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NOTE

Neurotropic mesomycetozoean-like infection in larvae of the southern toad *Anaxyrus terrestris* in Florida, USA

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ABSTRACT: As part of a state-wide multispecies survey of amphibian diseases, sampling was conducted at Archbold Biological Station, Venus, Florida, USA, on 15 April 2011. Gross examination of southern toad (*Anaxyrus terrestris*) larvae was unremarkable, but infections by a mesomycetozoean-like organism were observed in longitudinally sectioned routine haematoxylin and eosin-stained histologic slides. In 100% of the sectioned specimens examined (n = 5), a high density of the organism, representing several developmental stages, was found in the central nervous system, mainly in the spinal cord, brain, retina and optic nerve. No host inflammatory responses were found to be associated with the parasitic infection. Free, mature schizonts were occasionally found in the gill chamber and, more commonly, in the dorsal roof area. No organisms were found in other organs examined histologically, i.e. liver, kidney, heart, alimentary tract, exocrine pancreas and skeletal muscles. Presumptive mesomycetozoean ichthyophonids in anurans are usually reported to be pathogenic, especially affecting skeletal muscle tissue and causing death. To our knowledge, this is the first report of a similar organism infecting primarily the central nervous system in an amphibian.

KEY WORDS: Mesomycetozoean-like · Southern toad · Amphibian · Central nervous system · Larva

INTRODUCTION

Diseases are significant contributing factors in the global decline of amphibian populations (Gray et al. 2009, Schloegel et al. 2010, Lesbarrères et al. 2012). In Florida, as elsewhere, amphibian pathogens, such as frog virus 3 (FV3, the type species for the genus *Ranavirus*), protozoan alveolate parasites (Cook 2008, Rothermel et al. 2008, Landsberg et al. 2013) and chytrid fungi (e.g. *Batrachochytrium dendrobatidis* [*Bd*]; Rothermel et al. 2008, Rizkalla 2009, 2010) are considered important mortality factors (Rothermel et al. 2008, Landsberg et al. 2013).

During a 3 yr surveillance study of amphibian diseases, we incidentally observed a heavy mesomycetozoean-like infection reminiscent of piscine *Ichthyophonus* in histologic sections of the central nervous system (CNS, i.e. brain, spinal cord and eye) of larval southern toad *Anaxyrus terrestris* (n = 5), a species not frequently collected. *Ichthyophonus* spp. are mesomycetozoean ichthyophonid parasites of fish (Cavalier-Smith 1998, Mendoza et al. 2002, Adl et al. 2012, Glockling et al. 2013) that have also been reported but not definitively identified and characterized in amphibians. In amphibians, ichthyophonid-like infections (Herman 1984, Ware et al. 2008, Rowley et al. 2013) are typically found in the skeletal muscle and connective tissue, where they can cause granulomatous myositis (Green et al. 1995, Mikaelian et al. 2000), with occasional debilitation

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and emaciation causing mortality and morbidity (Green et al. 2002, Densmore & Green 2007).

Lacking molecular data for confirmation, we tentatively assigned the parasites infecting southern toads to the Mesomycetozoea, possibly in the Order Ichthyophonida, based on comparable morphology of other infections by these organisms in amphibians and fish. Herein we report a case study of an apparently unusual, nonpathogenic organotropism for these organisms using descriptive terms for piscine *Ichthyophonus* stages as proposed by Kocan (2013), and attempt to classify the cell stage types based upon size and morphology.

MATERIALS AND METHODS

Sampling

When sampling in ponds at the Archbold Biological Station (Venus, FL), we used 4.8 mm mesh (3/16 inch) dip nets (Model HDD-2, Memphis Net and Twine) to collect amphibian larvae (n = 5) for histopathology. At each pond, live larval specimens were immediately transferred into a Ziploc® bag or a plastic bucket of pond water, sorted by species and processed onsite. Dip nets, buckets, boots and other field gear were sprayed with 3% chlorine bleach following recommended disinfection procedures (Phillott et al. 2010).

Necropsy and histopathology

Live larvae were euthanized with 200 mg l^{-1} tricane methanesulfonate (Tricane S, Western Chemical) buffered with 400 mg l^{-1} sodium bicarbonate ($NaHCO_3$, Fisher Scientific) in pond water. Following anaesthesia, digital callipers were used to measure specimens (snout-to-vent length [SVL]) to the nearest 0.1 mm. Then, a midventral longitudinal incision was made to allow the fixative to penetrate into the abdominal cavity.

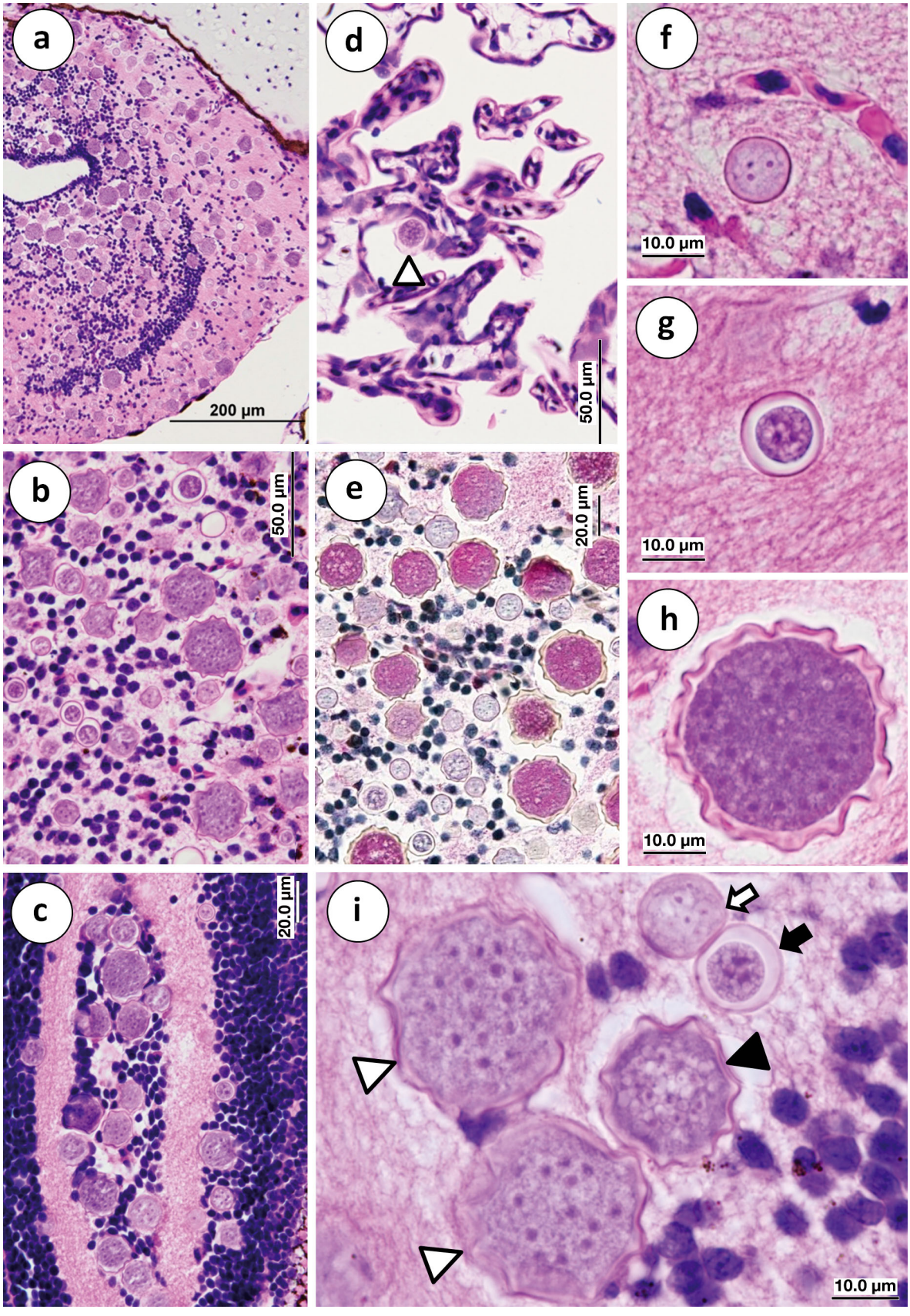
For histopathology, samples were fixed with 5% paraformaldehyde for at least 48 h, then rinsed with tap water and decalcified overnight with formic acid-sodium citrate (Luna 1968). Samples were dehydrated with an ascending-graded ethanol series to absolute ethanol, then embedded in paraffin and longitudinally sectioned at a thickness of 4 µm with a Leica rotary microtome. From each specimen, 2 types of sections were made: (1) deep midline (including the vertebral column, spinal cord and brain) and (2) shallow anterior, tangential cut (including the gill chamber and eye). Sectioned tissue ribbons were placed onto a glass slide and stained routinely with Mayer's haematoxylin and eosin (H&E), periodic acid Schiff (PAS) with metanil yellow counterstain, and thionin (Luna 1968). Light photomicrographs were captured with an Olympus BX51 microscope and DP71 digital camera. For each toad specimen, 10 organisms located in the spinal cord and brain were measured (maximum and minimum length diameters for both outer and inner cell membranes) using an ocular micrometer and a 100× oil immersion objective lens, and were then grouped by developmental stage.

RESULTS

Of 8 ponds surveyed at Archbold on 15 April 2011, southern toads (n = 5, mean SVL 11.4 mm ± 0.6 SD, range 10.5 to 12.0 mm) were collected and examined from only 1 pond (27.12925° N, 81.37747° W). Water and ground temperatures were 27.7 and 38.8°C, respectively. No dead or moribund larvae were observed. Other larval specimens collected from this pond and examined by histopathology were the little grass frog *Pseudacris ocularis* (n = 1, SVL = 3.0 mm) and the southern leopard frog *Lithobates sphenocephalus* (n = 4, SVL = 11.9 mm ± 1.0 SD, range 10.8 to 13.1 mm). No apparent abnormal clinical signs were noted, and no remarkable external lesions were found in any larva of a live-captured species.

All southern toad larvae examined were infected with mesomycetozoean-like organisms, while larvae of all other species were negative. A high density of the various stages of the organism (see results below) was found in the brain (Fig. 1a) of all southern toad

Fig. 1. Longitudinal histologic sections of a southern toad (*Anaxyrus terrestris*) larva infected with mesomycetozoean-like organisms. Sections are stained with Mayer's haematoxylin and eosin (H&E) or (e) periodic acid Schiff (PAS). (a) Brain tissue heavily infected with multiple life history stages; (b) spinal cord infected with various developmental stages without showing any host inflammatory response; (c) eye retina with immature schizont stages; (d) free schizont (▷) in the gill chamber; (e) spinal cord infected with various developmental life history stages showing a range in PAS reactivity from negative to positive. Type C schizonts exhibited a strong PAS-positive reaction; (f) Type A immature schizont with a few cell nuclei and thin cell membrane; (g) Type B schizont with a thick cell membrane and strongly basophilic cell cytoplasm; (h) Type C mature schizont with thick cell membrane and multiple nuclei located at the periphery of the inner cell; (i) various schizont stages. Type A (⇦), Type B (⬅) and Type D (▷) which are endospore-like structures showing multiple nuclei located near the centres of the inner cells, and 1 example (▶) with the centre of the cell remaining clear


a
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larvae and in the spinal cord (Fig. 1b) of 4 of the 5 specimens (the spinal cord of 1 specimen was not found in section). Despite heavy infections, no host inflammatory response, including exudates, was observed. In the 3 specimens for which it was possible to take good sections of the retina, all were infected with the organism, and stages tentatively termed immature schizonts (following Kocan 2013) were common (Fig. 1c; especially aggregated at the inner and outer nuclear layers, as well as in the inner plexiform layer). The optic nerve was infected in 4 specimens and the lens nucleus was vacuolated in one. In 3 specimens, free mature schizont stages were occasionally found in the gill chamber (Fig. 1d), while they were more common in the dorsal roof area.

Four presumptively different growth stages of the organism (schizonts; Kocan 2013) were found in the nervous tissues and were identified based on size and morphology:

(1) The Type A schizont. These were the smallest (equivalent to the immature spore stage of Mikaelian et al. 2000) and were rare. The cytoplasm was lightly basophilic to pale, PAS-negative and surrounded by a thin, eosinophilic-stained, PAS-positive single outer cell membrane (Fig. 1e,f,i). Type A schizonts (n = 50 cells) had a mean length of 10.1 to 10.6 µm ± 1.8 to 2.1 µm SD (range 6 to 17 µm) with an average of 4 (range 1 to 10) centrally located nuclei.

(2) The Type B schizont. These had multinucleate cells and the outer cell membrane (eosinophilic, PAS-positive) had begun to thicken. The cell membrane was separated from the inner cell by a vacuolar space (Fig. 1g,i). The shape of the outer cell membrane was oval to spheroid, but the inner cell was usually spheroid. The inner cell tended to be strongly basophilic, PAS-negative to lightly PAS-positive, with multiple centrally located nuclei. Type B schizonts were not measured.

(3) The Type C schizont. This largest stage (equivalent to the mature type of resting spores described by Mikaelian et al. 2000) possessed a thick outer cell membrane (an acellular, multilaminate membrane; Kocan 2013) with a rough, wrinkled irregular surface (a possible shrinkage artefact from slide processing). The outer cell membrane was thicker than that of Type B and eosinophilic. The outermost layer of the multilaminate cell membrane was much more PAS-positive than that of Types A and B. Inner multinucleate cells were strongly PAS positive (Fig. 1e). These had an average of 27 nuclei (range 14 to 45, n = 50 cells), which tended to be localised at the periphery of the inner cell (Fig. 1h). Schizont cells (n = 50 cells) had a mean length, including the outermost multilaminate cell membrane, of 33.0 to 35.3 µm ± 5.3 to 5.6 µm SD (range 24 to 48 µm) and a mean length of the innermost cell membrane of 28.9 to 30.4 µm ± 4.0 to 4.1 µm SD (range 22 to 39 µm).

(4) The Type D schizont. This was a stage intermediate in cell size between that of immature (Type A) and mature (Type C) schizonts. Type D schizonts possessed PAS-negative endospore-like structures with multiple centrally located nuclei (Fig. 1i). In some Type D schizonts, the centre of the cells remained clear after H&E staining (Fig. 1i). A thin outer cell membrane was eosinophilic and PAS-positive, and staining properties resembled those of Type A schizonts. Type D schizonts were not measured.

No infection by the putative mesomycetozoean-like organisms was found in the other organs examined. Notable pathological changes were found only in the liver and oesophageal epithelium. Hepatocytes were vacuolated and swollen in all specimens, and 1 liver exhibited focal necrosis. In another specimen, a large, unidentified metazoan parasite (75 × 85 µm; possibly a digenean) had encysted in the liver parenchyma and was associated with granulocytic infiltrations. The oesophageal epithelium exhibited a cystic appearance in 2 specimens. Nothing remarkable was found in the kidney, heart, alimentary tract, exocrine pancreas or skeletal muscle tissue.

DISCUSSION

Although ichthyophonid-like infections have been reported in ranids (Mikaelian et al. 2000), to our knowledge infections by putatively similar organisms have not been reported in toads. Jay & Pohley (1981) reported that the closely related mesomycetozoean dermocystid *Amphibiothecum penneri* (= *Dermosporidium penneri*, Feldman et al. 2005) in American toads *Anaxyrus americanus* was a subcutaneous skin parasite producing 10 to 12 µm spores with inclusion bodies in cysts different from those reported here. In addition, *A. penneri* has been reported in the skin, urostyle and mesonephros of Yosemite toads *Bufo canorus* (Green & Sherman 2001).

Heavy infections by the mesomycetozoean-like organism were found primarily in the CNS in southern toad larvae, without any signs of a host inflammatory response. Free mature schizonts were occasionally found in the gill chamber, suggesting that these stages or subsequent infective stages are released outside the host. This observation of organ-

otropism for primarily CNS tissue is new. For ichthyophonids, we found only 1 reference in the literature to CNS infections by *Ichthyophonus*, namely those of *I. hoferi* in the brain of Pacific herring *Clupea pallasi*, but in that case, other tissues were even more heavily affected (Marty et al. 1998).

Putative ichthyophonid infections in amphibians are usually found in the skeletal muscle and connective tissue in a variety of species and cause granulomatous myositis. For example, in Quebec, ichthyophonid-like infections were associated with granulomatous myositis in adult, juvenile and larval stages of the green frog *Lithobates clamitans*, wood frog *L. sylvaticus*, bullfrog *L. catesbeianus*, spring peeper *Pseudacris crucifer* and pickerel frog *L. palustris* (Mikaelian et al. 2000). Ichthyophonid-like infections have also been reported for the caudate red spotted newt *Notophthalmus viridescens* and spotted salamander *Ambystoma maculatum* (Herman 1984, Green et al. 1995, Raffel et al. 2006, Ware et al. 2008, Sherman et al. 2009, Glenney et al. 2010). In the USA, 2 presumptive ichthyophonid cases leading to the death of adult bullfrogs and wood frogs were reported in a review article by Green et al. (2002).

Apart from fish, *Ichthyophonus* reportedly has a wide host range, including marine and freshwater crustaceans, reptiles and piscivorous birds (McVicar 1999), but molecular characterisation of the nonpiscine species is needed to determine specificity and classification. In fish, *Ichthyophonus* sp. and *I. hoferi* (McVicar 1999) have been well studied in salmonids (Lauckner 1984), and the life cycle involves an infective motile plasmodium stage (Kocan et al. 2013; amoeboid stage, Mendoza et al. 2002), distinguishing this order (Ichthyophonida) from the Dermocystida (Mendoza et al. 2002). One of the presumptive diagnostic tests for *Ichthyophonus* requires that schizonts show a PAS-positive reaction in histology (Kocan et al. 2013), but confirmatory speciation requires a polymerase chain reaction with *Ichthyophonus*-specific primers (White et al. 2013, AFS-FHS 2014). The early schizont stages of the mesomycetozoean-like organism found in southern toads did not exhibit a strong PAS-positive reaction, suggesting that these organisms are not *Ichthyophonus*. Clearly, confirmation with molecular techniques and a reappraisal of amphibian ichthyophonids is necessary. In this study, it was not determined whether the infections were subclinical, or whether they might eventually affect the host if other triggering factors are involved in possible pathogenicity and disease. Perhaps southern toads are a reservoir or intermediate hosts, because the lack of a host response in this species (albeit in a small sample size) seems to be part of an adaptive life cycle. Southern leopard and little grass frogs residing in the same pond as the southern toads were apparently uninfected, though again the number of samples examined was small. Further study is necessary to classify this organism and to assess host species susceptibility and pathogenicity.

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