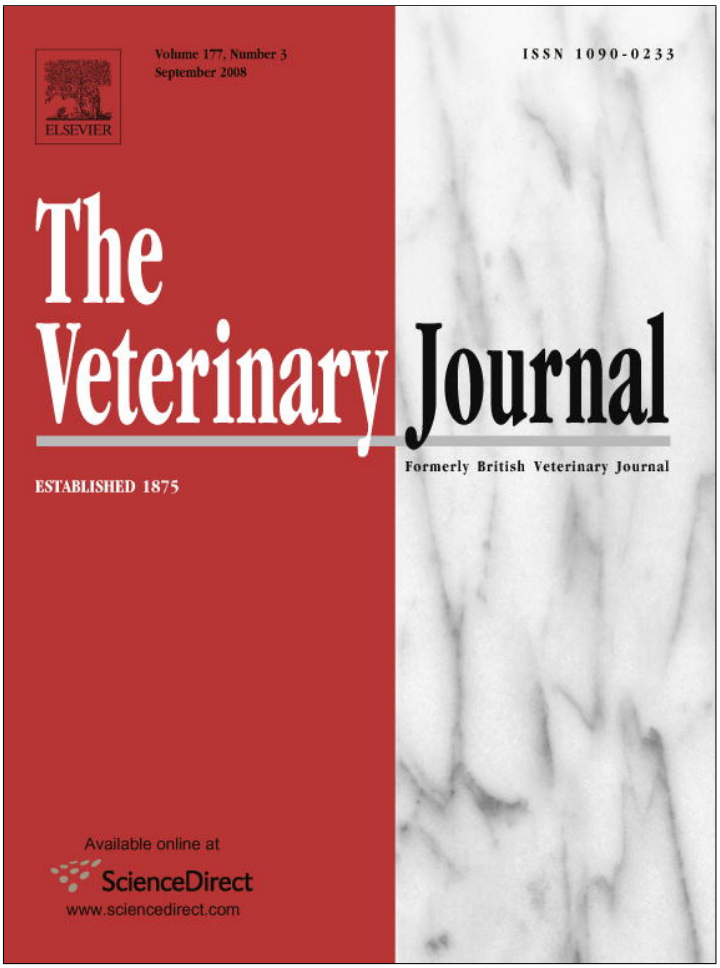

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Short Communication

Amphibian ocular malformation associated with frog virus 3

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Abstract

During an on-going amphibian ecology study, a free-ranging American bullfrog (*Rana catesbeiana*) metamorph was captured in a pitfall trap adjacent to a constructed farm pond at the Plateau Research and Education Center (PREC) on the Cumberland Plateau near Crossville, Tennessee, USA. Grossly, the right eye was approximately 50% the size of the left. Stereo and light microscopic examination revealed two granulomas within the orbit. Electron microscopic examination revealed virus particles scattered throughout one structure but mostly aggregated toward the center. Subsequent PCR and sequencing (GenBank accession Number EF175670) confirmed frog virus 3 (FV3). This represents the first report of a malformation in an anuran associated with FV3.

Keywords: Amphibian; FV3; Metamorph; Ocular malformation; *Rana catesbeiana*

Amphibian malformations may have various etiologies including parasites (e.g. *Ribeiroia*), UV exposure, chemical exposure and trauma (Blaustein and Johnson, 2003; Kiesecker, 2002). Ranaviral transmission in natural environments remains unknown, but based upon controlled studies, is thought to occur via water exposure, cannibalism and contact with sublethally infected individuals (Harp and Petranka, 2006). Frog virus 3 (FV3) is the type-species for *Ranavirus* and can be lethal to amphibians, targeting the renal tubular epithelium as well as resulting in multiorgan (liver, spleen) necrosis and lymphoid depletion (Converse and Green, 2005; Gantress et al., 2003; Miller et al., 2007; Robert et al., 2005). Viral inclusions or particles have been observed in necrotic areas of the kidney, liver, spleen, lymphoid and hematopoietic tissues of FV3-infected amphibians (Miller et al., 2007; Robert et al., 2005). Viral inclusions in other locations (e.g. the eye) have not been reported nor have ranaviruses been associated with malformations. We report here a case of FV3-associated ocular malformation in an American bullfrog (*Rana catesbeiana*) metamorph collected from a wetland accessed by cattle in Tennessee.

On 5 August 2005, an American bullfrog metamorph was captured in a pitfall trap at the Plateau Research and Education Center (PREC) on the Cumberland Plateau near Crossville, Tennessee, USA (36°00′59″N, 85°07′57″W). The trap was located adjacent to a constructed farm pond routinely accessed by beef cattle (on average, 53 cattle per 1 ha of wetland). Gross examination revealed an ocular malformation characterized by a right eye that was approximately 50% the size of the contralateral eye. The metamorph was humanely euthanased using benzocaine hydrochloride. Collection and euthanasia procedures followed approved University of Tennessee Institutional Animal Care and Use Committee protocol 1425.

The frog was formalin-fixed and subjected to clearing (Hanken and Wasserug, 1981) to rule-out *Ribeiroia ondatrae*, which is known to cause malformations in amphibians (Stopper et al., 2002). In brief, the skin and viscera were removed and the specimen was subjected to a series of acetic acid and ethanol solutions and Alcian blue and Alizarin red stains until the specimen was transparent except for cartilage and bone, which stained blue and red, respectively.

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Stereomicroscopic examination revealed two large (ca. 0.5 mm diameter) brown oval structures within the orbit of the right eye (Fig. 1A). One structure was removed and placed in McDowell and Trump's modified Karnovsky's fixative. It was then washed in buffer, dehydrated through a graded series of acetone solutions (70% to absolute acetone) and infiltrated with Spurr's resin. Sections 0.5 μm and 70 nm thick of the structure were cut on a Leica UC6 ultramicrotome for light microscopic and transmission electron microscopic (TEM) examination, respectively. Light microscopic sections were stained with toluidine blue and sections for TEM were stained with uranyl acetate followed by Reynold's lead citrate. Light microscopic examination revealed dense concentric fibrous tissue surrounding a central region of amorphous cellular and fibrillar material, consistent with a granuloma (Fig. 1B and C). Electron microscopic examination revealed virus particles with very dense nucleoids (maximum diameters of ca. 80 nm) scattered throughout the structure but present in aggregates toward the center (Fig. 1D).

The second structure was removed from the orbit and used for polymerase chain reaction (PCR). A heminested PCR targeting the major capsid protein gene of *Ranavirus* was performed (Kattenbelt et al., 2000), the PCR products resolved by gel electrophoresis, and the band isolated and sequenced. The PCR results were consistent with *Ranavirus* and sequencing (GenBank accession Number EF175670) confirmed FV3.

Based upon our findings, the structures were interpreted as FV3-containing granulomas and we suggest that the ocular malformation may have resulted from mechanical (space occupying) obstruction by the granulomas that

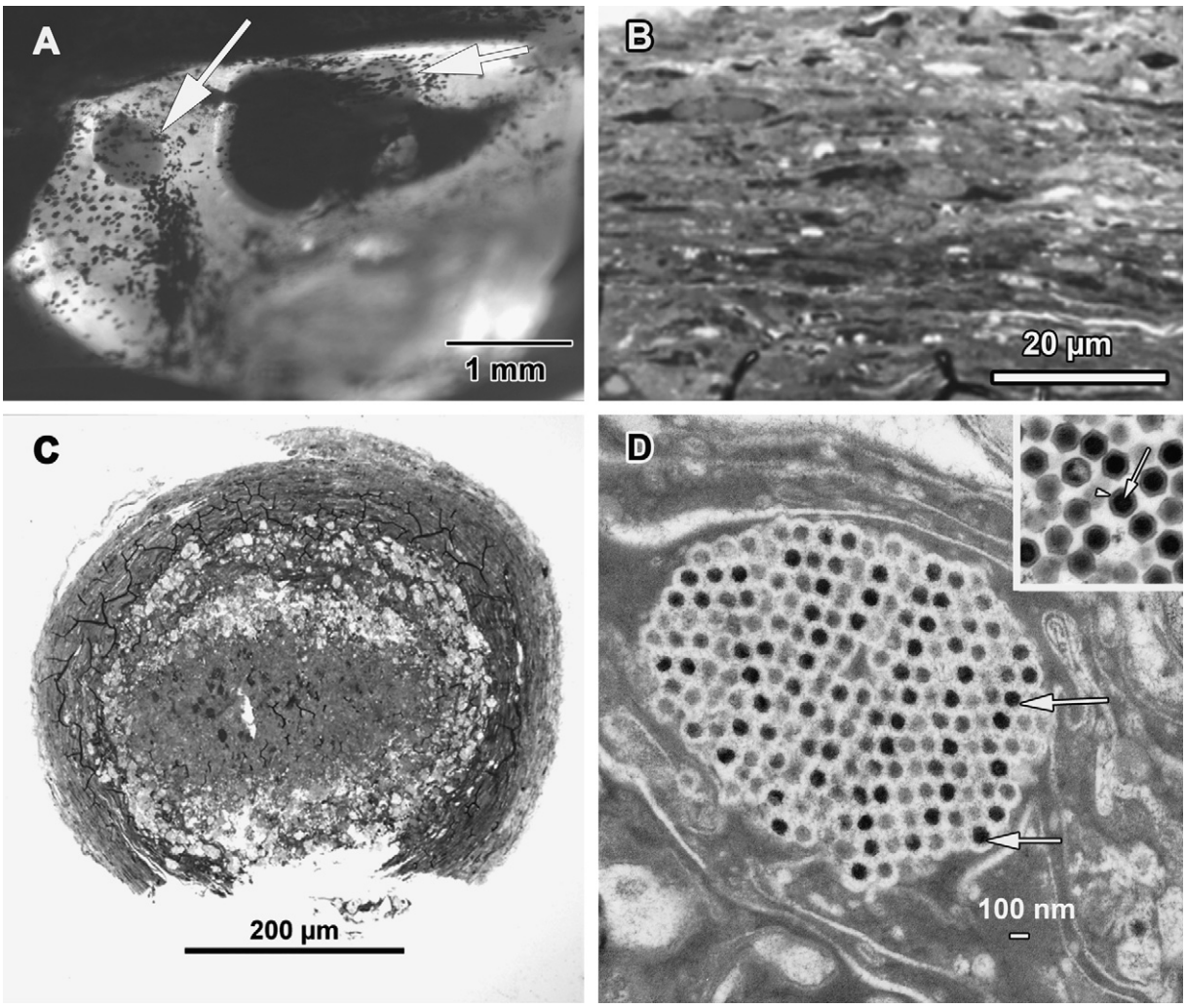

Fig. 1. Photomicrographs of a granuloma from the orbit of a malformed right eye of a bullfrog (*Rana catesbeiana*) metamorph collected from a cattle-access farm pond in Tennessee. (A) Stereomicroscopic view showing both granulomas (ca. 0.5 mm diameter each) within the orbit (translucent area). (B) and (C) Light microscopic view showing the concentric fibrous formation of the capsule (B). This capsule surrounded a central region of amorphous cellular debris (C). (D) Ultrastructure showing an intracellular viral aggregate within the center of the granuloma. Preservation was poor, presumably from the stringent processing necessary for the clearing protocol, and therefore only the viral cores (arrows) are evident. The inset is included for comparison and shows a viral aggregate (same magnification as larger view) from a cell culture (channel catfish ovary) inoculated with tissue from an FV3-infected bullfrog tadpole (GenBank accession Number DQ906048) collected from the same geographical location (Plateau Research and Education Center (PREC) on the Cumberland Plateau near Crossville, Tennessee, USA) as the metamorph. In this view the viral cores (arrow) are surrounded by the capsids and the internal membrane (arrowhead).

effectively restricted growth of the eye. We found subclinical FV3 infection via PCR and virus isolation in numerous bullfrog tadpoles collected at the same wetland (Gray et al., in press). Therefore, it is possible that this animal was infected with FV3 as a tadpole. We were unable to document viral infection within other organs of this metamorph, because the viscera were removed and discarded during processing to rule-out a trematode etiology. Alternatively, the granulomas may have been present for some other reason (e.g. parasite invasion) and the virus may have targeted fibroblasts within existing granulomas. It is possible that, in American bullfrogs, fibroblasts are an additional cell targeted by FV3. In fact, Gantress et al. (2003) found that in *Xenopus*, fibroblasts were targeted by FV3. If the same is true in bullfrogs, the virus may have targeted stromal cells within the eye, resulting in granuloma formation around the infected cells. Neither metazoan nor protozoan parasites were found on ultrastructural examination, despite documenting parasite-related malformations in metamorphs at this site previously (E.C. Burton, unpublished data).

This represents the first report of a malformation associated with FV3, and as such, suggests that FV3 should be included as a possible etiology when investigating amphibian malformations.

Acknowledgements

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