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NOTE

Fatal phaeohyphomycosis due to *Exophiala* sp. infection in a free-living common toad *Bufo bufo*

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ABSTRACT: A wild adult female common toad *Bufo bufo* found dead in Scotland in September 2016 was observed to have hepatomegaly, a large soft tissue mass in the coelomic cavity (2.7 g, 3.5 × 2.3 × 1.8 cm) and numerous dark-red papules (1–2 mm diameter) in the skin and subjacent tissue over the back and dorsal aspects of the limbs. Histopathological examination identified marked hepatitis and coelomitis associated with pigmented fungal hyphae, which are results consistent with a diagnosis of phaeohyphomycosis. Sequencing of the internal transcribed spacer region and the D1-D2 region of the large subunit of the ribosomal RNA gene from affected liver tissue identified the presence of *Exophiala* (Chaetothyriales) sp., a black yeast previously identified as a cause of amphibian phaeohyphomycosis. To our knowledge, this is the first published report of *Exophiala* sp. in a wild or captive amphibian in Europe and the first description of phaeohyphomycosis affecting a free-living amphibian in Great Britain. *Exophiala* spp. are saprobes and opportunistic pathogens. It has been postulated that phaeohyphomycosis is a disease of immunocompromised amphibians; however, we found no evidence of significant concurrent infection or generalised debility in this common toad. Phaeohyphomycosis appears to be a sporadic cause of mortality in amphibians, and this report adds to the growing list of pathogens known to affect wild amphibians in Europe.

KEY WORDS: Mycosis · Black yeast · Amphibian population declines · Wildlife disease surveillance

1. INTRODUCTION

In recent decades, there has been increased awareness of the importance of infectious disease as a cause of wild amphibian mortality, population declines and even species extinctions. *Batrachochytrium dendrobatidis* (*Bd*), *B. salamandrivorans* (*Bsal*) and *Ranavirus* are particularly well recognised pathogens in this regard (Skerratt et al. 2007, Blooi et al. 2013, Price et al. 2014). Surveillance of wild amphibian populations is an important means by which to monitor and better understand the diseases affecting them.

The common toad *Bufo bufo* is widespread in Europe, including Great Britain (GB); however, population declines have been observed in GB since the 1970s (Petrovan & Schmidt 2016). Although infectious disease has been hypothesised as a possible

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cause of common toad declines in southeast England (Cunningham et al. 2007), the reasons for common toad population declines in GB are currently unknown. Combined common toad population monitoring and pathogen surveillance are required to identify if a specific disease, or diseases, is/are implicated in the decline of this species or affecting animals on an individual level. Garden Wildlife Health (www.gardenwildlifehealth.org) is a collaborative citizen science project, which aims to conduct such a national disease surveillance scheme for amphibians and other wild fauna in GB.

Phaeohyphomycosis, a mycotic infection caused by saprobic dematiaceous hyphomycetes containing melanin in their cell walls, has rarely been reported in endothermic vertebrates but is increasingly recognised in ectothermic vertebrates and some invertebrates (Seyedmousavi et al. 2013). Approximately 60 genera and over 100 species of these opportunistic pathogenic fungi are currently known, with most cases of disease in animals and people being caused by *Bipolaris*, *Chaetomium*, *Curvularia, Exophiala*, *Exserohilum*, *Phoma* or *Wangiella*. The disease manifestation of phaeohyphomycosis ranges from colonisation of the skin and subcutaneous invasion to fatal disseminated infection (Seyedmousavi et al. 2013). Most reported cases of disseminated phaeohyphomycosis in amphibians have been caused by members of the genera *Fonsecaea*, *Phialophora* and *Rhinocladiella*, with pigmented hyphae invading multiple organs and causing tissue necrosis and inflammation (Cicmanec et al. 1973, Juopperi et al. 2002, Densmore & Green 2007).

The majority of confirmed cases of phaeohyphomycosis caused by *Exophiala* species involve captive fish (De Hoog et al. 2011, Seyedmousavi et al. 2013). Only 1 case of confirmed *Exophiala* sp. infection has been reported in wild amphibians, involving wild green toads *Bufo viridis* in Israel (De Hoog et al. 2011, A. A. Cunningham pers. obs.). Here we describe a fatal case of phaeohyphomycosis in a wild common toad in GB caused by infection with *Exophiala* sp.

2. MATERIALS AND METHODS

Scanning disease surveillance of wild amphibians has been conducted in GB through the Garden Wildlife Health project that solicits reports of sick or dead animals throughout the calendar year. A single adult common toad found dead in a garden pond, shared with common frogs *Rana temporaria*, smooth newts *Lissotriton vulgaris* and three-spined stickleback *Gasterosteus aculeatus*, in Ayrshire, Scotland, in September 2016 was submitted by a member of the public for post mortem examination. External and internal examination was conducted following a systematic protocol (Franklinos et al. 2018), with appraisal of body condition based on subjective assessment of hind limb muscle condition and of fat bodies within the coelomic cavity. Parasitological investigation involved microscopic examination of a physiological saline mount of small intestinal contents for protozoan and metazoan parasites. A dry cotton ventral skin swab was collected to screen for chytrid fungi (*Bd* and *Bsal*) using a real-time duplex polymerase chain reaction (rtPCR) (Blooi et al. 2013). A liver sample was taken for *Ranavirus* testing using a quantitative rtPCR (Leung et al. 2017). A suite of tissue samples and macroscopic lesions were collected and stored at −20°C and/or −80°C with parallel samples being placed in 10% neutral-buffered formalin. Once fixed, the latter were processed for routine histopathological examination, embedded in paraffin wax and sectioned before being stained with haematoxylin and eosin (H&E). In addition, where indicated on the basis of H&E examination, some abnormal tissues were stained using periodic acid-Schiff (PAS) stain to better visualise fungal elements.

Impression preparations of the liver were taken and stained with either Gram or Ziehl-Neelsen stains. Bacteriological culture was conducted on a fresh liver sample using Columbia blood +5% horse blood agar (Thermofisher Scientific), aerobically at 25°C and anaerobically at 25 and 37°C. Mycological culture was conducted on a fresh liver sample using both Sabouraud dextrose agar with chloramphenicol (100 mg l^{-1}) and Dermasel agar (Thermofisher Scientific) incubated aerobically at 25°C and observed on Days 1, 2, 5, 7, 14 and 28. Cultured fungi were analysed for their colonial and Gram staining morphology. Phase contrast direct microscopy was used for phenotypic identification of filamentous fungi.

Pan-fungal PCRs targeting the internal transcribed spacer (ITS) region 1 and the D1-D2 region of the large subunit (LSU) of the ribosomal RNA gene (Borman et al. 2006) with subsequent sequencing of amplicons were performed on DNA extracted from frozen samples of multiple abnormal tissues using the QIAamp® DNA mini kit (Qiagen). The sequence data were screened against GenBank entries using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned with an internal *Exophiala* data set using ClustalW (Borman et al. 2017).

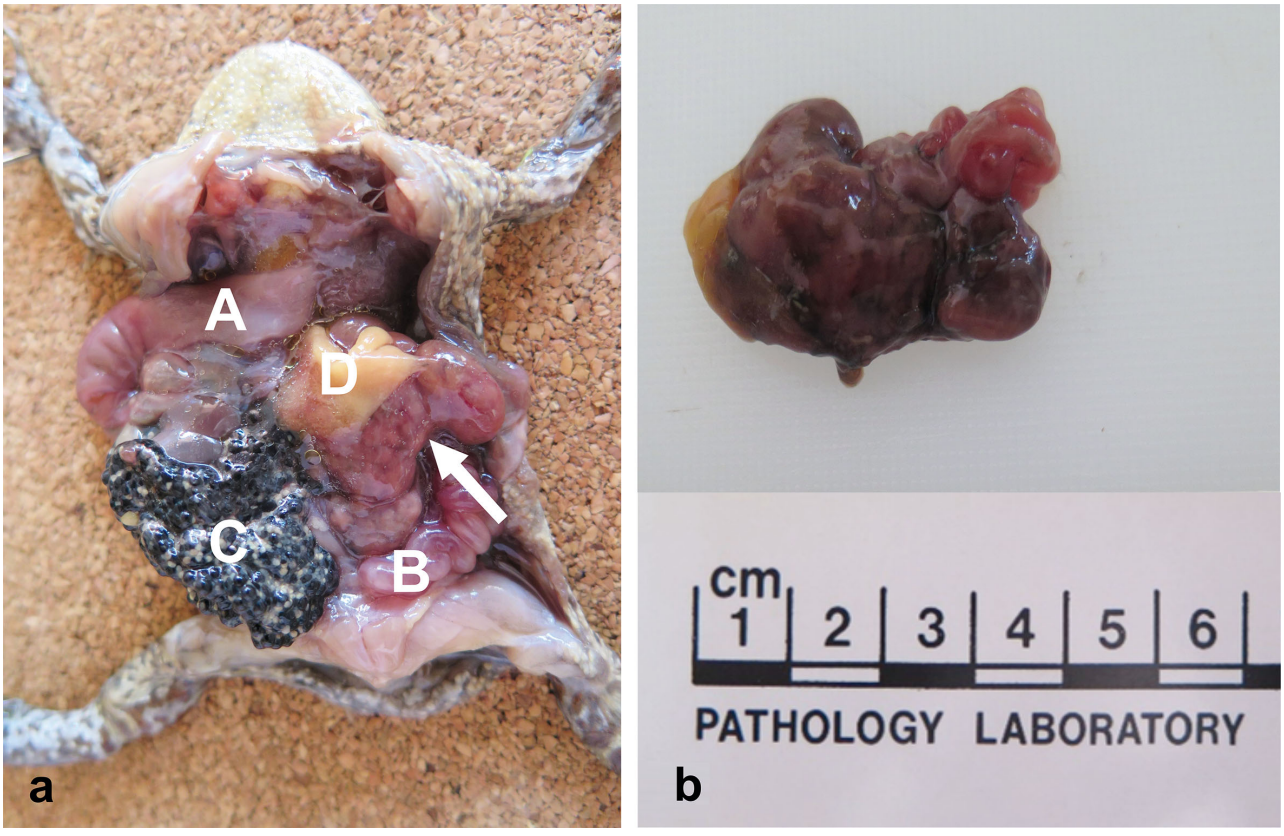

Fig. 1. (a) Common toad *Bufo bufo* with opened coelomic cavity (liver and heart removed) showing a large undifferentiated soft tissue mass *in situ* (arrow), adherent to the left ovary and kidney (not visible); A: stomach, B: left oviduct, C: spawn, D: fat body. (b) Excised coelomic mass weighing 2.7 g and measuring 3.5 × 2.3 × 1.8 cm

3. RESULTS

The common toad was an adult female weighing 46.3 g and in normal body condition. Macroscopic abnormalities included marked hepatomegaly with a liver weight of 4.9 g (mean weight of apparently normal livers of submitted adult toads of similar body weight = 0.8 g, SD = 0.36), constituting 10.6% of the total body weight (mean = 1.8%, SD = 0.9). The liver had a heterogeneous dark red-brown discolouration and a slightly firm texture with a single 1.5 mm beige-coloured spherical nodule in the left lobe. In addition, there was a 3.5 × 2.3 × 1.8 cm, 2.7 g, soft tissue mass of undetermined origin in the coelomic cavity, which was adherent to the left ovary and kidney (Fig. 1). Further, numerous dark red papular lesions of approximately 1–2 mm in diameter were observed in the skin and subjacent tissue over the back extending to the dorsal aspects of the limbs (Fig. 2).

Skin swabs and liver tissue samples were rtPCR-negative for chytrid fungi (*Bd* and *Bsal*) and *Ranavirus*, respectively. Parasitological examination detected a moderate burden of acanthocephalan parasites in the small intestinal contents. A moderate number of Gram-positive, rod-shaped bacteria (suspected *Bacillus* and *Clostridium* spp.) were observed on the Gram-stained impression preparation of the liver. The Ziehl-Neelsen-stained preparation was negative for acid-fast organisms. Bacteriological culture isolated a *Clostridium* sp. at 37°C under anaerobic conditions, with no bacterial growth observed at 25°C.

Mycological culture of the liver yielded a predominant fungal isolate, with 32–35 mm diameter colonies observed 7 d post incubation. These colonies had a rosy vinaceous colour and an off-white reverse. Conidiophores on hyphae with phialides and conidia were observed with 100× and 400× magnification using phase contrast microscopy. Based on its morphology, this fungus was identified as *Purpureocillium lilacinum*, an environmental saprophyte.

Histopathological examination of the liver revealed severe, multifocal to coalescing, necrotising hepatitis with intra-lesional brown pigmented fungal hyphae

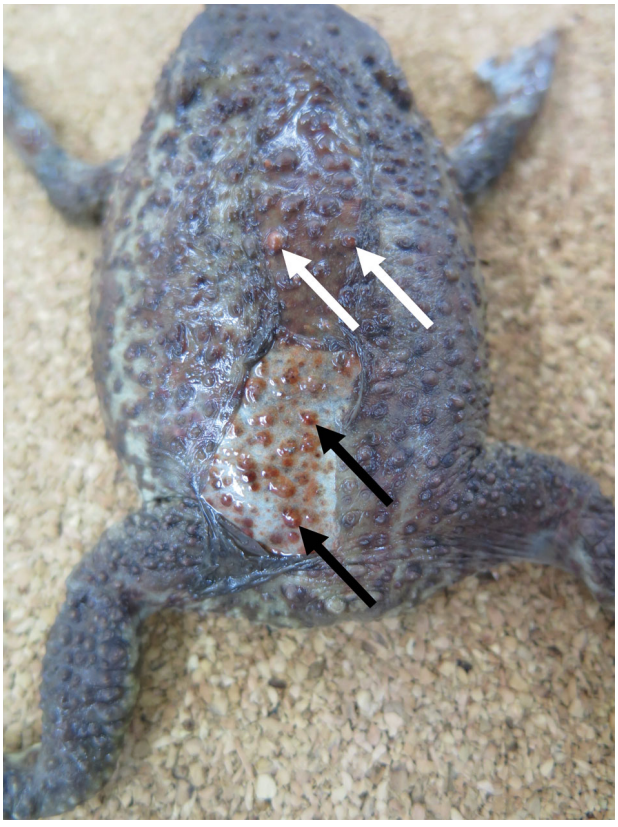

Fig. 2. Common toad *Bufo bufo* with numerous dark red papular lesions in the skin (white arrows) and subjacent tissue (black arrows) of approximately 1–2 mm in diameter over the back extending to the dorsal aspects of the limbs. Skin partly resected during post mortem examination

throughout the affected tissue, confirmed by an additional PAS stain. The hyphae were septate, with parallel walls, a diameter of approximately 2–3 µm and with infrequent, predominantly 90°, branching (Fig. 3). The coelomic mass comprised inflammatory cells, consisting of numerous granulocytes and mononuclear cells, and pigmented fungal hyphae, also confirmed by PAS staining, similar to those observed in the liver. These observations are consistent with a diagnosis of phaeohyphomycosis.

The majority of other tissues examined histologically were diffusely and markedly autolysed, hindering interpretation of microscopic changes. The papular lesions in the skin and subjacent tissue comprised moderate multifocal epidermal hyperplasia with superficial bacterial and non-pigmented fragmented hyphae on H&E, Gram stains and PAS. Infrequent sections of nematode parasites were detected in the alveolar spaces of the lung, and these were considered to be incidental to the cause of death. No significant abnormalities were detected in the other tissues examined, which comprised adipose tissue, gastrointestinal tract, brain, heart, oviduct and skeletal muscle.

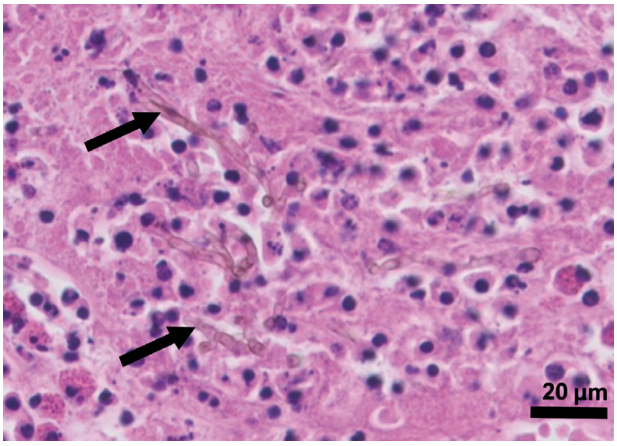

Fig. 3. Photomicrograph showing severe necrotising hepatitis with intralesional pigmented fungal hyphae of approximately 2–3 µm in diameter and infrequent branching (arrows) consistent with phaeohyphomycosis in the liver of a common toad *Bufo bufo*. H&E stain

The ITS (234 bp) and LSU (333 bp) sequence data obtained from liver tissue identified the presence of intra-lesional fungi of the genus *Exophiala*. The ITS sequence was more discriminatory, with a 95% identity to *E. cancerae* (GenBank accession number NR_137766.1). Further, alignment of the sequence with an *Exophiala* database found that the detected *Exophiala* sp. grouped with other *E. cancerae* strains (Borman et al. 2017). The aligned sequences are published in GenBank under accession numbers MK141029 (ITS) and MK141030 (LSU). PCR analysis of the coelomic mass was negative. Analysis of the skin lesion sample obtained weak results of mixed sequence from which individual species could not be identified.

4. DISCUSSION

This report describes a case of phaeohyphomycosis due to *Exophiala* sp. infection in a wild common toad. To our knowledge, this is the first report of a wild amphibian affected by this disease in GB and the first time *Exophiala* sp. has been described in an amphibian, captive or wild, in Europe. Whilst other possible reports of *Exophiala* sp. in amphibians exist, the primary literature sources for these cases are scattered, and sequence analysis to confirm the fungal pathogen identity is often lacking (De Hoog et al. 2011).

Exophiala is an anamorph genus phylogenetically affiliated to the ascomycete order Chaetothyriales,

comprising the black yeasts, first described in the 1960s (Carmichael 1966). It has been noted that waterborne animals are much more frequently infected by chaetothyrialean fungi than are terrestrial animals (De Hoog et al. 2011). The genus *Exophiala* contains numerous potential opportunistic pathogens (De Hoog et al. 2011).

In our study, *Exophiala* sp. infection was detected in association with hepatitis and coelomitis, with this host inflammatory response associated with the fungal hyphae in both the liver and coelomic mass, indicative of an ante mortem fungal infection. The ITS region sequence data confirmed the presence of *Exophiala* sp. in the liver, but we were unable to discriminate between infection with *E. cancerae* and *E. salmonis* or a novel species. Since *E. salmonis* most commonly causes superficial, minimally invasive, infections (Saunte et al. 2012), and because the aligned ITS sequence grouped with other *E. cancerae* strains (Borman et al. 2017), we postulate that *E. cancerae* is most likely to be the cause of the disease in this toad. The negative PCR result for the coelomic mass does not preclude the presence of invasive *Exophiala* sp. infection, as pigmented fungal hyphae were detected on microscopic examination of this structure. The poor quality mixed DNA sequences derived from the skin lesion sample could indicate the presence of multiple non-pigmented fungal species, perhaps including environmental saprophytes or secondary organisms.

Mycological culture of the liver isolated the fungus *Purpureocillium lilacinum*, which is a common environmental opportunist. However, histopathological examination revealed pigmented hyphae consistent with *Exophiala* sp. in the liver and coelomic mass; nevertheless, it is not possible to exclude the concurrent presence of non-pigmented hyphae such as *P. lilacinum*. It was therefore deduced that *Exophiala* sp. is most likely to be the primary pathogen in this common toad, with a secondary infection or post mortem invasion by *P. lilacinum* and possibly other fungi. In ectotherms, *P. lilacinum* infection has been reported only sporadically when it has affected immunocompromised reptiles (Schumacher et al. 2014). Competitive inhibition of the fastidious *Exophiala* sp. by organisms such as *P. lilacinum* on media could partly be responsible for the limited detection of *Exophiala* sp. in culture to date (Kondori et al. 2011). Whilst Gram-positive bacilli were noted on the impression preparation of the liver, and a *Clostridium* sp. was isolated on anaerobic culture at 37°C, histological examination did not detect bacterial colonies or lesions associated with bacteria; given these findings, the bacilli present are considered likely due to post mortem invasion.

The only confirmed report to date of a wild amphibian being infected with *Exophiala* sp. is of *E. cancerae* (strain CBS 119920; ITS sequence, GenBank accession number JF747065) infection detected in a green toad with systemic phaeohyphomycosis when it was examined as part of an investigation into green toad mass mortality in the Jordan Valley, Israel, in the late 1990s (De Hoog et al. 2011, A. A. Cunningham pers. obs.).

In amphibians, exposure to fungal pathogens can occur via contact with a contaminated environment or infected animals, including other amphibians (Juopperi et al. 2002). Whilst there was no evidence of significant concurrent infection or generalised debility in the common toad described in this study, it has been postulated that immunocompromised amphibians are most susceptible to infection with opportunistic fungal pathogens such as *Exophiala* spp., and that traumatic injury to the skin may predispose the animals to infection with these environmental fungi (Otis et al. 1985, Juopperi et al. 2002). Based on available information, *Exophiala* sp. seems most likely to be a sporadic cause of mortality in individual wild amphibians. This study adds to our knowledge of the growing list of infectious diseases that affect amphibians in Europe and illustrates the value of scanning disease surveillance and detailed pathological examination.

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