelonae* complex (either *M. chelonae* or *M. abscessus*). This was confirmed by 5’16sRNA sequencing. In 17 cases mycobacterial lesions were present only in the joints and skeleton highlighting the importance of not ruling out mycobacterial infection on the basis of lack of cutaneous or visceral lesions.

Although mortality rates associated with mycobacteriosis are generally low in captive anurans, it has been reported as the leading cause of death in some research frog colonies (Fremont-Rahl *et al*., 2011; Chai, 2012). Mature amphibians are affected more often than tadpoles and mycobacterial infection is often subclinical, with disease developing mainly in immunocompromised animals (Clark and Shepard, 1963; Ramakrishnan *et al*., 1997; Ferreira *et al*., 2006; Chai, 2012; Martinho and Heatley, 2012). Anuran families known to be susceptible to mycobacteriosis include Bufonidae, Hylidae, Leptodactylidae, Pipidae, Pseudidae, Dendrobatidae and Ranidae with most infections reported in African clawed frogs (*Xenopus* species) (Green *et al*., 2000; Trott *et al*., 2004; Godfrey *et al*., 2007; Suykerbuyk *et al*., 2007; Reavill and Schmidt, 2012).

Mycobacteria are ubiquitous in soil and aquatic environments (Chai, 2012, Martinho and Heatley, 2012) and have been isolated from both wild anurans and their environments (Mok and Carvahlo, 1984; Willson *et al*., 2013; Garchitorena *et al*., 2014). The mycobacterial
species affecting amphibians are water-borne and can infect frogs through skin lesions or wounds. Ingestion is also a potential route of infection in both tadpoles and adults (Nonidez and Kahn, 1937). Vectors such as protozoa may transmit mycobacteria to amphibians (Gauthier and Rhodes, 2009). As in other species, infection results in chronic granulomatous inflammation. Granulomata may be irregular and poorly demarcated, or large and encapsulated. Early granulomata are composed of mostly epithelioid macrophages and progress to form encapsulated foci consisting of variable numbers of macrophages, lymphocytes, epithelioid cells, fibroblasts and melanocytes surrounding a necrotic centre (Asfari 1988; Bouley et al., 2001; Trott et al., 2004). Early granulomata have been confused for neoplastic lesions such as lymphosarcoma (Asfari 1988; Green, 2001), however confirmation of the diagnosis is simple as they usually contain large numbers of acid-fast bacilli. Caseation is a variable and unreliable feature and mineralisation and cavitation are not reported (Green, 2001). Cutaneous and visceral mycobacteriosis are the most common forms reported in anurans. In the visceral form granulomata develop in the liver, spleen, kidneys and gastrointestinal tract. In the cutaneous form skin lesions include hyperaemia, petechiae and ulcers, as well as miliary grey or whitish nodules (Shiveley et al., 1981; Taylor et al., 2001; Trott et al., 2004; Ferreira et al., 2006; Hill et al., 2010). Clinical signs vary, with some individuals asymptomatic and others presenting with sudden death. Weight loss, anorexia and emaciation may occur but are not consistent clinical signs. Other reported clinical signs include coelomic swelling, bloating and abnormal buoyancy, subcutaneous oedema, ulcerative or nodular dermatitis, ataxia and mucopurulent nasal and oral secretions. A pathological tibial fracture attributed to mycobacterial infection has been described in a marine toad (Bufo marinus) (Fitzgerald et al., 2004). Mycobacterial arthritis however has rarely been reported in amphibians. There is an anecdotal report of mycobacteriosis causing spinal lesions in Wyoming toads (Bufo baxteri) and mountain yellow-legged frogs (Rana
muscosa) (Pessier, 2014) and a description of mycobacterial infection affecting the cervical vertebrae and a tibiotarsal joint in an American toad (Bufo americanus) (Done et al., 1993). In this paper we report mycobacterial arthritis and synovitis affecting both the appendicular and axial skeleton of painted reed frogs, Hyperolius marmoratus, a small insectivorous anuran species from sub-Saharan Africa.

Thirty six adult painted reed frogs were collected from the wild in Mpumalanga province, South Africa in order to initiate a captive breeding programme. The purpose of this project was to develop husbandry protocols for the painted reed frog as an analogue species for future maintenance of a population of the critically endangered Pickersgill’s reed frog (Hyperolius pickersgilli). Adults were housed in 5 litre transparent plastic jars with a paper towel substrate and 8ml of reverse osmosis (RO) water or in plastic tanks (320 x 220 x 250mm) containing smooth aquarium gravel and moss. The tanks were housed on racks with a recirculating RO water system connected to a shared biological filtration system as described by Van der Spuy and Krebs (2008). Diet consisted of appropriately sized crickets, fruit flies, silkworms and termites all dusted with calcium powder. Tadpoles were housed in glass tanks (912 x 325 x 325mm) with an air pump and canister filter with the inlet spray bar placed on the wall so that water flowed down the glass into the tank. Tadpoles were fed on spirulina tablets and cos lettuce. Painted reed frogs were successfully bred to the F3 generation in these systems.

The study animals consisted of 20 frogs which were found dead or humanely euthanased due to cachexia, limb paresis, paralysis, deformity or ‘spindly leg syndrome’, or because they were in contact with other affected frogs. They included some wild caught adults and some captive bred frogs from the F1 and F3 generations. The first cases occurred in wild caught frogs 14 months after capture. Frogs were preserved in 10% buffered formalin and multiple
serial sections made through the entire carcass for histopathology. In addition joint samples from nine of the frogs were submitted as fresh unfixed tissue for mycobacterial culture. Tissues from each of these nine were homogenised and decontaminated in equal volumes of 1% hydrochloric acid and 2% sodium hydrochloride followed by neutralisation in double distilled sterile water, centrifugation and inoculation on Loewenstein-Jensen medium containing glycerol. Medium slopes were incubated at 25°C, 37°C and 42°C and monitored for colony growth twice per week. Mycobacterium colonies were subjected to Ziehl-Neelsen staining and acid-fast isolates were subcultured and subjected to biochemical characterisation including 3-day arylsulfatase acitivty test and nitratase reductase activity. 5'-16S ribosomal ribonucleic acid (rRNA) PCR-sequencing was carried out on two isolates (Rogall et al., 1990).

The most significant findings on histopathology were in the appendicular and spinal intervertebral joints. All the frogs had multifocal histiocytic to granulomatous synovitis affecting at least one and often several distal appendicular joints, rib joints or spinal intervertebral joints (Figures 1-3 with a normal joint for comparison). The synovial membranes were greatly expanded by aggregates of large foamy macrophages mixed with a few heterophils. In one case there was osteolysis of distal bone epiphyses. In addition, periarticular granulomata consisting of loose aggregates of large, reactive macrophages mixed with fewer lymphocytes and occasional necrotic heterophils were present surrounding the joint cartilages in affected joints, sometimes obliterating the joint space and extending into the subcutis and skeletal muscle. Small granulomata in the spinal canal at intervertebral joints, sometimes compressed the spinal cord. In one case granulomatous spinal osteomyelitis was present. Rostral spinal column bone and cartilage was multifocally necrotic and replaced by loose aggregates of macrophages (Figure 4). Ziehl-Neelson (ZN) stains showed small to large numbers of acid fast bacteria in macrophages in synovial
Figure 1: Normal appendicular limb joint in a painted reed frog. Note clear articular space between cartilage caps (*). Haematoxylin and eosin, Bar = 0.3mm

Figure 2: Histiocytic arthritis in stifle joint of a painted reed frog. Loose aggregates of large foamy macrophages (*) containing myriad acid fast organisms (Ziehl Neelsen, insert) fill the joint space between cartilage caps which show degeneration and necrosis (arrows). Haematoxylin and eosin, Bar = 0.3mm
**Figure 3:** Histiocytic arthritis in stifle joint of a painted reed frog. Loose aggregates of large foamy macrophages (*) and one multinucleate giant cell (arrow) fill the joint space. Haematoxylin and eosin, Bar = 10 µm

**Figure 4:** Histiocytic osteomyelitis, cellulitis and myositis (arrows) of the spinal column of a painted reed frog. Large numbers of macrophages, mixed with fewer lymphocytes and plasma cells infiltrate and expand the spinal cord (*), dura mater (arrow) and surrounding bone and skeletal muscle (arrowheads). Haematoxylin and eosin, Bar = 0.8mm
membranes, cellulitis, skeletal muscle and spinal canal (Fig 2, insert). One frog had moderate
diffuse heterophilic, histiocytic and lymphocytic interstitial nephritis with acid-fast organisms
in renal histiocytes, two had multifocal hepatic granulomata and in another case, small to
medium numbers of acid-fast bacterial rods consistent with mycobacteria occurred in
macrophages in the meninges. Associated histopathology showed hepatic atrophy and
lipidosis, skeletal muscle atrophy and pancreatic zymogen granule depletion. In addition, all
frogs displayed prominent hepatic melanomacrophage hyperplasia. Other additional
findings, each seen in only one or two frogs included acute renal tubular necrosis, renal
haemosiderosis, diffuse membranous glomerulonephritis, microfilarial hepatitis, hepatic
karyomegaly, meningeal melanosis, cerebral protozoa, mild bone marrow myelopoiesis,
skeletal muscle nematodes and intestinal mucosal associated lymphoid hyperplasia. Apart
from congestion all other tissue samples were unremarkable histologically. Mycobacterium
isolates were identified as *Mycobacterium chelonae-abscessus* complex organisms based on
their non-pigmented colony appearance, rapid growth rate (<7 days), ability to grow at 25°C,
arylsulfatase activity and negative nitrate reduction. 5'-16S rRNA PCR-sequencing was
successful on one of two isolates submitted and confirmed it to be a member of the
*Mycobacterium chelonae* complex (either *M. chelonae* or *M. abscessus*).

Mycobacterial arthritis and synovitis have rarely been reported in amphibians. Cutaneous
and visceral lesions were conspicuous by their absence in this case series. Death in those
frogs that died spontaneously was likely caused by the metabolic effects of inadequate food
intake, due to locomotor dysfunction as a result of involvement of the axial and/or
appendicular skeleton. Hepatic lipidosis and pancreatic zymogen granule depletion were
likely attributable to anorexia. Some affected juvenile frogs presented as cases of ‘spindly
leg syndrome’, a relatively common presentation in anurans characterized by limb
deformities, muscle atrophy and sometimes absence of limb bones (Hakvoort et al., 1995; Claunch and Augustine, 2015). The aetiology is unknown but nutrition, genetics, environment and trauma are potential contributing factors (Marlett et al., 1988; Wright, 2001). This case series highlights the need to consider mycobacterial infection as a differential diagnosis for muscle atrophy of the limbs in anurans.

Mycobacteriosis has been reported in several wild anuran species with prevalences varying from 2% in common lesser toads (Bufo granulosus) and 19.6% in four-eyed frogs (Pleurodema cinerea and P.marmoratus) to 100% in South American bullfrogs (Leptodactylus pentadactylus) from an urban area of Brazil (Darzins, 1952; Machicao and LaPlaca, 1954; Mok and Carvahlo, 1984). It is therefore possible that the painted reed frogs were infected with mycobacteria when they were caught, however given that the first mortalities in wild caught adults occurred 14 months after capture and that cases also occurred in F1 and F3 generations, it is more likely that the infection was contracted in captivity. The most likely source was the water; M. liflandii has been isolated from water in an epizootic of mycobacteriosis in Xenopus tropicalis and an atypical mycobacterium was found in tap water used to house Xenopus laevis (Kirsch et al., 2008; Chai, 2012). M. chelonae and M. fortuitum have also been isolated from water samples taken from amphibian breeding grounds in Brazil (Mok et al., 1987). Ingestion is one potential route of entry. Entry through skin lesions is another and although no skin lesions were present in any of the frogs at post mortem examination, one individual had nematode larvae in the muscle indicating that larval penetration of the skin might have occurred suggesting a potential route of invasion for mycobacteria. Once mycobacteria had entered the body, haematogenous spread to the joints may have occurred, as suspected in a sea turtle with mycobacterial arthritis (Greer et al., 2003). Lymphatic spread is also possible. In one study, after
experimental infection of tadpoles, mycobacteria were assumed to have spread within
macrophages migrating via the lymphatics, since none were detected in serial sections of the
heart and major blood vessels (Nonidez and Kahn, 1937).

Mycobacterial arthritis and/or osteomyelitis has been reported in humans, horses and reptiles
(Kelly et al., 1972; Shu et al., 2009; Gardam and Lim, 2005; Greer et al., 2003; Hewes et al.,
2005; Kramer, 2006; Winthrop and Iseman, 2013). In all species, immunosuppression is a
significant predisposing factor. Immunosuppressive drugs used to treat rheumatoid arthritis
and infection with human immunodeficiency virus have been implicated in human cases and
a horse with septic arthritis caused by M. avium complex had previously received multiple
cortisol injections into the affected joint (Shu et al., 2009; Gardam and Lim, 2005; Hewes et
al., 2005; Winthrop and Iseman, 2013). A bearded dragon (Pogona vitticeps) with
osteomyelitis due to atypical mycobacteriosis was assumed to be immunocompromised due
to inadequate husbandry (Kramer, 2006). Immunosuppression is well recognized as a
predisposing factor for mycobacteriosis in amphibians and it is likely to have played a role in
this case series (Clark and Shepard, 1963; Ramakrishnan et al., 1997; Ferreira et al., 2006;
Chai, 2012; Martinho and Heatley, 2012). Immunosuppression was also thought to be a
factor in 15 cases of spinal arthropathy due to the bacterium Ochrobactrum anthropi in cane
toads (B. marinus) (Shilton et al., 2008).

The reason for the unique site predilection of the mycobacterial infection identified in these
painted reed frogs is unclear. There may be a genetic predisposition or the particular
mycobacterial species involved may have a predisposition for joint tissues. It is important
that mycobacterial infection is not ruled out on the basis of lack of cutaneous or visceral
lesions in anurans and that husbandry protocols for all captive amphibians are designed to minimise stress and consequent immunosuppression.

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References


