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Dermocystid infection and associated skin lesions in free-living palmate newts (*Lissotriton helveticus*) from Southern France

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Since the early 1900s, mesomycetozoan parasites have been reported in both European anuran and caudate species. These reports have primarily been descriptive, which has made assessing the impact of these parasites on host populations difficult. Anecdotal reports of dermocystidium-like parasites are becoming widespread across Europe, possibly indicating that these mesomycetozoan parasites are increasing in distribution and/or abundance. This highlights the need for further investigations into the occurrence, pathogenesis and effects on host health of these parasitic infections for free-living amphibian populations, particularly those which are already stressed or threatened by other factors. Here we report the results of pathological, microbiological and molecular investigations used to characterize unidentified skin lesions in palmate newts (*Lissotriton helveticus*) from Larzac, France. We confirm that the lesions are the result of infection with a novel dermocystidium-like parasite, which is related to *Amphybiocystidium ranae*. We also show that the same parasite is distributed across several newt breeding sites. The lesions that result from infection with this parasite range from single or few vesicular or nodular cutaneous lesions to multiple coalescing skin ulcers with extensive hemorrhages. The latter have not been previously described in amphibians due to mesomycetozoan parasitic infection. Dermocystid DNA was detected only in newts that showed lesions, providing comparative evidence of the parasite's pathogenicity. We discuss the potential significance of the presence of this pathogen in the context of the population health of palmate newts.

Keywords: Amphibian Dermocystida, Emerging infectious disease, Mesomycetozoa, Palmate newt, Skin, *Lissotriton helveticus*

1. Introduction

Dermocystidians form one of two branches of the class Mesomycetozoea, a protozoan lineage often cited as the ancestor to all metazoans [1,2]. While the true relationships between the Fungi, Animalia and Mesomycetozoea remain unresolved, it is clear that mesomycetozoans, along with the choanoflagellates, occur somewhere at the boundary between animals and fungi [3] and [4]. Members of the order Dermocystida infect hosts from nearly all the vertebrate orders and several genera utilize amphibian hosts, in some cases exclusively [3,5,6]. Gross signs of infection of amphibian hosts can appear severe (Fig. 1), yet the pathogenesis and impact of dermocystidian parasites in amphibian hosts remain poorly studied. While Pascolini et al. [6] described a dermocystidian affecting an apparently declining amphibian population; they avoided invoking cause and effect in their descriptive study [7]. Raffel et al. [8] reported an increased mortality of red-spotted newts (*Notophthalmus viridescens*) visibly infected with *Amphibiocystidium viridescens*, however they, too, avoided making a case for cause and effect due to the correlative nature of their data. Green et al. [9] provided further correlative evidence that cannot unambiguously attribute the cause of mortality or morbidity to a dermocystidian parasite. Nevertheless, all these studies make strong cases for dermocystidian parasites causing clinical signs of

disease and leading to amphibian mortality, mirroring that seen in fish hosts lethally affected by dermocystidian infections [3,10].

Since the beginning of the twentieth century, dermocystid mesomycetozoan parasites have been reported in a number of European amphibian species (e.g. marbled newts (*Triturus marmoratus*) [11,12]; common frogs (*Rana temporaria*) [13,14] and *Pelophylax esculentus* complex [6,15]). Most reports of infections in Europe are primarily descriptive, lacking robust and comparative sampling efforts, unlike efforts made by researchers outside the European continent (e.g. [8,16,17], but see [6]). This is unfortunate, as amphibian parasites, specifically ranavirus and *Batrachochytrium dendrobatidis*, have been linked to mass mortality events and declines of European amphibians [18,19,20,21,22,23]. Extensive and/or robust sampling for *B. dendrobatidis* has shown that this lethal amphibian parasite has not reached equilibrium in Europe, and continues to extend its range in both space and host [24]. A similar situation is thought to occur for ranavirus infection, at least in the United Kingdom [18], but little research has been conducted on this pathogen in amphibians in Europe [see 25]. Anecdotal reports of dermocystidian-like infections of European amphibians appear to be wide-ranging. Such observations may indicate dermocystidian parasites are extending their ranges in Europe and highlight the need for further investigations into the occurrence, pathogenesis and health significance of mesomycetozoan parasites in free-living amphibian populations. In particular, dermocystid parasites might present a threat to the survival of amphibian populations that are already stressed or threatened by other factors [26,27].

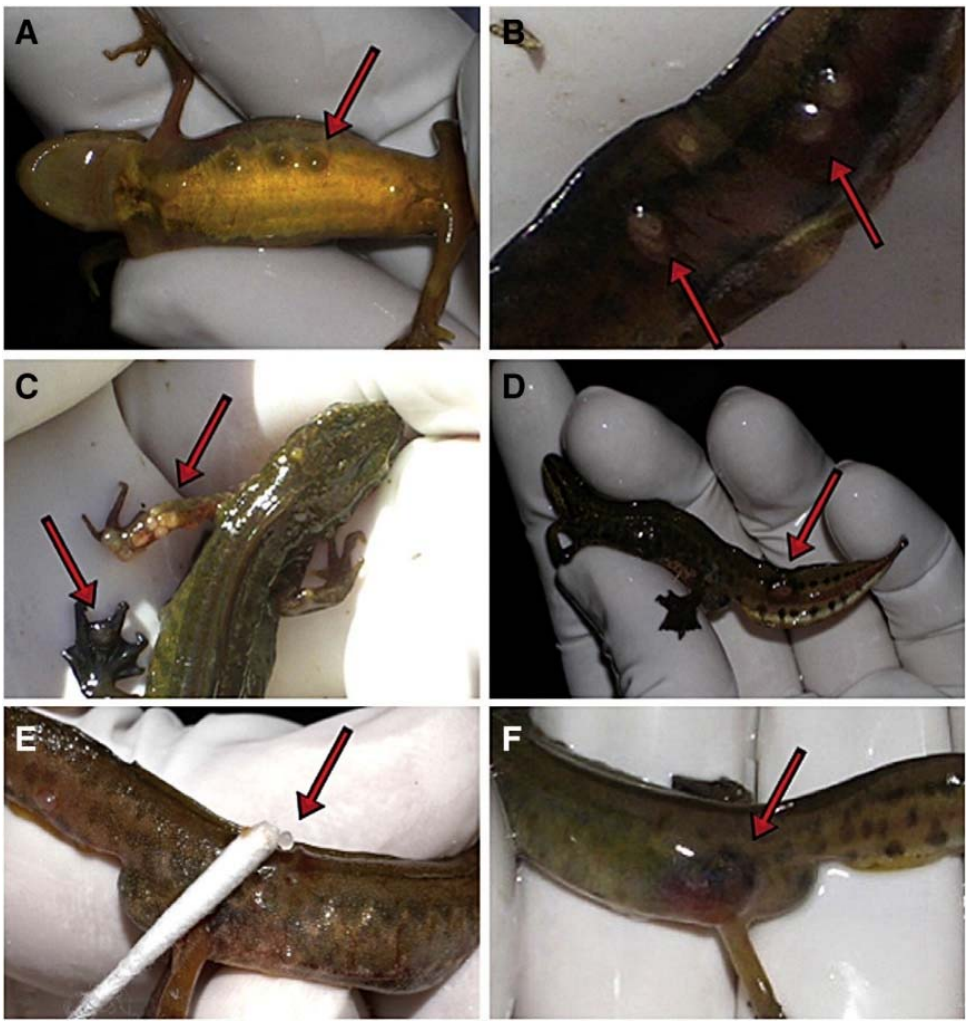

Fig. 1. Gross skin lesions in the palmate newt. A) Vesicles, B) carbuncle-like preulcerative lesion, C) multifocal preulcerative lesions, D) cluster of preulcerative lesions, E) ulcer and F) spherical white cyst detached from a preulcerative lesion.

Recently a high frequency of vesicular skin lesions in palmate newt (*Lissotriton helveticus*) in populations in Southern France was observed (M. Denoël, pers. obs.). Superficially, these lesions appeared similar to those generated by dermocystidian infections of other amphibian hosts. However, the etiology of these lesions and their health significance to palmate newts has not yet been established. Here, we report the results of pathological, microbiological and molecular investigations used to characterize these skin lesions, identify the causal and associated agents, confirm infection with a dermocystidian parasite using a comparative framework, show infection occurs at multiple newt breeding locations and discuss the potential significance to the palmate newt population's health.

2. Materials and methods

2.1. Study site

The study was conducted at the Larzac limestone plateau, located in the south of France (Department of Herault, Languedoc-Roussillon), where there are numerous temporal ponds inhabited by the native palmate newt (*L. helveticus*), and two other newt species [alpine newts (*Mesotriton alpestris*) and marbled newts (*T. marmoratus*)], in addition to several species of anurans [28,29,30]. On the basis of previous observations (M. Denoël, unpubl. data), three ponds were selected as sampling sites: Pond "A" Bagnelades (1 × 1 km UTM coordinates: 0529–4855), Pond "B" Pioch de Labro (0523–4859) and Pond "C" Les Labres (0530–4856).

2.2. Collection of samples

A total of 40 live palmate newts (Pond A = 14, Pond B = 14 and Pond C = 12) were captured by sweeping the ponds with handheld nets and were placed in plastic containers to be transported to a field station a few kilometers from the study sites. All individuals were sexed, measured for snout to vent length (SVL) and weighed with an electronic scale (Ohaus Scout Pro, Switzerland) to the nearest 0.01 g. The entire integument of each animal was searched for the presence of lesions. When found, lesions were classified based on their size, shape, coloration, texture, distribution and abundance. Two impression slides were taken from the skin (dorsal surface of each newt or at the site of lesions if present) and from the liver, kidneys and reproductive organs. Slides were air-dried and fixed with 70% ethanol at the field station for examination using light microscopy. Two sets of skin swabs were collected from the dorsal surface of each newt or at the site of lesions when these were present. One set of swabs was conserved at 4 °C in charcoal transport medium and the other set was preserved in 96% ethanol for molecular analyses.

A subset of the captured newts (n = 21), of which 10 showed macroscopic skin lesions, was euthanized by immersion in MS – 2,2,2 (< 1 g/L tricaine methane sulphonate, Thompson and Joseph Ltd., Norwich, UK) aqueous solution buffered to pH 7 with sodium bicarbonate in accordance with approved regulations on euthanasia of amphibians [31]. A systematic post-mortem examination was conducted on each euthanized newt and two sets of samples from the liver, lung, kidney, spleen, gastrointestinal tract, gonads and skin were stored in 96% ethanol and in 10% buffered formalin, respectively. Samples that encompassed skin lesions were collected when present. Triplicate impression slides of internal organs were obtained prior to formalin fixation and fixed with 70% ethanol.

2.3. Microbiology

One set of skin-impression slides was stained with Gram's stain and one set with Ziehl-Neelsen's stain to aid the detection and identification of bacteria and acid-fast bacilli respectively. Each swab stored in charcoal transport medium was streaked on xylose lysine (XLD) agar for bacterial culture and on Sabouraud's medium for fungal culture. Plates were incubated at 25 °C for 3 days. Cultured organisms were identified using microscopic appearance, Gram staining characteristics and/or colony morphology and biochemical tests using a commercially-available system for bacterial identification (API 20NE, bioMérieux UK Ltd., Basingstoke, Hampshire).

2.4. Histology

Formalin-fixed tissue samples were processed and stained with routine H&E (hematoxylin and eosin) stain or with Giemsa using standard methods [32]. A small number of formalin-fixed skin sections that contained lesions were deparaffinized and processed for electron microscopy (EM). Sections for EM were counterstained with uranyl acetate and lead citrate and examined with a Philips 400 TEM at 60 kV.

2.5. Molecular analyses

DNA was extracted and purified from the ethanol-preserved skin swabs using an automated workstation (BioSprint 15, Qiagen, UK). Swabs were vortexed vigorously for 3 min to ensure the release of any parasites. Following centrifugation and removal of the ethanol, each pellet was resuspended in 100 μL of TE before extracting DNA using a commercially-available kit (BioSprint Blood DNA, Qiagen, UK) following the manufacturer's instructions. Skin sections (including dissected dermal cysts and ulcers) collected from the euthanized newts were macerated in liquid nitrogen, and processed for DNA extraction as previously described. Purity (A260:A260 = 1.75–1.90) and concentration were determined for each DNA extract using a spectrophotometer (Eppendorf UK Ltd., Cambridge, UK). A series of polymerase chain reactions (PCR) was used to investigate the infectious etiology of the skin lesions. Each ethanol-fixed skin swab was also screened for the presence of *B. dendrobatidis* using real-time PCR [35], as were the skin samples from which DNA was extracted. We also screened for the presence of dermotropic mycobacteria and mesomycetozoan parasites, both of which can induce the vesicular, ulcerative and/or cyst-like lesions in amphibians [10]. Primers specific to the class Mesomycetozoea (AmgF 5′-GTAGTCATATGCTTGTCTC; AmgR 5′-TATTGCCTCAAACTTCCAT) were designed based on the alignment of the entire 18S rRNA sequence from seven species from the orders Dermocystida (*Dermocystidium* spp., GenBank accession number AF533950; *Rhinosporidium seeberi*, AF118851; *Amphibiothecum penneri*, AY772001 and *Amphibiocystidium ranae*, AY692319) and Ichthyophonida (*Ichythophonus irregularis*, AF232303; *Ichthyophonus hoferi*, U25637 and *Pseudoperkinsus tapestis*, AF192386). The seven sequences were aligned using the program ClustalW [33] to identify conserved regions. These were in turn aligned with the newt (*Cynops pyrrhogaster*, AB239574) and frog (*Xenopus laevis*, X04025.1) 18S rRNA gene sequences, to ensure that the primers were designed to not amplify host (amphibian) DNA. Primer specificity was tested in silico using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nih.gov).Reactions were performed in 25 μl total volumes containing < 1 μg DNA, 0.3 μM of each primer, 200 μM dNTPs (Bioline, London, UK), and 2.5 units of HotStarTaq Plus (Qiagen, Crawley, UK). To account for possible contamination, reactions containing RNAase-free water as templates were run alongside the samples. Amplified products were electrophoresed on 0.7% agarose gels stained with SYBRgold (Invitrogen, Paisley, UK) and visualized under UV illumination (Synoptics, UK). All reactions were conducted in duplicate.

Hi-fidelity PCR (HotStar HiFidelity Polymerase, Qiagen, UK) was used to repeat amplification reactions in three of the DNA samples that had yielded a fragment of the expected size. The resulting amplicons were gel-excised, column purified (QiaQuick, Qiagen, Crawley, UK) and commercially sequenced. Chromograms were visually inspected for errors and aligned using BioEdit (Ibis Biosciences, California, USA). Identity was confirmed by in-silico alignment with known sequences using the algorithm megablast within BLAST. The statistical significance threshold (Expected value) was set at 1 [34].

2.6. Statistical analyses

Alignment of the three forward sequences, and separately for the three reverse sequences, was performed using ClustalW (full alignment algorithm; single CPU mode). Phylogenetic analysis included consensus sequences from the same organisms used for primer design (see Section 2). A phylogenetic tree was created in Mega v4 [36] using the neighbor-joining (NJ) method on the basis of the number of nucleotide substitutions per site and the Kimura model with homogeneous patterns among lineages [37]. Reliability of clustering patterns was tested by bootstrapping (1000 iterations).

Table 1. Description and localization of external skin lesions in the palmate newts

Lesion	Description	Number of lesions and distribution
Vesicle	1–2 mm in diameter, no particular coloration	One or more lesions, scattered or clustered, distributed anywhere on the body[a]
Preulcerative	Inflamed circular area measuring between 5 and 7 mm with one or more openings (resembling a carbuncle). The epidermis was typically white and appeared friable noticeably thinned. Lesions contained numerous spherical cysts filled with a clear fluid	One or more lesions, scattered or clustered, distributed anywhere on the body[a] but most commonly on the flanks and base of the tail
Ulcer	3–4 mm in diameter, a considerable amount of dermal and epidermal tissue was absent. Occasionally, several lesions coalesced	One or more lesions, scattered or clustered, distributed anywhere on the body[a]

[a] No lesions were observed surrounding eyes, nostrils or mouth

3. Results

Three distinct types of skin lesion, not necessarily mutually exclusive, were observed in the captured newts (Table 1, Fig. 1). While skin lesions could be observed on any part of the body, they were typically distributed dorso-laterally and particularly prevalent at the junction between the torso and extremities. In addition to the three types of discrete skin lesion, two newts had extensive epidermal hemorrhages. Skin lesions were present in newts from all sampling sites.

Acid-fast bacilli were observed in skin-impression slides of twelve (31.58%) newts and in reproductive organs of three newts (7.89%). A number of environmental and commensal bacteria were isolated from the skin of 75% of the newts, the most frequent being *Pseudomonas fluorescens* (50%), followed by *Aeromonas hydrophila/caviae* (40%). *Weeksella virosa*, *Empedobacter brevis* and *Bacillus* sp. were isolated from the skin swabs of a small number of newts (< 5%).

Histologic examination of skin sections revealed encysted spore-like forms (hereafter spores) which were, in all cases, associated with the macroscopic lesions. Large basophilic spheroid cyst-like structures (hereafter cysts), surrounded by a translucent double membrane with some invaginations, were present in the stratum spongiosum of the dermis (Fig. 2A). Mild neutrophil infiltration in the subcutaneous layer (Fig. 2B) was occasionally associated with the lesions. The cysts were packed with spores forming a reticulated pattern (Fig. 2C), and occasionally protruded towards the skin surface (Fig. 2D). Ultrastructural examination showed that each spore had a well-defined nucleus, multiple basophilic/osmiophilic inclusion bodies, mitochondria and food vacuoles (Fig. 3). Neither flagella nor apical complexes nor polar bodies were observed in any of the spores.

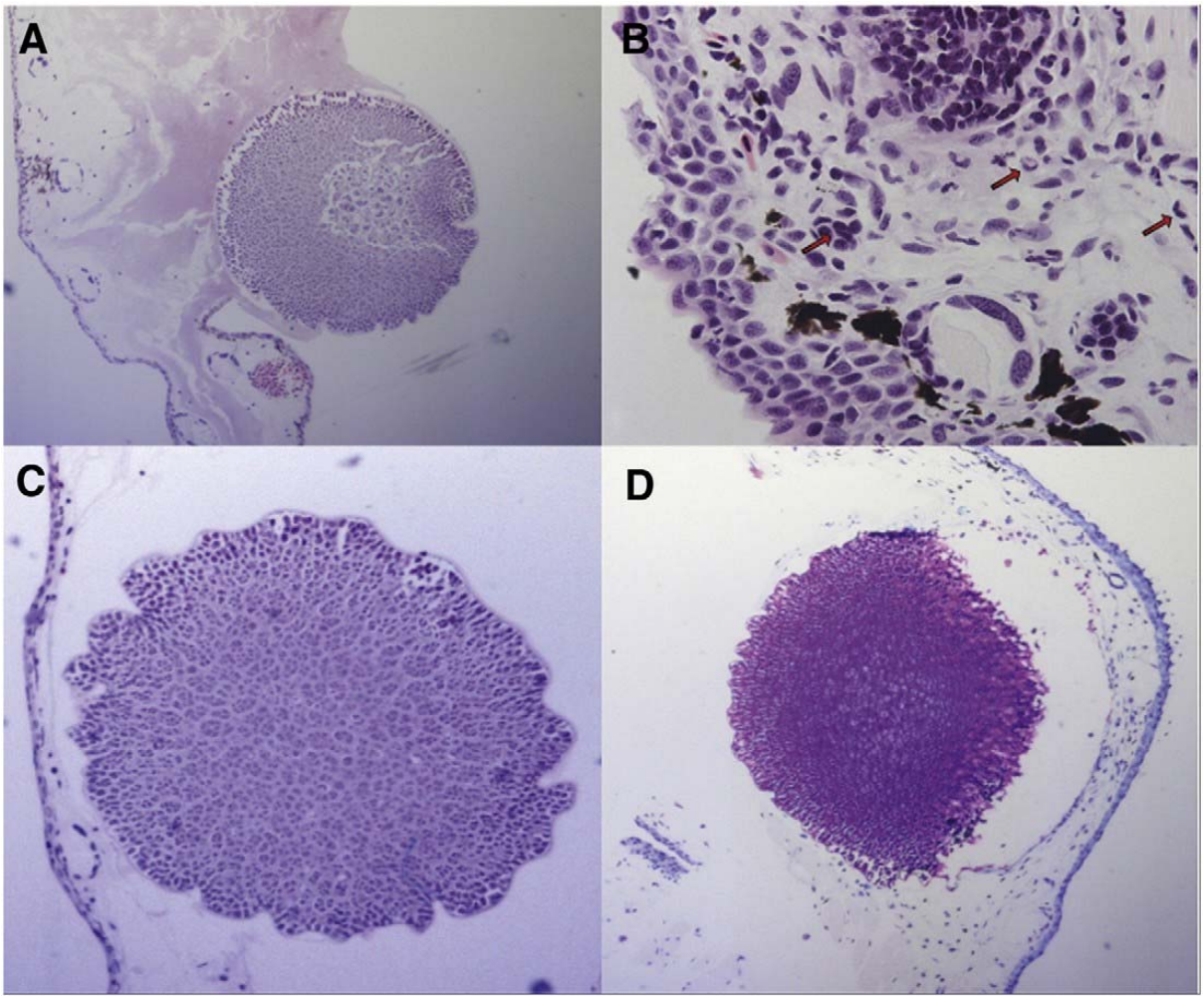

Fig. 2. H&E (hematoxylin and eosin) photomicrographs of palmate newt skin sections showing (A) large spheroid basophilic cyst, (B) mild neutrophil infiltration of the subcutaneous connective tissue associated with the cyst and (C) translucent membrane with invaginations projecting into the cyst. (D) Giemsa-stained photomicrograph of palmate newt skin section showing a cyst protruding towards skin surface.

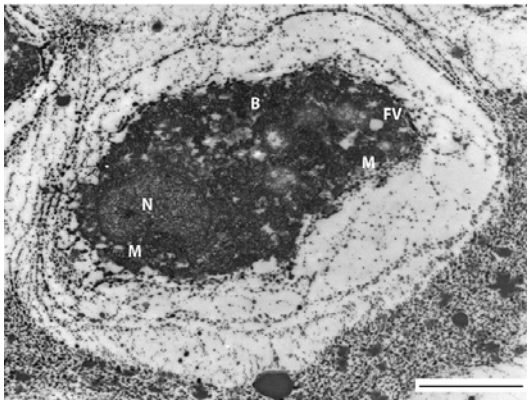

Fig. 3. Dermocystid parasite infecting palmate newt skin. Transmission electron micrograph of plastic-embedded tissue. Photomicrograph shows a spore with an evident and well-defined nucleus (N), multiple inclusion bodies (B), mitochondria (M) and food vesicles (FV). Scale bar = 2 μm

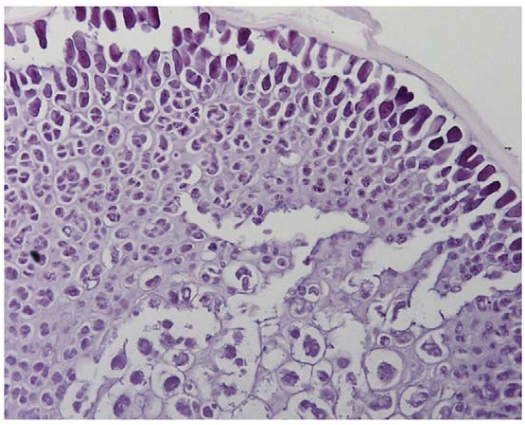

Fig. 4. H&E (hematoxylin and eosin) photomicrograph of a parasite cyst showing spore architecture. Spores found close to the centre of the cyst tended to have two to four nuclei, while those located closer to the cyst wall showed a single membrane-bound nucleus with a large nucleolus, denser basophilic–osmiophilic granules and a thicker membrane.

4. Discussion

Members of the class Mesomycetozoea are typically parasitic and infect a diverse range of hosts, from humans [e.g. 38] to fish [e.g. 39]. Since the early twentieth century, dermocystid mesomycetozoans have been detected in a range of European amphibian species in which infection is typically associated with nodular-type skin lesions [e.g. [6,11,12,13,14,15]], however, their biological significance remains mostly unknown. Over recent years, an increased frequency of skin vesicles and cysts has been observed in palmate newts in Southern France (M. Denoël, pers. obs.). Based upon the gross and microscopic lesions found in affected newts, the ultrastructural composition of the parasite, and our microbial and molecular analyses, these skin lesions are likely caused by a mesomycetozoan parasite within the genus *Amphibiocystidium*, order Dermocystida.

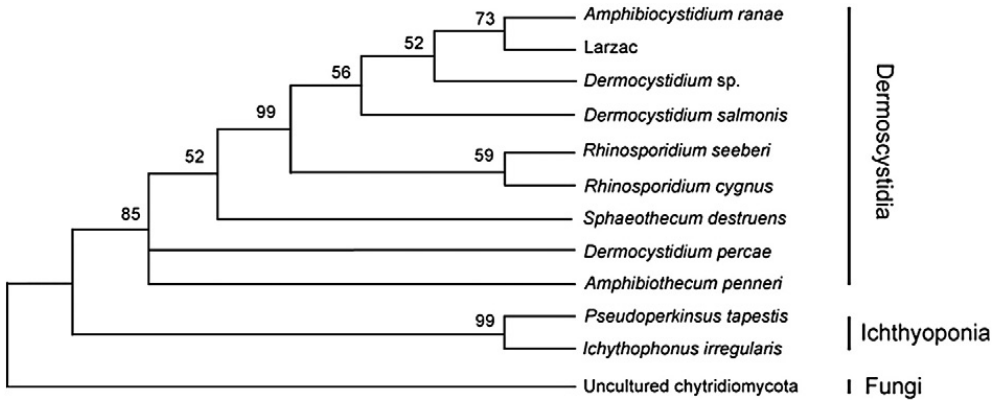

Fig. 5. Phylogram based on small ribosomal subunit sequences (817 bp) of the identified parasite and Mesomycetozoa organisms. The phylogeny test was run using a Minimum Evolution (ME) model with an interior branch test (1000 replicates). Sample from this study is referred to in the figure as Larzac.

The gross and microscopic lesions and the structure of the parasite are similar to those described previously for amphibian dermocystid infections, but some of our findings differ from prior descriptions and warrant further consideration. Previously, gross dermal lesions have been described as nodular cysts that vary in shape (spherical, ovoid or U-shaped) and size [6,12,14,40] and which present a single opening or depression on the external surface [14]. Interestingly, a number of the infected newts from Larzac revealed lesions that resembled carbuncles, with up to four openings. The cysts were divided by thin translucent septa into small, spore-filled chambers [see [14,40]], but we observed that the chambers in the centre of the cyst were further subdivided into smaller chambers containing basophilic granular nuclei. The spore or 'spore-like' elements within the cysts appeared to be at different stages of maturation depending on their location: those at the centre showed the typical sub-chamber division and a single large basophilic granular nucleus in each chamber [as described by [14,40]], while those located closer to the cyst membrane had a dense basophilic multi-granular spore surrounded by a thick wall. Our findings challenge previous observations that describe how "younger immature spores" (sporoblasts) are located at the periphery and the "ripe" spores at the centre of the cysts [14]. They also add support to a previous study that describes the parasite as a multinuclear 'plasmodium' that subsequently divides into numerous spherical spores [41].

The taxonomic affinities of the mesomycetozoa have revealed that they are an early branch of the holozoan tree that diverged slightly after the split between animals and fungi [42]. This has caused a range of classification problems as they contain characteristics of both fungi and animals [e.g. [11,12]] and are extremely difficult to identify to the species level without using phylogenetic classification [see 5]. Phylogenetic analysis of a fragment of the 18S rRNA gene suggests that the parasite found infecting the palmate newts is closely related to *A. ranae*. Unfortunately, due to the high sequence similarity exhibited among dermocystid parasites for this gene, we were unable to increase the phylogenetic resolution. Sequence analysis of a different genetic region might allow this to be accomplished in the future, but our attempts to amplify other regions were met with difficulties, such as cross-amplification with newt DNA (data not shown). Until more dermocystid parasite sequence data are available, it will be challenging to identify these parasites to species level using PCR and sequencing. To date, the pathogenic potential and host-population significance of dermocystidian parasitism in amphibians remain uncertain. Reports in the literature are contradictory, with some reports suggesting that infection is not harmful and self limiting [e.g. [31,43]], and others stating that the parasites can be debilitating to the amphibian host, particularly when infection intensity is

high [e.g. 44]. There is some indication that probability of survival is reduced in infected amphibians [8]. Pascolini et al. [6] found that the incidence of parasitism was significantly higher in a declining population of parental common European frog, *Pelophylax lessonae*, compared to a stable hybrid population (*P. esculentus*), suggesting that these parasites may pose a threat to population health. In addition, red-spotted newt (*N.viridescens*) mortalities in the USA have been associated with mesomycetozoan infections [8]. Our molecular analyses provide comparative evidence of the parasite's pathogenicity in the palmate newt. Firstly, *Amphybiocystidium* sp. DNA was detected only in newts with skin lesions or histological abnormalities characteristic of dermocystid infection, while *Amphybiocystidium* sp. DNA was not detected in apparently healthy individuals. This would suggest that there were no asymptomatic or carrier-state infections in the palmate newts, but in order to unequivocally determine this, a larger sample size would be required. Secondly, lesion intensity varied between individuals, ranging from a few small vesicles to multiple, coalescent ulcerous lesions involving a relatively large percentage of the newt's integument. For the severely-affected newts, the loss of a significant amount of tissue may interfere with skin functions such as osmoregulation and gas exchange [45]. Also, alteration of epidermal integrity increases the risk of secondary infections by opportunistic microorganisms. Environmental and opportunistic bacteria, including *P. fluorescens* and *A. hydrophila/caviae*, were found in the skin of more than 75% of the newts studied. Although common in aquatic environments and generally innocuous, these bacteria can cause disease in fish in which the integument or immune system is compromised [46]. Our data are insufficient for determining the impact of dermocystid infections on newt survival, but indicate a need to investigate this question further.

5. Conclusions

Here we report the presence of a dermocystid mesomycetozoan parasite in populations of palmate newts from Southern France. Recent correlative studies have found dermocystidium related mortality events in larval and adult stages of various amphibian species in the USA [8,9]. This illustrates the need to evaluate the potential threat of dermocystid infections to amphibians. Our results indicate that parasitism with *Amphibiocystidium* sp. and other dermocystid organisms has the potential to incur a high cost to infected newts, but determining the impact of this parasitism at the individual animal level or at the population level requires further investigation. Long-term monitoring of palmate newts and other amphibians that are affected by dermocystidium-like parasites will increase our understanding of the biological significance of these parasites and their relevance to amphibian conservation.

Statement of contributions

M.G. and M.D. conducted fieldwork. M.G., A.D. and A.C. analyzed microscopy sections. M.G. conducted microbiology tests. M.G. and K.A performed molecular assays. M.D. was at the initiative of this study and was responsible for all fieldwork logistics and animal sampling procedures. All authors were involved in the preparation of the manuscript.

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References

[1] Cavalier-Smith T. Neomonada and the origin of animal and fungi. In: Coombs GH, Vickerman K, Sleigh MA, Warren A, editors. Evolutionary relationships among protozoa, Vol. 56. Dordrecht: Kluwer; 1998. p. 375–407.

[2] Margulis L. Archael-eubacterial mergers in the origin of eukarya: phylogenetic classification of life. Proc Natl Acad Sci USA 1996;93:1071–6.

[3] Mendoza L, Taylor JW, Ajello A. The class Mesomycetozoea: a heterogenous group of microorganisms at the animal-fungal boundary. Ann Rev Microbiol 2002;56:213–344.

[4] Ragan MA, Goggins CL, Cawthorn RJ, Cerenius L, Jamienson AVC, Plourde SM, Rand TG, Söderhäll K, Gutell RR. A novel clade of protistan parasites near the animal-fungal divergence. Proc Natl Acad Sci USA 1998;93:11907–12.

[5] Feldman SH, Winsatt JH, Green E. Phylogenetic classification of the frog pathogen *Amphibiothecum* (*Dermosporidium*) *penneri* based on small ribosomal subunit sequencing. J Wildl Dis 2005;41:701–6.

[6] Pascolini R, Daszak P, Cunningham AA, Tei S, Vagnettie D, Bucci S, Fagotti A, di Rosa I. Parasitism by *Dermocystidium ranae* in a population of *Rana esculenta* complex in Central Italy and description of *Amphibiocystidum* n. gen. Dis Aquat Org 2003;56:65–74.

[7] Simoncelli F, Fagotti A, Dall'Olio R, Vagnetti D, Pascolini R, di Rosa I. Evidence of *Batrachochytrium dendrobatids* infection in water frogs of the *Rana esculenta* complex in Central Italy. EcoHealth 2005;2:307–12.

[8] Raffel TR, Bommarito T, Barry DS, Witiak SM, Shackelton LA. Widespread infection of the Eastern red-spotted newt (*Notophthalmus viridescens*) by a new species of *Amphibiocystidium*, a genus of fungus-like mesomycetozoan parasites not previously reported in North America. Parasitology 2008;135:203–15.

[9] Green DE, Converse KA, Schrader AK. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Ann N Y Acad Sci 2002;969:323–39.

[10] Pessier AP. An overview of amphibian skin disease. Sem Avian Exotic Pet Med 2002;11:162–74.

[11] Pérez C. *Dermocystis pusula*, organisme nouveau parasite de la peau des tritons. Reunion Biol Bordeaux 1907;5:445–6.

[12] Pérez C. *Dermocystis pusula*, parasite de la peau des tritons. Arch Zool Exp Gén 1913;52:343–57.

[13] Guyénot E, Naville A. Un nouveau protiste, du genre *Dermocystidium*, parasite de la grenouille. *Dermocystidium ranae* nov. spec. Rev Suisse Zool 1922;29:133–45.

[14] Broz O, Privora M. Two skin parasites of *Rana temporaria*: *Dermoscystidium ranae* Guyénot & Naville and *Dermosporidium granulosum* n. sp. Parasitology 1952;42:65–9.

[15] Pereira CN, di Rosa I, Simoncelli F, Pascolini R, Mendoza L. The pathogen of frogs *Amphibiocystidium ranae* is a member of the order Dermocystidia in the class Mesomycetozoea. J Clinical Microbiol 2005;43:192–8.

[16] Berger L, Speare R, Thomas A, Hyatt A. Mucocutaneous fungal disease in tadpoles of *Bufo marinus* in Australia. J Herpetol 2001;35:330–5.

[17] Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 1998;95:9031–6.

[18] Teacher A. G. F. Population and immunocompetent genetic variation: a field-based study. PhD thesis. University of London 2008; xxx p.

[19] Ariel E, Kielgast J, Svart HE, Larsen K, Tapiovaara H, Jensen BB, Holopainen R. Ranavirus in wild edible frogs Pelophylax kl. esculentus in Denmark. Dis Aquat Org 2009;85:7–14.

[20] Bielby J, Bovero S, Sotgiu G, Tessa G, Favelli M, Angelini C, Doglio S, Clare F, Gazzaniga E, Lapietra F, Garner TWJ. Fatal chytridiomycosis in the Tyrrhenian painted frog. Ecohealth 2009;6:27–32.

[21] Garner TWJ, Walker S, Bosch J, Leech S, Rowcliffe JM, Cunningham AA, Fisher MC. Life history trade-offs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. Oikos

2009;118:783–91.

[22] Bosch J, Martinez-Solano I, Garcia-Paris M. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstricans*) in protected areas of central Spain. Biol Conserv 2001;97:331–7.

[23] Cunningham AA, Langton TES, Bennett PM, Lewin JF, Drury SEN, Gough RE, Macgregor SK. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Phil Trans R Soc Lond B 1996;351:1539–57.

[24] Fisher MC, Garner TWJ, Walker SF. The global emergence of *Batrachochytrium dendrobatidis* in space, time and host. Ann Rev Microbiol 2009;63:291–310.

[25] Duffus ALJ. The chytrid blinders: what other disease risks to amphibians are we missing? EcoHealth, doi:10.1007/s10393-009-0271-8.

[26] Smith KF, Acevedo-Whitehouse K, Pedersen AB. The role of infectious diseases on biodiversity. Anim Conserv 2009;21:1–12.

[27] Acevedo-Whitehouse K, Duffus AJL. Effects of environmental change on wildlife health. Phil Trans Roy Soc Lond B Biol Sci 2009;95:9031–6.

[28] Denoël M. Persistance et dispersion d'une population introduite de triton alpestre (*Triturus alpestris*) dans les causses du Larzac (sud de la France). Rev Ecol 2005;60: 139–48.

[29] Denoël M. Priority areas of intraspecific diversity: Larzac, a global hotspot for facultative paedomorphosis in amphibians. Anim Cons 2007;10:110–8.

[30] Denoël M, Lehmann A. Multi-scale effect of landscape processes and habitat quality on newt abundance: implications for conservation. Biol Conserv 2006;130:495–504.

[31] AVMA [American Veterinary Medical Association] Guidelines on euthanasia. 2007;20.

[32] Luna LG. Manual of histologic staining methods of the Armed Forces Institute of Pathology. New York: McGraw-Hill; 1968.

[33] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. ClustalW2 and ClustalX version 2. Bioinformatics 2002;23:2947–8.

[34] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

[35] Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 2004;60:141–8.

[36] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–9.

[37] Page RDM, Holmes EC. Molecular evolution: a phylogenetic approach. Oxford: Blackwell Science; 1998.

[38] Silva V, Periera CN, Ajello L, Mendoza L. Molecular evidence for multiple host-specific strains in the genus *Rhinosporidium*. J Clin Microbiol 2005;43: 1865–8.

[39] Pekkarinen M, Lom J, Purphy CA, Ragan MA, Dyvoká I. Phylogenetic position and ultrastructure of two *Dermocystidium* species (Ichthyosporea) from the common perch (*Perca fluvaitilis*). Acta Protozool 2003;42:287–307.

[40] Jay JM, Pohley WJ. *Dermocystidium penneri* sp. n. from the skin of the American toad, *Bufo americanus* (Amphibia: Bufonidae). J Parasitol 1981;67:108–10.

[41] Reichenbach-Klinke HH, Elkan E. The principle diseases of lower vertebrates. California: Academic Press; 1965.

[42] Baldauf SL. An overview of the phylogeny and diversity of eukaryotes. J System Evol 2008;46:263–73.

[43] Densmore CL, Green DE. Diseases of amphibians. ILAR J 2007;48:235–54.

[44] Poynton SL, Whitaker BR. Protozoa and metazoa infecting amphibians. In: Wright KM, Whitaker BR, editors. Amphibian medicine and captive husbandry. Malabar: Krieger Publishing Co; 2001. p. 111–22.

[45] Beebee TJC, Griffiths R. Amphibians and reptiles. London: Harper Collins Publishers; 2000.

[46] Mastan SA. Incidences of dermatomycosis in fishes of Larpur reservoir, Bhopal (M.P.). J Herbal Med Toxicol 2001;2:37–40.

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