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PATHOLOGIC FINDINGS IN LARVAL AND JUVENILE ANURANS INHABITING FARM PONDS IN TENNESSEE, USA

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ABSTRACT: Amphibian populations are declining globally, yet general pathologic surveys for free-ranging amphibians are uncommon. Pathologic surveys are necessary to provide insight into the impacts of humans on emergence of pathogens in amphibian populations. During 2005, 104 American bullfrog (*Rana catesbeiana*) and 80 green frog (*Rana clamitans*) larvae and 40 green frog juveniles were collected from farm ponds in Tennessee, and complete necropsies were performed. Diagnostic testing included bacterial culture, virus testing, fecal parasite analysis, and histologic examination. Gross and histologic examination revealed that all individuals, except one bullfrog tadpole, could be classified as clinically normal. The clinically abnormal tadpole had swollen erythemic legs, and was positive for *Aeromonas hydrophila* but negative for *Ranavirus*. Parasites were common (43%) among specimens, with myxosporidium and trematodes most often noted. Commensal and opportunistic microorganisms were cultured from the tissues. *Ranavirus* was detected in 29% of individuals but generally not associated with significant histopathologic changes. Myxosporidia and *Ranavirus* coinfections occurred in 7 and 26% of green and bullfrog tadpoles, respectively, with the highest coinfection rate (83%) in bullfrog tadpoles during winter. Protozoans were most common in fecal examination. These data can serve as a baseline to evaluate the presence of clinical disease in larval and juvenile amphibians.

Key words: Anura, bacteria, histopathology, icthyophonus, Iridoviridae, parasite, *Ranavirus*, virus isolation.

INTRODUCTION

Amphibian populations are declining globally, and many factors have been implicated in die-offs, including habitat loss or fragmentation, toxins, and pathogens (Daszak et al., 2000; Whiles et al., 2006; Becker et al., 2007). Further, we hypothesized that anthropogenic stressors, such as cattle grazing wetlands, may make amphibians more susceptible to infections because of immune system suppression (Gray et al., 2007; Gray et al., 2007). To date, few studies (e.g., Green and Sherman, 2001; Green et al., 2002; Nieto et al., 2007) exist that report general histopathologic findings in an amphibian population, and these reports often have focused on a particular pathogen (e.g., *Batrachochytrium dendrobatidis*) or on identifying the etiologic agent of a die-off. Reports are needed on clinically normal free-ranging amphibians to provide a reference for clinical disease. In addition, investigations that focus on only one pathogen ignore the possibility of multiple pathogens interacting to induce a diseased state (Miller et al., 2007, 2008).

Larval and juvenile anurans are susceptible to a variety of pathogens, including internal and external parasites, viruses, bacteria, and fungi (Converse and Green, 2005; Green and Converse, 2005). External parasites reported in anurans include saprolegnia, leeches, and anchorworms, whereas internal parasites include various trematodes, cestodes, and nematodes (Poynton and Whitaker, 2001). *Ranavirus*, herpesvirus, adenovirus, and West Nile virus have been reported in amphibians, although West Nile virus has not been associated with disease (Green and Converse, 2005). Numerous bacteria may be cultured from anurans (Mauel et al., 2002), but *Aeromonas hydrophila* and *Mycobacterium liflandii* (e.g., a mycolac-

tone-producing mycobacteria) tend to be recognized most often because the former can be associated with red-leg disease (Green and Converse, 2005) and the latter is closely related to the human pathogen *Mycobacterium ulcerans* (Yip et al., 2007). Many species of helminths have been documented in anurans, and often they are considered incidental (Miller et al., 2004), but their presence may be an indicator of stress related to land use (Gray et al., 2007). Finally, numerous fungal and fungallike organisms (Green and Converse, 2005) and newly characterized pathogens (Davis et al., 2007) are known to result in catastrophic mortality of amphibian populations, and in the case of the fungus *B. dendrobatidis*, extinction of species (Berger et al., 1998). General pathologic surveys for amphibian populations are lacking, but are needed to provide insight into the impacts of human activity and the status of amphibian population health. We describe the pathologic findings in two anuran species collected from farm ponds in Tennessee, USA as part of two large-scale ecologic studies.

MATERIALS AND METHODS

Study site

Eight farm ponds located at the University of Tennessee Plateau Research and Education Center on the Cumberland Plateau near Crossville, Tennessee, USA (36°00′57″N, 85°07′56″W) were sampled for this study. The ponds ranged in size from 0.15 ha to 1.29 ha, were permanently flooded (<2 m in depth), and contained emergent shoreline vegetation (Burton et al., 2008). Four of these ponds were accessed by beef cattle (average density of 13 head/0.1 ha), whereas the other four ponds had not been accessed by cattle in over 10 yr (Burton et al., 2008).

Sample collection

Ponds were sampled for tadpoles with the use of dip nets and seines (Schmutzer et al., 2008); and juveniles were collected in pitfall traps (Burton et al., 2008). Sampling events for larvae occurred on 15 February (winter), 15 June (summer), and 14 October (fall) of 2005. Sampling events for juveniles were during the week of 15 June 2005. American bullfrogs (*Rana catesbeiana*) and green frogs (*Rana clamitans*) were chosen as target species because they were abundant at our study site and have a widespread geographic distribution in eastern North America. A total of 104 American bullfrog larvae and 80 green frog larvae were collected. Only American bullfrog larvae ($n=40$) were captured during the February sampling event. Forty larvae of each species were collected in June. In October, 40 green frog and 24 bullfrog larvae were collected. For juveniles, only green frogs ($n=40$) were collected.

At each sampling event, larvae or juveniles were collected alive, rinsed with sterile water, placed in individual glass jars, and transported to the University of Tennessee. Animals were humanely euthanized within 24 hr of collection by transdermal exposure to buffered benzocaine hydrochloride (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA). An internal coelomic swab was collected for microbial (bacterial and fungal) culture. A subset of select tissues (liver, kidney, intestine, skin) was collected and frozen for virus isolation and molecular testing. A subset of each tissue (brain, skin, eye, thymus, heart, lung, gill, spleen, intestine, kidney, and liver) was preserved in 10% buffered formalin for histologic analysis and molecular testing. Feces were collected for parasite analysis and electron microscopic examination for virus shedding. All collected tissues were transported to the University of Georgia, College of Veterinary Medicine, Veterinary Diagnostic and Investigational Laboratory, Tifton, Georgia, USA.

Diagnostic testing

Formalin-fixed tissues were routinely processed and embedded in paraffin. One or more 4-μm-thick sections were cut from each paraffin block and placed on glass slides. The slides were stained with hematoxylin and eosin, coverslipped, and viewed by light microscopy for histopathologic changes in tissues.

Swab and fresh tissue samples were processed for microbial (bacteria and fungi) and viral testing, respectively. For bacterial culture, swab specimens were inoculated into blood and MacConkey agar and incubated at 37 C. For fungal culture, swab specimens were inoculated into Sabourand dextrose agar and incubated at 29 C. All isolates were identified either by using an automated identification system (Sesititer, Trek Diagnos-

tic Systems, Westlake, Ohio, USA) or conventional biochemical testing. For virus isolation, fresh tissue specimens from all organs were used. In brief, a 10% tissue homogenate was made and filtered directly onto confluent monolayers of a variety of cell lines, including fathead minnow, white sturgeon skin, channel catfish ovary, and epithelioma papilloma cyprini cells. Cultures demonstrating viral cytopathic effect were harvested and random isolates verified by electron microscopy (EM) and molecular testing. Virus culture was examined by negative stain EM. Grids were examined for viruses or viruslike particles with the use of a Zeiss 900 transmission electron microscope at 12,000× or greater. Fecal samples were similarly processed to examine for viral shedding. For molecular testing, the polymerase chain reaction (PCR) was used for molecular analysis of the tissues (including virus culture). Paraffin-embedded and/or fresh tissues were used for amplification of the major capsid protein gene of *Ranavirus* with the use of a PTC-200 Peltier Thermal Cycler (MJ Research, Incline Village, Nevada, USA) and following the heminested procedure described by Kattenbelt et al. (2000). For fresh tissue, approximately 1 ml of a fresh tissue homogenate was centrifuged to pellet the tissue. The pellet was processed with the use of the QIAamp DNA mini kit (QIAGEN, Valencia, California, USA). Similarly, for paraffin-embedded tissue, genomic DNA was extracted following the protocol of Kattenbelt et al. (2000).

For fecal parasite analysis, feces were mixed with Sheather's sugar solution in a conical tube and the tube filled until a meniscus formed. A coverslip was placed on top of the tube and the tube was allowed to sit for approximately 1 hr. The coverslip was then placed on a glass slide and examined for parasite ova and oocysts with the use of light microscopy. Parasites observed during fecal examinations or in histologic sections were identified with the use of descriptions and keys in various texts, including Poynton and Whitaker (2001), Gardiner et al. (1998), Gardiner and Poynton (1999), Hoffman (1999), Mader (2006), and Jacobson (2007). Organisms observed in the fecal examination included food items, commensals, and potential pathogens. Fecal organisms that were consistent with those documented as pathogenic in amphibian fecal specimens submitted for diagnostic testing (Miller, unpubl.) or reported in the literature as potentially pathogenic to amphibians (Poynton and Whitaker, 2001) were identified with general classifications of ciliates, flagellated protozoans, and nematodes.

RESULTS

Gross findings

Individuals collected during the sampling events appeared grossly within normal limits. However, one tadpole collected during the June sampling event from a pond without cattle access had severe edema and erythema. The legs of this tadpole were erythemic and swollen two times normal size (Fig. 1).

Histology

Larvae: Renal changes were the most apparent histopathologic changes noted in the larvae. The most consistent finding in all larvae was the presence of parasites in the renal tubules, and associated eosinophilic (hyaline) droplet degeneration of the cytoplasm of the surrounding tubular epithelium (Fig. 2A). The parasites were 5–8-μm-diameter, round-to-oval spores containing two refractile spherical polar capsules, and they stained with acid-fast. These parasites were consistent with myxosporidia. Additionally, there were infiltrates of eosinophils and occasional increased granulopoiesis noted in the surrounding renal interstitium of more severely affected individuals (Fig. 2A). Myxosporidia were found in 13 (16%) green frog larvae and 43 (41%) bullfrog larvae. Vacuolar degeneration of the renal tubular epithelial cells (Fig. 2B) was noted in 19 larvae.

Overall, parasites (platyhelminths and myxosporidia) were observed most often in histologic sections of larvae from nonaccess ponds (46%) compared to cattle-access ponds (36%). However, for June, myxosporidia were observed more often in histologic sections of larvae from ponds with cattle access (50% versus 40% in nonaccess ponds). Identifiable platyhelminths were consistent with encysted trematodes and occasionally mild inflammatory cell infiltrates surrounded the cysts. Encysted trematodes observed in tadpole kidneys (Fig. 3A) were ca. 100 μm in diameter, and were consistent with

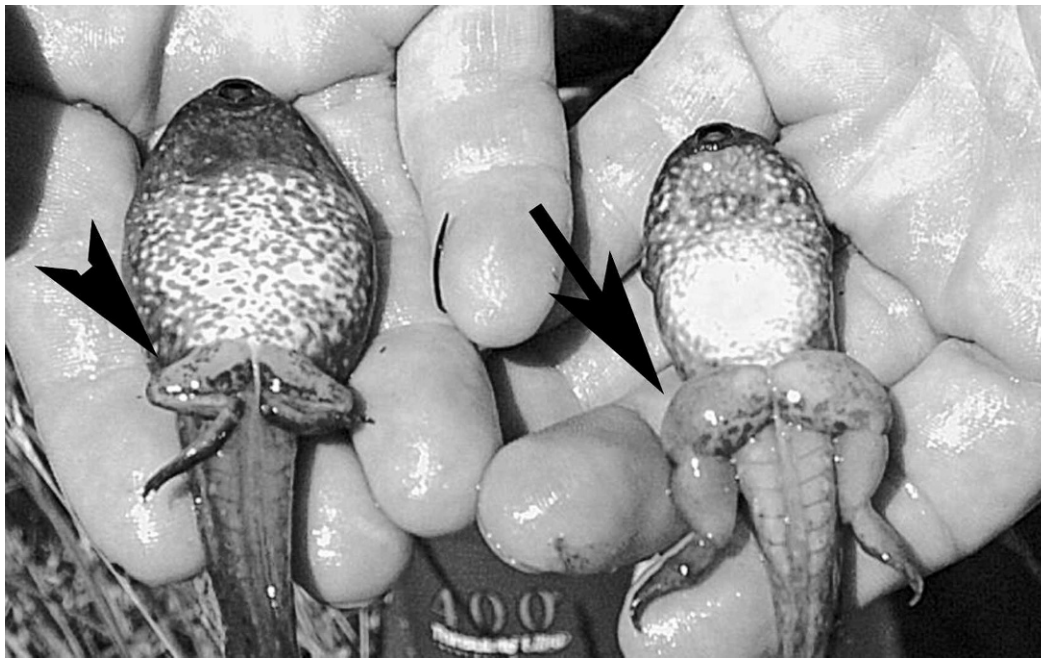

FIGURE 1. Photograph of an American bullfrog (*Rana catesbeiana*) larvae showing severe swelling in the limbs (arrow). A normal larva is shown for comparison (arrowhead).

echinostomatid metacercaria (Martin and Conn, 1990). Granulomas were observed in multiple organs, including the liver, spleen, and kidney. Occasionally, granulomas contained parasites (trematodes and cestodes) but often they contained no discernible agents (Fig. 3B).

Other changes noted were either infrequent or of minimum severity. Minimal changes were noted in the intestines, and these were consistent throughout individuals in all sampling periods. Myositis was noted in eight larvae, with 88% of these collected during June (Fig. 3C). Mild numbers of inflammatory cells were observed within the intestinal wall (Fig. 3D). Lymphoid depletion was occasionally noted in the thymus but was generally mild and rarely moderate (Fig. 3E). Hepatocellular swelling and vacuolar degeneration was a common finding in most larvae (Fig. 3B). Histologic evidence of *B. dendrobatidis* was not found.

Juveniles: Parasites and ichthyophonus were the primary findings noted in juvenile green frogs, and Burton (2007) reported that prevalence did not differ ($P \geq 0.23$) between cattle-access and non-access ponds. Metazoans (primarily platyhelminthes) were observed in 33% of the juveniles. One individual had encysted trematode metacercaria in the skin near the site of tail resorption (Fig. 4A). Encysted metacercaria also were observed in the kidney, skeletal muscle, and base of the lungs. Granulomas were occasionally observed (primarily in the liver), and were either associated with metazoan parasites or did not contain any discernible etiology. Ichthyophonus were present in varying numbers and had invaded the skin and underlying skeletal muscle of 8% of the juveniles (Fig. 4B). Myxosporidia were observed in 30% of the juveniles. As in the larvae, these parasites were present in varying numbers within the renal tubules, and generally the tubular epithelium was characterized by eosinophilic (hyaline) droplet degeneration. Eosinophilic infiltrates were present in the surrounding interstitium of only the more severely

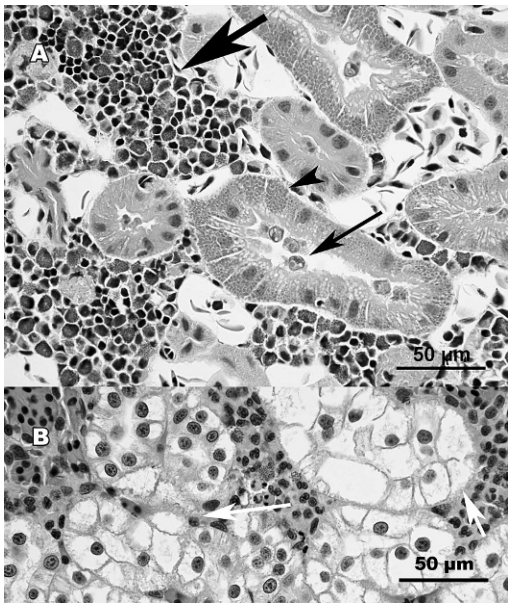

FIGURE 2. Photomicrograph of a hematoxylin and eosin–stained kidney from a green frog (*Rana clamitans*) larvae collected from a farm pond in Tennessee. A. Myxosporidal parasites were often present in the lumens of the renal tubules (small arrow) and the surrounding tubular epithelium generally contained eosinophilic (hyaline) droplets (arrowhead). Granulopoiesis is present in the surrounding interstitium (large arrow). B. Vacuolar degeneration of the renal tubular epithelium was often observed in larvae and may be associated with *Ranavirus*.

affected individuals. Similar to the larvae, only mild numbers of inflammatory cell infiltrates consisting of mononuclear cells and granulocytes were noted in the gastrointestinal lamina propria. Granulopoiesis was common in the liver and kidneys. Histologic evidence of *B. dendrobatidis* was not found in any juveniles.

Virus testing

Ranavirus was isolated on cell culture, confirmed by EM or detected by PCR, and sequenced. The results of these tests have been reported previously (Gray et al., 2007); however, a few coinfections were observed. Specifically, only one of the 13 green frog larvae infected with myxosporidia was also positive for *Ranavirus*, whereas 11 of the 43 bullfrog larvae infected with myxosporidia were coinfected with *Ranavirus*. Of the 43 bullfrog larvae with myxosporidia, six were collected during February and five of these (83%) were positive for *Ranavirus*. Although viral inclusions were not observed, 80% of the larvae with vacuolar degeneration of the renal tubules were positive for *Ranavirus*. The tadpole with grossly swollen legs was negative for *Ranavirus*. Particles consistent with *Iridoviridae* were not found in fecal specimens that were examined with the use of EM.

Microbial culture and fecal parasites

Numerous microorganisms were cultured from the tissues of the larvae and juveniles (Table 1). The tadpole with the swollen legs was positive for *A. hydrophila*. In general, cultured bacteria could not be related to histologic changes noted in the larvae and juveniles and thus it remains uncertain if they were commensals or pathogens in these animals.

Parasites found in examination of fecal specimens were not identified to species; however, ciliates most often appeared to be *Nyctotherus* and flagellated protozoans were most often trichomonad type (see Poynton and Whitaker, 2001). Nematode ova were most often identified as strongyloid and oxyurid (found in larvae) nematodes. The oxyurid ova observed in the larvae were presumed to be *Gyrinicola batrachiensis*, previously reported in many anuran larvae in North America (see Pryor and Greiner, 2004).

DISCUSSION

Similar to other wildlife species, pathogens may be common in clinically normal amphibians and only become pathogenic when pathogen numbers increase beyond a threshold for which the host can compensate. In addition, intrinsic or extrinsic factors (e.g., environmental, nutritional, immune function, coinfections) can negatively impact the ability of a host

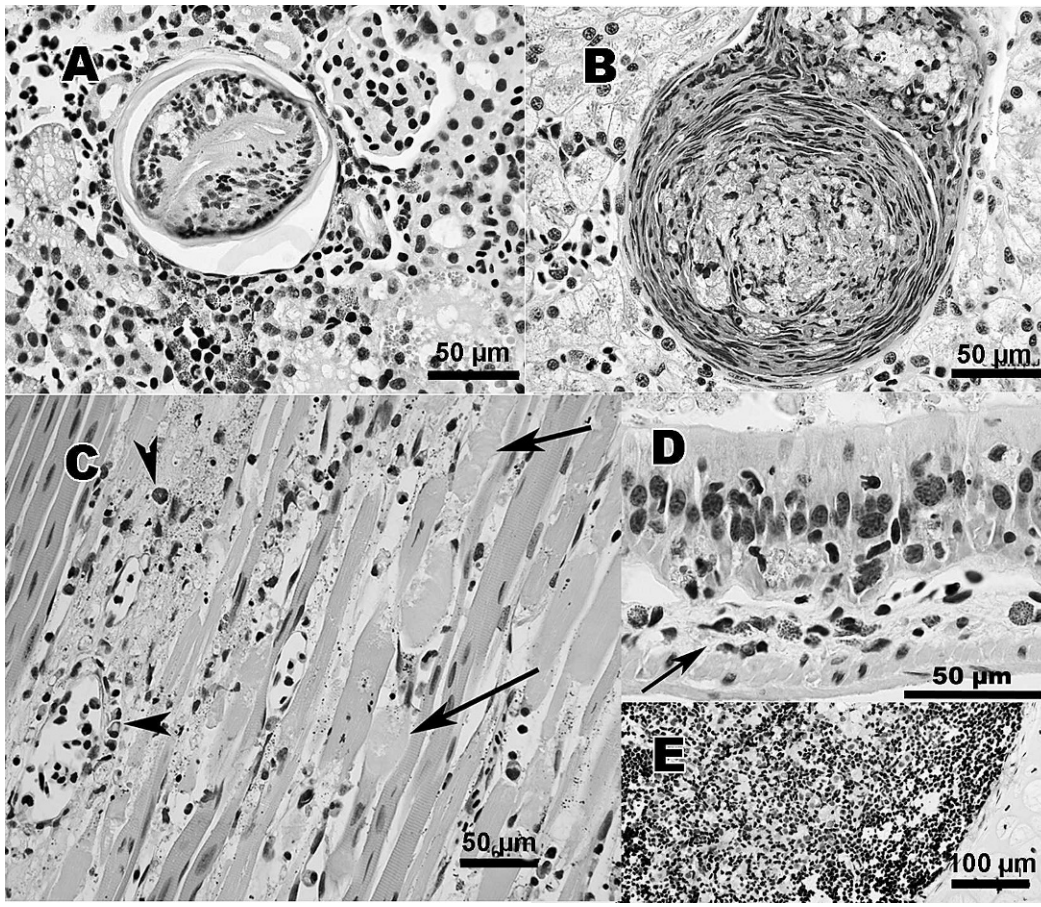

FIGURE 3. Photomicrograph of a hematoxylin and eosin–stained tissues from anuran larvae collected from farm ponds in Tennessee. A. Trematodes were often noted in various organs and occasionally were surrounded by inflammatory cells. B. Granuloma in the liver of an American bullfrog (*Rana catesbeiana*) larvae. Within the surrounding liver parenchyma, hepatocytes are swollen and vacuolated, which was commonly seen in most individuals. C. Myositis observed in the swollen legs of the American bullfrog larvae shown in Figure 1. Myofibers were often separated, swollen, and fragmented (arrows), and infiltrated by mixed populations of inflammatory cells (arrowheads). D. Mild numbers of mixed inflammatory cells (arrow) were occasionally seen in the intestines. E. Thymus of a green frog larvae showing moderate lymphoid depletion.

to fight infection. For example, *A. hydrophila*, *Chryseobacterium indologenes*, *Citrobacter braakii*, *Edwardsiella tarda*, *Flavobacterium* spp., and *Pseudomonas* spp. have been implicated as significant bacterial pathogens (Mauel et al., 2002; Forbes et al., 2004; Green and Converse, 2005) and trichomonad flagellates and strongyloid and oxyurid nematodes may be significant intestinal parasites. Although these organisms were documented in this survey, only *A. hydrophila* was associated with pathologic changes that appeared to impact the organism significantly (e.g., edema and erythema noted grossly). The causes for an amphibian to succumb to disease are complex, but likely include the interaction of anthropogenic and natural stressors, which are the topic of current investigations.

The swollen erythemic legs noted in one tadpole are consistent with red-leg disease. Although historically associated with *A. hydrophila*, *Ranavirus* also causes edema

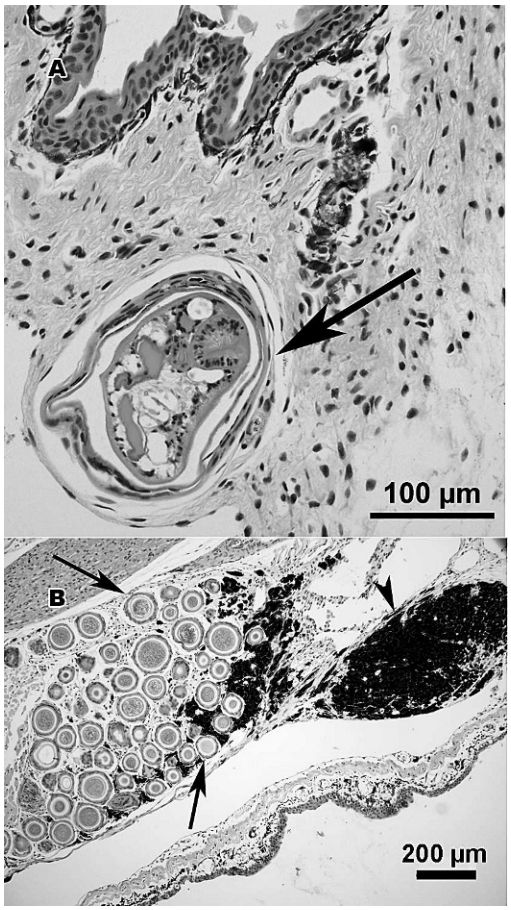

FIGURE 4. Photomicrograph of hematoxylin and eosin–stained skin sections from green frog (*Rana clamitans*) juveniles collected from farm ponds in Tennessee. A. Encysted trematodes (arrow) were commonly found and were observed in the skin of one juvenile. B. Ichthyophonus (arrows) was common and, when numerous organisms were present, they appeared to incite pigmentation in the surrounding tissue (arrowhead).

and erythema (Converse and Green, 2005; Miller et al., 2007). Virus isolation and molecular testing of this tadpole were negative, but bacterial culture was positive for *A. hydrophila*. Thus, *A. hydrophila* likely was the etiologic agent in this tadpole. The findings from this tadpole emphasize the need for complete diagnostic testing to identify causes of pathologic changes properly, because different pathogens may cause similar lesions.

In this investigation, the kidneys appeared to be the organ with the most significant changes, followed by the liver. The changes in the kidney may have been related to infection by parasites and *Ranavirus*. An association between parasite and *Ranavirus* occurrence was not observed in bullfrog and green frog larvae ($P \geq 0.19$; Schmutzer, 2007). However, a relationship existed between myxosporidia and *Ranavirus* during February, as 83% of bullfrog larvae with myxosporidia simultaneously tested positive for *Ranavirus*. It is possible that the cooler temperatures in winter may have served as an environmental stressor that predisposed the tadpoles to both pathogens (Raffel et al., 2006; Gray et al., 2007).

Vacuolar tubular degeneration of renal tubules is generally due to damage to mitochondrial membranes. The degree of degeneration observed in this study generally was mild and likely did not result in significant loss of renal function. This change may be seen in *Ranavirus* infection, which likely was the cause for this change in the juveniles, given that 80% of the juveniles with vacuolar tubular degeneration were positive for *Ranavirus*. However, the same was not found in the tadpoles, and many *Ranavirus*-negative tadpoles had vacuolar tubular degeneration.

Eosinophilic (hyaline) droplet degeneration was the type of epithelial cell degeneration observed in the renal tubules containing myxosporidia. Although not known to be specific for a particular etiology, hyaline droplets are generally associated with increased glomerular permeability (Maxie and Newman, 2007). Hyaline droplets are swollen lysosomes containing protein. This protein is meant to be returned to the general circulation as amino acids but becomes trapped if the circulation becomes saturated or the mechanism is impaired (Maxie and Newman, 2007). It is possible that the parasites interfere with this mechanism, and in severe infections, may result in a protein losing nephropathy. Given infection was in

TABLE 1. Bacteria isolated from intracoelomic swab specimens collected from American bullfrogs (*Rana catesbeiana*) and green frogs (*Rana clamitans*) at necropsy immediately posteuthanasia.

Bacteria	Type	Development stage
Achromobacter xylosoxidans	Aerobic	Larvae
Achromobacter xylosoxidanaerobics	Aerobic	Larvae, juvenile
Acinetobacter spp.	Aerobic	Larvae, juvenile
Acinetobacter baumanaerobicnii	Aerobic	Larvae, juvenile
Acinetobacter lwoffi	Aerobic	Larvae, juvenile
Aeromonas hydrophila	Facultative	Larvae, juvenile
Aeromonas sobria	Facultative	Larvae
Alcaligenes spp.	Anaerobic	Larvae
Agrobacterium radiobacter	Anaerobic	Larvae
Bacillus spp.	Facultative	Larvae
Bacillus cereus	Facultative	Larvae
Bacillus myroides	Facultative	Larvae
Burkholderia cepacia	Aerobic	Larvae
Chromobacterium violaceum	Aerobic	Larvae
Chryseobacterium spp.	Aerobic	Larvae
Chryseobacterium indologenes	Aerobic	Larvae
Chryseobacterium meningosepticum	Aerobic	Larvae, juvenile
Citrobacter braakii	Aerobic	Larvae
Comamonas acidovoranaerobics	Anaerobic	Larvae
Corynebacterium spp.	Aerobic	Larvae
Corynebacterium auris	Aerobic	Larvae
Delftia acidovoranaerobics	Aerobic	Larvae, juvenile
Edwardsiella tarda	Facultative	Larvae
Empedobacter brevis	Facultative	Larvae
Enterobacter species	Facultative	Larvae, juvenile
Enterobacter cloacaerobic	Facultative	Larvae
Escherichia coli	Facultative	Larvae
Flavobacterium spp.	Facultative	Larvae
Hafnia alvei	Facultative	Larvae, juvenile
Moraxella osloensis	Aerobic	Larvae
Myroides odoratus	Anaerobic	Larvae
Ochrobactrum anaerobicthropi	Aerobic	Larvae, juvenile
Oerskovia spp.	Anaerobic	Larvae
Oligella urethralis	Aerobic	Larvae
Panaerobictoea agglomeranaerobics	Anaerobic	Larvae, juvenile
Pasteurella spp.	Facultative	Larvae
Plesiomonas shigelloides	Anaerobic	Larvae
Pseudomonas spp.	Facultative	Larvae, juvenile
Pseudomonas stutzeri	Aerobic	Larvae
Pseudomonas aerobicruginosa	Aerobic	Larvae
Pseudomonas alcaligenes	Aerobic	Larvae
Pseudomonas fluorescens	Facultative	Larvae
Pseudomonas fluorescens-putida	Aerobic	Larvae
Pseudomonas mendocina	Aerobic	Larvae
Pseudomonas pseudoalcaligenes	Aerobic	Larvae
Psychrobacter phenylpyruvica	Anaerobic	Larvae
Sphingomonas paucimobilis	Facultative	Larvae
Staphylococcus epidermis	Aerobic	Larvae
Staphylococcus paucimobilis	Aerobic	Larvae
Vibrio spp.	Facultative	Larvae
Vibrio fluvialis	Facultative	Larvae

the renal tubules, the myxosporidia are most likely *Sphaerospora* (formerly Leptotheca) *ohlmacheri*. However, *Sphaerospora* are considered nonpathogenic to amphibians (Green and Converse, 2005); therefore, future investigations need to evaluate renal function changes due to the presence and numbers of *Sphaerospora*.

Considering that renal tubular epithelium is a target cell for *Ranavirus* (Gantress et al., 2003; Converse and Green, 2005; Robert et al., 2005), it is logical to assume that *Ranavirus* may be more likely to invade damaged cells. Such a relationship was expected in organisms with hyaline droplet degeneration of the renal tubules (e.g., observed with myxosporidia) but not found. Future study might investigate the ability of *Ranavirus* to infect individuals with preexisting renal tubular epithelium changes to shed light on the pathogenesis of *Ranavirus* infection.

Some degree of vacuolar degeneration was observed in the livers of all animals. In lower vertebrates, vacuolar degeneration of hepatocytes may be a nonspecific finding or associated with inanition (Green, 2001) or high-lipid diet (Roberts, 2001). However, in cases of inanition, this may be indicative of normal cyclic nutritional phases rather than a pathologic change (Roberts, 2001). Similarly, granuloma formation in lower vertebrates is a common response to various agents, including bacteria, fungi, parasites, foreign bodies, and occasionally viruses. Thus, unless discernible agents were observed within the granulomas, the cause of the granuloma was unknown.

Herein we report histopathologic findings from clinically normal free-ranging amphibians collected from ponds with no previously reported history of mass amphibian morbidity or mortality. These data serve as a baseline from which to evaluate clinical disease and a base upon which to build future studies. Thorough surveys of amphibian health are necessary to understand the impact of disease on amphibian populations. This is a critical need in times of declining populations and species extinction.

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