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Co-infection by alveolate parasites and frog virus 3-like ranavirus during an amphibian larval mortality event in Florida, USA

Jan H. Landsberg[1,*], Yasunari Kiryu[1], Maki Tabuchi[1], Thomas B. Waltzek[2], Kevin M. Enge[3], Sarah Reintjes-Tolen[4], Asa Preston[5], Allan P. Pessier[5]

[1]Fish and Wildlife Research Institute, Florida Fish and Wildlife Conservation Commission, St. Petersburg, Florida 33701, USA
[2]Department of Environmental and Global Health, College of Public Health Professions, and [4]Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, Florida 32611, USA
[3]Fish and Wildlife Research Institute, Florida Fish and Wildlife Conservation Commission, Gainesville, Florida 32601, USA
[5]Amphibian Disease Laboratory, Wildlife Disease Laboratories, Institute for Conservation Research, San Diego Zoo Global, San Diego, California 92112, USA

ABSTRACT: A multispecies amphibian larval mortality event, primarily affecting American bullfrogs *Lithobates catesbeianus*, was investigated during April 2011 at the Mike Roess Gold Head Branch State Park, Clay County, Florida, USA. Freshly dead and moribund tadpoles had hemorrhagic lesions around the vent and on the ventral body surface, with some exhibiting a swollen abdomen. Bullfrogs (100%), southern leopard frogs *L. sphenocephalus* (33.3%), and gopher frogs *L. capito* (100%) were infected by alveolate parasites. The intensity of infection in bullfrog livers was high. Tadpoles were evaluated for frog virus 3 (FV3) by histology and PCR. For those southern leopard frog tadpoles (n = 2) whose livers had not been obscured by alveolate spore infection, neither a pathologic response nor intracytoplasmic inclusions typically associated with clinical infections of FV3-like ranavirus were noted. Sequencing of a portion (496 bp) of the viral major capsid protein gene confirmed FV3-like virus in bullfrogs (n = 1, plus n = 6 pooled) and southern leopard frogs (n = 1, plus n = 4 pooled). In July 2011, young-of-the-year bullfrog tadpoles (n = 7) were negative for alveolate parasites, but 1 gopher frog tadpole was positive. To our knowledge, this is the first confirmed mortality event for amphibians in Florida associated with FV3-like virus, but the extent to which the virus played a primary role is uncertain. Larval mortality was most likely caused by a combination of alveolate parasite infections, FV3-like ranavirus, and undetermined etiological factors.

KEY WORDS: Alveolate parasite · Ranavirus · Frog virus 3 · Amphibian mortality · Bullfrog · Southern leopard frog · Gopher frog · Tadpole

INTRODUCTION

Global extinctions and mass mortalities of amphibian species have led to increased awareness of the need to investigate and diagnose the causes of local die-offs. The roles of various pathogens such as ranavirus, chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), and protistan parasites in amphibian declines have raised concerns about the increasing spread of these pathogens as well as the urgent requirement for management strategies to address the spread and control of amphibian dis-

*Email: jan.landsberg@myfwc.com

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eases (Ouellet et al. 2005, Robert 2010, Lesbarrères et al. 2012). The risk of pathogen transfer from free-ranging to captive amphibians and vice versa is of particular concern. The World Organization for Animal Health (OIE) recently listed *Bd* and amphibian ranaviruses as reportable diseases (Schloegel et al. 2010). Iridoviruses of the genus *Ranavirus* infect poikilothermic vertebrates across 3 taxonomic classes: anuran and caudate amphibians, squamate and testudine reptiles, and bony fishes (Chinchar 2002, Chinchar et al. 2009). Frog virus 3 (FV3), the type species for the genus *Ranavirus*, is pathogenic to both larval and adult amphibians and has been responsible for frog population declines and die-offs worldwide (Gray et al. 2009b, Lesbarrères et al. 2012).

Another parasite that appears to be of high pathogenic significance but has yet to achieve reportable status is the alveolate protistan parasite responsible for larval amphibian die-offs throughout the USA (Green et al. 2003, Davis et al. 2007, Cook 2008, 2009). This parasite, the cause of an emerging disease in amphibians, has not yet been officially named. Herein we refer to the pathogen as an alveolate parasite, but it has been reported as *Dermomycoides* sp., a *Perkinsus*-like parasite, and *Anuraperkinsus* (Green et al. 2003, Davis et al. 2007, Cook 2009). The alveolate parasite reported by Davis et al. (2007) was the first published record preliminarily describing the molecular classification of this parasite with the 18S rRNA gene sequence (1583 bp, GenBank EF675616). The alveolate organism reported as a *Perkinsus*-like parasite in amphibians is phylogenetically distinct from *Perkinsus* sensu stricto (a genus commonly found in mollusks; Davis et al. 2007), but both are currently placed in the class Perkinsea with other marine alveolate parasites such as *Parvilucifera*, which infects dinoflagellates (Hoppenrath & Leander 2009, Bråte et al. 2010).

In Florida, USA, amphibian mortality incidents have rarely been documented, with major events having been attributed to a few cases of alveolate parasites (Rothermel et al. 2008). The normal distribution of *Bd* has been addressed on a limited scale (Rothermel et al. 2008, Rizkalla 2009, 2010), but this pathogen has not been associated with acute mortality events, other than in a few individuals. Little is known regarding FV3 (Gray et al. 2009a). An unpublished record for the presence of FV3 in larval bronze frogs *Lithobates clamitans* and an adult green tree frog *Hyla cinerea* in northern and southern Florida, respectively, is known from a mortality event (M. Gray pers. comm.).

During an amphibian disease surveillance survey at Mike Roess Gold Head Branch State Park (GHBSP), Clay County, Florida, 2 of us (K.M.E. and S.R.T.) coincidentally observed an ongoing larval mortality event. Herein, we report this mortality affecting multiple host species and putatively associate with the die-off 2 co-occurring etiological pathogenic agents, the alveolate parasite and the FV3-like ranavirus.

MATERIALS AND METHODS

Field observations, sample collections, and transport

While conducting an amphibian disease survey on 7 April 2011, an ongoing tadpole mortality event was observed in GHBSP's Pebble Lake (29.8254° N, 81.9535° W; hereinafter referred to as the pond) and specimens were collected. This semipermanent sinkhole pond has a total open water area of 9 acres (ca. 3.64 ha; when the lake level is approximately 35 m above mean sea level), surrounded by xeric hammock/scrub (longleaf pine, hardwoods, saw palmetto, and sandhill; FDEP 2010). During the sampling period, the pond had a maximum depth of about 4 m. On the following day, environmental data were obtained, and additional specimens were collected for histopathology and further diagnostics. A handheld YSI multiparameter water-quality meter (Model 650MDS, Yellow Springs Instrument Company) was used to measure dissolved oxygen, pH, temperature, and conductivity. Water samples were fixed with Lugol's solution for plankton analysis by light microscopy.

Freshly dead and moribund tadpoles were collected by hand with disposable latex gloves, individually placed in ziplock bags, and necropsied on site. When live tadpoles were observed in the pond, specimens were collected with a dip net and immediately placed in a container of freshly collected pond water. These were transported to the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute (FWRI) in St. Petersburg in a 19 l bucket partly filled with pond water and aerated with a portable, battery-operated air pump (Marine Metal Products) and examined 12 to 48 h after capture. The site was revisited on 25 April and 28 July. Appropriate biosecurity measures for disinfection were taken before and following sampling by treating nets and field gear with 3% chlorine bleach (Phillott et al. 2010).

Necropsy and histopathology

Following sample collection, necropsies were conducted either in the field or in the laboratory, employing a variety of techniques to identify potential etiological agents. Specimens were processed as was appropriate to their condition at collection and with the objectives of conducting gross and histopathology, transmission electron microscopy (TEM), and molecular diagnostics; therefore, not all specimens were used for all assays (see Table 1). Prior to necropsy, all live tadpole specimens were anesthetized with 200 mg l^{-1} of tricaine methanesulfonate (Tricaine S, Western Chemical) buffered with 420 to 1050 mg l^{-1} sodium bicarbonate solution (Fisher Scientific). Following anesthesia, digital calipers were used to measure specimens (snout to vent length, SVL) to the nearest 0.1 mm. Then a midventral incision was made either to excise tissue or to allow the fixative to penetrate into the abdominal cavity.

For the 2 field visits in early April, larger freshly dead or moribund tadpoles of the American bullfrog *Lithobates catesbeianus* and southern leopard frog *L. sphenocephalus* were necropsied on site to obtain fresh tissues for histopathology and molecular analysis (see below). Livers either were preserved immediately in 70% ethanol or were transported on ice to the laboratory and frozen at –80°C.

Additional specimens returned to the laboratory were either dissected or fixed whole. For dissected specimens, liver tissue was divided as appropriate in triplicate for wet-mount observation (samples collected in July only), and for preservation or fixation for use in further diagnostic tests. For histopathology, samples were generally fixed in 5% paraformaldehyde for 11 to 18 d. Whole specimens were decalcified in a formic acid–sodium citrate mixture overnight after fixation (Luna 1968). Fixed samples were rinsed with tap water, dehydrated with a graded ethanol series, embedded in paraffin, and sectioned at a thickness of 4 µm with a rotary microtome (Leica).

Three additional samples (*Lithobates sphenocephalus*, n = 2; gopher frogs *L. capito*, n = 1) collected on 8 April were fixed for histopathology in Bouin's solution (Luna 1968) for 48 h, and also used for paraffin-embedded tissue DNA extraction to test for ranavirus. Although there are difficulties in using formalin- or Bouin's-fixed samples to reliably extract DNA (Nuovo & Silverstein 1988, Ben-Ezra et al. 1991, Kattenbelt et al. 2000, Bonin et al. 2005, Santos et al. 2008), molecular diagnosis was attempted to increase the sample size for ranavirus assays. Tissue samples were first serially sectioned for 8 ribbons at a thickness of 10 µm, deparaffinized with xylene (modified from Kattenbelt et al. 2000), and then processed following the manufacturer's instructions for the QIAamp DNA formalin-fixed paraffin-embedded tissue kit (Qiagen). Extracted DNA samples were processed for ranavirus detection as described below.

For routine histopathology, sectioned slides were stained with Mayer's hematoxylin and eosin (H&E) and thionin (Luna 1968). Light photomicrographs were captured with an Olympus BX51 microscope equipped with a Nomarski prism (differential interference contrast) and DP71 digital camera.

Transmission electron microscopy

A subset of liver tissues was fixed in Trump's fixative (4% formaldehyde, 1% glutaraldehyde, 50 mM sodium phosphate, pH 7.2) followed by fixation in 1% osmium tetraoxide for 1 h. Tissues were subsequently dehydrated in a graded ethanol series, infiltrated with epoxy propylene oxide, and embedded in epoxy resin. The epoxy block was then sectioned with a Leica EM UC6 ultramicrotome, stained with uranyl acetate followed by lead citrate, and examined with a JEM-1400 transmission electron microscope (JEOL) equipped with a digital photomicrograph Orius SC1000 CCD camera (Gatan). Semithin (1 µm) sections of the epoxy block were also stained with toluidine blue for detection and confirmation of alveolate spores. We used digitized TEM images and an adjusted automated scale to measure length and width of alveolate spores (endospores and exospores) from the liver tissue of 1 live-captured gopher tadpole. Mature spore measurements were taken when the cross section of the spore was cut through 9 channels in the outer wall and in which the spore was presumptively sectioned through the spore center.

Molecular diagnostics

On 7 April, tadpoles were sampled for ranavirus (Table 1) and chytrid fungus (*Bd*). Liver samples and skin swabs to be tested for each pathogen were pooled by species for analysis. For *Bd*, skin swabs were taken from the mouthparts of additional live tadpoles of the southern cricket frog *Acris gryllus* (n = 5), southern leopard frog (n = 5), and bull-

Table 1. Species, sample dates, identification accession number of specimens, condition, morphometric data (SVL: snout to vent length), and diagnostic tests for alveolate spores and frog virus 3 (FV3)-like ranavirus. D: dead; L: live and presumably healthy; M: moribund; TEM: transmission electron microscopy; –: not detected, +: positive; na: not applicable; blanks: no data

Species	Date (mm/dd/yy)	No.	Condition	SVL (mm)	Alveolate parasite		FV3-like ranavirus		
					Histology spores	TEM	Histology inclusion body	PCR	TEM
Lithobates catesbeianus	04/07/11	1–6	D, M	na				+	
(American bullfrog)	04/08/11	1	D	36.1	+[a]			–	
		2	D	34.0	+[a]				
		3	D	30.8	+[a]				
		4	D	35.7	+[a]			–	
		5	D	36.2	+[a]			+	
		6	D	29.0	+[a]	+[a]			–
		7	M	40.7	+	+	–	–	–
	07/28/11	1	L	35.0	–		–	–	
		2	L	25.1	–		–	–	
		3	L	31.5	–		–	–	
		4	L	33.7	–		–	–	
		5	L	26.9	–	–	–	–	–
		6	L	26.0	–	–	–	–	–
		7	L	21.3	–		–	–	
L. sphenocephalus	04/07/11	1–4	D, M	na	–			+	
(southern leopard frog)	04/08/11	1	L	22.9	–	–	–	–	–
		2	L	17.6	–		–	–	
		3	L	14.9	–		–		
		4	L	15.5	+		–		
		5	L	11.8	–		–	+[b]	
		6	L	11.1	+		–	–[b]	
	07/28/11	1	L	22.9	–		–	–	
		2	L	26.5	–		–	–	
L. capito (gopher frog)	04/08/11	1	L	7.2	+		–	–[b]	
	07/28/11	1	L	34.8	+	+	–	–	–
Acris gryllus	07/28/11	1	L	11.2	–		–		
(southern cricket frog)		2	L	12.3	–		–		
		3	L	12.1	–		–		
Hyla cinerea	07/28/11	1	L	17.6	–		–	–	
(green tree frog)		2	L	17.5	–		–	–	
		3	L	17.5	–		–	–	

[a]Although the quality of the tissues from the dead specimens was not ideal for describing histopathological changes, they were in a condition sufficient for verifying a high incidence of alveolate parasites
[b]PCR was conducted on paraffin-embedded, tissue-extracted DNA

frog (n = 3), using Rayon-tipped swabs (Dryswab Fine Tip MW113, Advantage Bundling SP/Medical Wire & Equipment Company). Samples were later analyzed at the San Diego Zoo (SDZ) Institute for Conservation Research, Amphibian Disease Laboratory, for molecular diagnostic (real-time PCR) testing following the methods of Pallister et al. (2007) for ranavirus, specifically using the conserved probe (CON) and primers, and the methods of Hyatt et al. (2007) for *Bd*. DNA was extracted from both swabs using DNeasy Blood and Tissue Kits (Qiagen) with spin columns, following the manufacturer's protocol, and frozen at –80°C. Additional liver samples were collected on 8 April and 28 July. These were taken from bullfrog, southern leopard frog, green tree frog, and gopher frog tadpoles and stored frozen at –80°C, until they were analyzed for ranavirus detection and confirmatory sequencing at FWRI and the University of Florida (UF). Liver samples (approximately 10 µg) were cut into small pieces, and DNA was extracted with a commercially available DNA extraction kit (QIAamp DNA Mini Kit, Qiagen). A portion (532 bp) of the viral major capsid protein (MCP) gene was amplified with the MCP gene primer no. 4 (forward), no. 5 (reverse; Mao et al. 1997), and the con-

served region of the MCP gene (Geng et al. 2011) on an MBS0.2S system (Thermo-Hybaid). Thermocycler conditions followed Robert et al. (2011). DNA products were separated on 1.0% agarose gel and visualized with ethidium bromide staining. The DNA band representing ranavirus was excised and isolated with a commercially available DNA extraction kit (QIAquick Gel Extraction Kit, Qiagen). DNA sequencing was performed at FWRI's genetics laboratory on a 3130xl genetic analyzer (Applied Biosystems) and confirmed by BLASTn analysis (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

General pond observations and environmental data

On 7 April, most of the larger bullfrog tadpoles were observed to be dead or moribund, while most leopard frog tadpoles appeared active and healthy. The only bullfrog tadpole that appeared healthy was the smallest one; all others were moribund or dead. A few small, apparently healthy gopher frog tadpoles were also present.

On 8 April, recently dead as well as older decomposed and decomposing tadpole carcasses were observed. Eastern mosquitofish *Gambusia holbrooki* were numerous and apparently healthy. The water in the pond proper appeared normal, but a green scum was present on the surface in a shallow area at the northeastern edge. A water sample taken from the vicinity of the scum mat was dominated by *Gonyostomum* sp. at more than 1.1×10^6 cells l^{-1}. Water quality results in a clear area of the pond at a depth of approximately 0.5 m were as follows: temperature 29.75°C; dissolved oxygen 6.48 mg l^{-1}; pH 7.35; conductivity 0.0064 mS cm^{-1}.

On 25 April, mostly healthy tadpoles of leopard frog, bullfrog, gopher frog, and southern toad *Anaxyrus terrestris* were observed. Only a few moribund or dead specimens were present, all of them leopard frogs. The only bullfrog tadpole found had well-developed hind legs. All of the gopher frog tadpoles collected appeared healthy, with 2 size classes present (ca. 25 and 55 mm total length). Southern toads had bred since the last visit, with tens of thousands of tadpoles observed.

On 28 July, the water level was substantially lower than on previous visits. Most ranid tadpoles had apparently metamorphosed, but a few bullfrog, leopard, and gopher frog (1 tadpole with hind legs) tadpoles were captured. Southern cricket frogs and green tree frogs had bred since the most recent visit, with tadpoles present. No dead or moribund tadpoles were observed.

Gross observations

Gross external observations of several bullfrogs collected at the ongoing kill event (7 and 8 April) demonstrated reddened (petechial) areas along the ventral surface from head to abdomen and a reddened swollen vent (Fig. 1), but not all specimens had these external lesions. Internally, the liver of bullfrog and southern leopard tadpoles exhibited a creamy mottled brown-pink color.

Histopathology

Of the tadpoles processed for histopathology from the 7 and 8 April samplings, 100% of bullfrogs (n = 7), 33.3% of leopard frogs (n = 6), and 100% of gopher frogs (n = 1) had been infected by the alveolate parasite. The liver tissue of dead tadpoles collected on 8 April exhibited deterioration due to post-mortem changes. Alveolate spores also exhibited some deterioration, but their condition in the moribund individual was still good (Fig. 2a). In July specimens (examined in fresh liver wet mounts and by histopathology), no bullfrogs (n = 7), leopard frogs (n = 2), green tree frogs (n = 3), or cricket frogs (n = 3) were positive for alveolate parasites. The 1 gopher frog (SVL = 34.8 mm) sampled was found to be infected (Fig. 2b,c), with the tissue in relatively good condition.

Fig. 1. *Lithobates catesbeianus*. Bullfrog tadpole with petechial haemorrhaging, swollen abdomen, and reddened vent

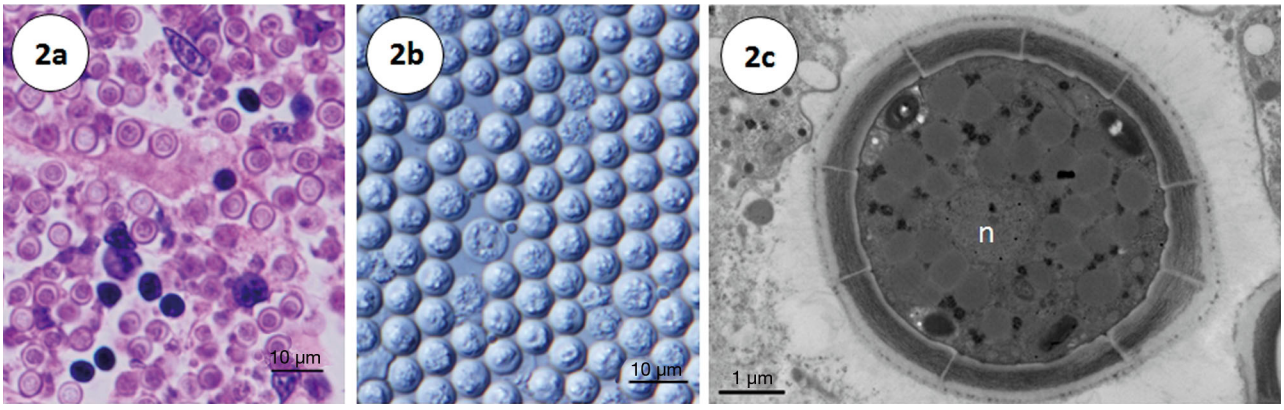

Fig. 2. *Lithobates catesbeianus* and *L. capito*. (a) Histological section of liver from a moribund bullfrog showing heavy parasitism by alveolate parasite spores (H&E staining). (b) Fresh, alveolate spores in wet mount from liver of gopher frog (Nomarski optics). (c) Transmission electron micrograph of gopher tadpole liver infected with alveolate pathogens. Mature spore stage with thick cell wall possessing 9 channels, n = nucleus

The alveolate parasite commonly had thick-walled, subspherical to spherical spores that exhibited a granular, purple to light-grey appearance in H&E-stained slides and in fresh wet mounts (Fig. 2a,b). Life history stage of the parasite appeared to be homogeneously consistent, with all bullfrog tadpoles and 1 leopard frog tadpole having mature parasite cells with endospores in the liver tissues. The livers of 1 southern leopard tadpole and 1 gopher frog tadpole contained more abundant parasite cells but no endospores.

Infections in bullfrogs examined in April were extensive, with spores present in multiple tissues: liver (parenchyma), kidney (glomeruli and interstitial tissue), spleen, gastrointestinal tract (submucosa), skeletal muscle (myosepta), mesentery (adipose tissue), heart (ventricle), brain (capillary lumen), thymus, eyes (outer basement membrane of retina), gills (filaments and lamellae), gonads, and cartilage. The liver was a primary target organ and had been almost entirely parasitized by alveolate spores, with little parenchymal tissue remaining (Fig. 2a). Because the liver tissues from the 8 April bullfrogs had been almost obliterated by alveolate parasite spores, it was impossible to assess whether there had been other, earlier pathological changes associated with ranavirus infection in these specimens.

Two leopard frogs negative for alveolate spores demonstrated focal inflammatory responses and multifocal areas of coagulative necrosis in the liver (Fig. 3), but the hematopoietic tissue was intact. No clearly discernible viral inclusion bodies were noted. One of these specimens was confirmed to be positive for FV3-like ranavirus by PCR from DNA extracted from paraffin-embedded tissue.

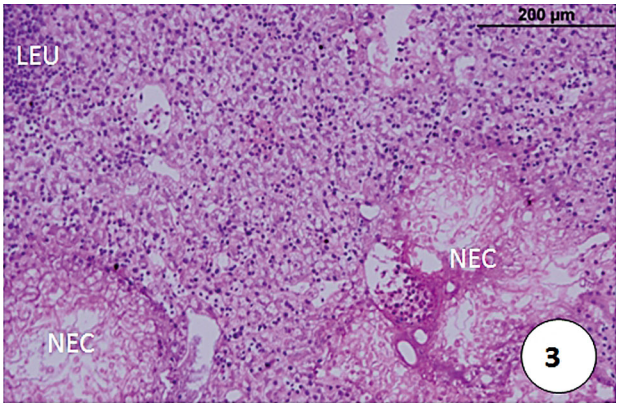

Fig. 3. *Lithobates sphenocephalus*. Histological section of liver, from a live-collected southern leopard frog, exhibiting multifocal liquefactive necrosis (NEC) and focal leucocytic infiltration (LEU). Note that the tadpole was negative for alveolate parasites by histopathology (H&E staining)

Other parasites detected in 8 April specimens were *Ichthyophonus*-like organisms in 57% (n = 7) of bullfrog skeletal muscle and 16.7% (n = 6) of leopard frog connective tissues; *Trichodina* in the gills of 40% of leopard frogs (n = 5); and opalinids in the intestinal lumen of 1 gopher frog tadpole. No bacteria were detected in the tissues of moribund and live tadpoles examined by histopathology. In all 6 dead bullfrog tadpoles collected on 8 April, rod-shaped bacteria were detected in thionin-stained tissues, especially the livers, but these bacteria were considered to have grown postmortem and are not further discussed.

Transmission electron microscopy

Most mature alveolate spores have a thick cell wall, whereas immature spores have a thin, 1-electron-dense layer of cell wall. In the gopher frog tadpole, spores had been phagocytosed by macrophages and melanomacrophage aggregates. The hepatocytes appeared degenerate, but the hepatic cords seemed to be in good condition. Most alveolate parasites (>90%) were mature spores with a single nucleus and a thick cell wall (exospore) covered by an outer glycocalyx-like layer possessing at least 9 channels, each located diagonally to the center of the spore (Fig. 2c). Spore sizes (n = 7 spores, reported in µm) were: mean ± SD exospore length, 5.8 ± 0.36, range 5.4–6.5; exospore width, 5.5 ± 0.21, range 5.2–5.7; endospore length, 4.2 ± 0.32, range 3.7–4.8; and endospore width, 4.1 ± 0.31, range 3.7–4.6. A few parasites at an earlier stage of development, which had a thin cell wall, were also found. Several mature spores and some immature cells with thin cell walls had coalesced and were surrounded by fluid-like material. A few spores had been phagocytosed by mononuclear cells. Isolated mononuclear leucocytes possessing active pseudopodia were found in the sinusoidal space and blood vessels. Viral particles were not observed in any of the specimens (Table 1).

Molecular diagnostics

Pooled liver tissue samples from bullfrogs and leopard frogs (7 April) were confirmed positive at SDZ for ranavirus DNA by quantitative PCR (qPCR). The liver tissue of an additional bullfrog tadpole (8 April) was confirmed to be FV3-like ranavirus-positive by 2 independent laboratories (UF and FWRI). Interlaboratory comparisons of the partial MCP gene sequences (496 bp after exclusion of primer binding sites) for bullfrog (UF and FWRI) and leopard frog (SDZ) were identical to each other and to the sequence for FV3 (GenBank AY548484). Three additional bullfrog tadpoles and 2 leopard frog tadpoles (8 April) were negative for FV3-like ranavirus. Twelve follow-up specimens (28 July; bullfrog, leopard frog, gopher frog, and green tree frog) were also negative for FV3-like ranavirus (the 3 cricket frogs were not tested by PCR).

From the 3 Bouin's-preserved tadpoles (8 April), we extracted DNA from the paraffin-embedded tissue block and tested for ranavirus by PCR. One leopard frog tadpole was positive for FV3-like ranavirus, while 2 other samples (leopard and gopher frog) were negative. All tadpoles tested for *Bd* by PCR were negative.

DISCUSSION

Based on this limited survey, we propose that the larval amphibian mortality event was likely caused by alveolate parasite infections that could, in some individuals, have been exacerbated by FV3-like ranaviruses, together with as yet undefined etiological factors. Increasing spring temperatures following an unusually cold winter in Florida may have also contributed to the April die-off. Pathogen infections in amphibians (Miller et al. 2008, Romansic et al. 2011) in conjunction with environmental and anthropogenic stressors (Gray et al. 2007, Gahl & Calhoun 2010) are increasingly being recognized as contributing to synergistic multifactorial etiologies in multispecies die-offs, which are an important aspect of disease ecology and amphibian conservation and health management. Based on the evidence presented here, alveolate parasites seem to be the most likely primary pathogen, while the role for FV3-like ranavirus in the die-off remains equivocal.

Alveolate parasite-related amphibian mortalities and morbidities have been documented in several locations in northern and central Florida in southern leopard frog, gopher frog, American bullfrog, and southern cricket frog (Dodd et al. 2004, Rothermel et al. 2008). First reported in New Hampshire (Green et al. 2003), such alveolate-associated amphibian mortalities were subsequently recorded in Mississippi, Georgia, Virginia, Alaska, Minnesota, North Carolina, and Maine, USA (Gahl 2007, Cook 2009). The spore size of the Florida alveolate parasite noted here by TEM (diameter of exospore 5.8 µm and endospore 4.2 µm) falls into the range of other reports (Davis et al. 2007, Cook 2008, 2009, Jones et al. 2012). Presumably, the channel-like structures that we and Jones et al. (2012) observed in the outer glycocalyx-like covering are the same as those observed by scanning electron microscopy in spores described as having a polyhedral surface (Fig. 2C in Davis et al. 2007). The pathology of the alveolate pathogen can be severe, and infections are threatening the federally endangered Mississippi gopher frog *Lithobates sevosus* (Cook 2008, 2009). Such pathogenicity and disease risk raise concerns regarding the extent to which this parasite affects Florida gopher frogs, which are designated as a species of greatest conservation need (Florida Fish and Wildlife Conservation Commission 2005).

To our knowledge, apart from 1 unpublished report (M. Gray pers. comm.), FV3-like ranavirus has not been associated with amphibian die-offs in Florida. Throughout the rest of North America, FV3 and FV3-like viruses have been identified as the cause of widespread amphibian mortality events (Chinchar 2002, Green et al. 2002, Converse & Green 2005, Fauquet et al. 2005, Greer et al. 2005, Harp & Petranka 2006, Miller et al. 2007, Duffus et al. 2008, Torrence et al. 2010, Brunner et al. 2011, Lesbarrères et al. 2012). In Florida, FV3-like viruses have been reported from a gopher tortoise *Gopherus polyphemus* and a Florida box turtle *Terrapene carolina bauri* (Johnson et al. 2008) but not from amphibians. FV3 and FV3-like viruses are generally globally widespread among anurans (Chinchar et al. 2011, Lesbarrères et al. 2012). Further, Ranidae (comprising all species affected in this reported mortality event) are more susceptible than are other amphibian families to FV3-like viruses (Hoverman et al. 2010, 2011).

Of growing concern globally is the indication that these viruses have caused significant epizootics in a variety of reptile and fish species (Chinchar et al. 2009, Gray et al. 2009b, Lesbarrères et al. 2012). Whether amphibian, reptile, and fish populations transfer these viruses among one another remains an important unanswered question, but the possibility broadens the concern for ranavirus risk assessment and disease management. Another complication is that ranavirus can be widespread in amphibian populations without causing any obviously associated disease or mortalities (Miller et al. 2009, Uyehara et al. 2010, Hoverman et al. 2012), and FV3 has been considered minimally pathogenic (Majji et al. 2006). Further, in the asymptomatic carrier form, host populations may also differ in their responses to infection (Robert et al. 2011). Our histopathology findings for FV3-infected southern leopard frogs were inconsistent with previous descriptions of FV3 (Gray et al. 2009b), in which the hematopoietic tissue has usually been necrotic (Daszak et al. 1999, Greer et al. 2005) and infections appeared to be subclinical.

For bullfrogs, the extensive alveolate parasite infections suggest that they likely had a stronger role than other potential pathogens in the mortality event, although it was impossible to identify the parasites as primary pathogens given the co-occurrence of FV3-like ranavirus in some specimens. Possibly, due to their age or parasite tolerance, older bullfrogs were the most intensely infected species. Bullfrog tadpoles had hatched the previous summer, whereas leopard and gopher frog tadpoles were less than 3 mo old in April. It was not clear whether smaller, younger gopher frog tadpoles were affected; if so, they might have been overlooked due to sinking and rapid decomposition. The high incidence of spore infections in bullfrogs with homogeneously mature stages in multiple organs, especially in the liver tissue, supports previous reports (Green et al. 2003, Davis et al. 2007, Cook 2008, 2009). Gahl (2007) also found infections during the summer in Maine, when larvae were nearing metamorphosis. Presumably most of the premetamorphic bullfrog tadpole specimens we examined in April were in latent terminal stages of infection, and once the disease had progressed that far, it is likely that it would be fatal. Older, potentially recovered bullfrogs were not located in July, suggesting that all diseased tadpoles in the latent stages observed in April were either dead or had metamorphosed with reduced infections. This was further determined in that young-of-the-year bullfrogs collected 3 mo later were not infected, although gopher frog tadpoles (albeit a small sample) were infected with alveolate parasites. Cook (2008) observed that alveolate parasitism in most post-metamorphs had not persisted and that when it did, it was usually limited to the intestinal lumen. However, in an infected adult southern leopard frog in South Carolina, the skeletal muscle tissue was associated with granulomatous myostis (Jones et al. 2012). While alveolate infections in our study may have remained somewhat latent the previous year, with possible ongoing low-level chronic mortalities, an increase in infection was evidently triggered later, and a lethal threshold for parasitism had been reached by early April, contributing to a die-off in the pond.

The incidental protistan and fungal parasites we observed are commonly reported (Densmore & Green 2007). These parasites were found at low incidence, but together with the other pathogens present they may be of significance as additional stressors and may influence the overall health of amphibians in Pebble Lake. Although bacteriological isolation tests were not performed, no bacteria were histologically detected in tadpoles collected live or moribund. Thus, bacteria, such as *Aeromonas hydrophila* or other pathogens involved in red leg syndrome (bacterial dermatosepticemia, Densmore & Green 2007), can be ruled out as causative factors in this case.

The presence of live, apparently healthy fish and relatively normal water quality conditions did not indicate an overall environmental issue, but it is difficult to extrapolate from a one-time survey. The presence of algal scum, although at the time in only 1 area of the pond, may indicate eutrophic conditions,

but nutrient levels were not measured. The presence of *Gonyostomum* sp., a nuisance alga, is of interest in that it discharges mucilaginous strands upon contact and is associated with itching skin and other allergic reactions in recreational bathers (Cronberg et al. 1988). Although we are not aware of any documented evidence, this raphidophyte could have acted as a dermal irritant and stressor to tadpoles already debilitated by parasitism.

FV3-like ranavirus and alveolate parasites are easily transmitted on nets, boots, and other objects, but the former is destroyed by 3% Chlorox Bleach® at 1 min exposure (Bryan et al. 2009). These pathogens are highly pathogenic to amphibians when favorable environmental conditions prevail, especially for the alveolate parasites (e.g. determined by cycling of dry and rainy seasons). Although not a public health threat, it is advisable to minimize human traffic between Pebble Lake and other GHBSP ponds to reduce risk of transmission and spread to amphibians.

It is of interest for state agencies to further discuss the implications of these pathogens for wildlife management. Further, the confirmation of pathogenic alveolate parasites and detection of FV3-like ranavirus poses concerns for Florida's amphibian populations, which are already at risk from habitat loss, invasive species, and climate change. Further research is needed to evaluate the role of alveolate parasites and FV3 in amphibian mortality events in Florida.

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