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PRESENCE OF AMPHIBIAN CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS* AND OTHER AMPHIBIAN PATHOGENS AT WARM-WATER FISH HATCHERIES IN SOUTHEASTERN NORTH AMERICA

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Abstract.— **We conducted health screenings for infectious diseases of amphibians at four warm-water fish hatcheries and one National Wildlife Refuge in the southeastern United States. We confirmed the presence of *Batrachochytrium dendrobatidis* (amphibian chytrid fungus) in *Rana catesbeiana* (American Bullfrog) from one hatchery, as well as potentially new species of microsporidian and myxozoan parasites infecting all 10 amphibian species sampled. Viruses were not found in tissue cultures or histologically. Tens of thousands of individual amphibians may breed in outdoor warm water fish-rearing ponds. Although there have been no reports of disease outbreaks at the sampling sites, the potential transmission of infectious diseases between amphibians and fishes could have serious consequences for amphibian populations at recipient sites.**

Key Words.—amphibian larvae; *Batrachochytrium dendrobatidis*; disease spread; emerging disease; fish hatchery

INTRODUCTION

Amphibian populations and species are declining or disappearing from many regions throughout the world (Stuart et al. 2004). No single cause has been demonstrated, although a number of emerging infectious diseases have been suggested as primary etiologic agents (Berger et al. 1998; Daszak et al. 2003; Lips et al. 2006). Several factors, including climate change, parasite infestation or compromised immune systems may interact locally or regionally to threaten species and populations (Carey and Bryant 1995; Parris and Beaudoin 2004; Pounds et al. 2006). Still, the disease model of amphibian decline may not be universally applicable (Daszak et al. 2005; McCallum 2005).

The impacts of disease can devastate anuran populations, and declines due to disease, particularly amphibian chytrid fungus (*Batrachochytrium dendrobatidis,* "BD") and ranaviruses (Berger et al. 1998; Chinchar 2002), are well documented (Daszak et al. 2003; Kiesecker et al. 2004). In addition to the better-known fungi and viruses, an undescribed *Perkinsus*-like organism also has had serious localized effects on populations of ranid frogs in southeastern North America (e.g. *Rana sevosa* in Mississippi, various Florida species; unpublished data).

In North America, warm water fish hatcheries supply stock for sport fishing, ecological restoration, and endangered species management. Several million fish may be transported across multiple regions and river drainages in a single restocking event. For example, in 2004 three million bluegill (*Lepomis macrochirus*), originating from Orangeburg National Fish Hatchery (NFH), South Carolina were stocked at Harris Neck National Wildlife Refuge (NWR), Georgia as food for a nesting colony of endangered wood storks. This stocking in 2004 transported fish from the upper coastal plain across the Savannah River to the lower coastal plain, and may be responsible for mixing different larval phenotypes of *Rana catesbeiana* at Harris Neck (Dodd and Barichivich 2007).

Our objective was to determine whether diseases known to have detrimental effects on amphibians (ranavirus, BD, mesomycetozoa, protozoa and helminths) are present in amphibian larvae living in warm-water fish hatcheries in the southeastern United States. We further examined hatchery records to assess the extent to which amphibian larvae have been transported throughout various regions and potentially contribute to spreading emerging infectious diseases.

MATERIALS AND METHODS

National fish hatcheries (Warm Springs, Georgia; Orangeburg, South Carolina; Welaka, Florida; Edenton, North Carolina) were sampled in June 2005, and Harris Neck National Wildlife Refuge, Georgia, was sampled in July 2005. Tadpoles were collected using dip nets and sent alive to the USGS National Wildlife Health Center for screening within 24-hr of capture. Amphibians dead on arrival were necropsied the same day as received. Live larvae were euthanized in 1:500 solution of methanesulfonate salt (Sigma Chemical Co., St. Louis, Missouri). External and internal examinations were performed using a dissecting microscope equipped with a digital camera. Euthanasia, necropsies, viral and bacterial cultures, parasite examinations and histology were performed as detailed in Green and Muths (2005).

We examined 152 anuran larvae of 10 species from the four national fish hatcheries and National Wildlife Refuge. Ten larval American Bullfrogs (*Rana catesbeiana*) captured at Harris Neck NWR were included because these larvae likely were transported to the refuge from Orangeburg NFH with stocked bluegill fish (Dodd and Barichivich 2007).

Samples of the liver, mesonephros and spleen were pooled for virus cultures and isolations were carried out on fathead minnow cell lines (Docherty et al. 2003). Samples of liver, urine, mesonephros, bile, spleen or lung were examined for aerobic bacterial cultures. A 2 mm × 3 mm segment of cloaca and a 2-4 mm segment of distal toe were used for fungal cultures. Tissues and body fluids for routine aerobic bacterial cultures (ca. 1 mm^3) were placed into vials of 2 ml tryptic soy broth with glycerine (TSB) and incubated at room temperature (25-27°C). Cultures for Salmonella spp. were prepared in Rappaport-Vassiliadis R10 broth (Becton, Dickinson and Co., Cockeysville, Maryland,

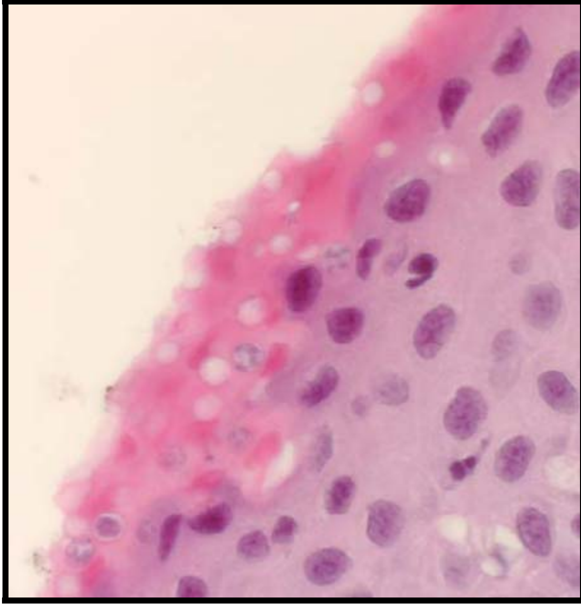

FIGURE 1. Photomicrograph of 4912-156, larval *Rana catesbeiana*, jaw sheath. The clear and pale blue circular vacuoles in the surface (red) cells at left are thalli of *B. dendrobatidis*. Hemotoxylin and Eosin stain, 400x.

USA). Subcultures were performed on 5 % sheep blood agar plates and eosin methylene blue plates. Biochemical identifications of bacterial isolates were performed using the Biolog MicroStation Microbial Identification System (Hayward, California, USA). Fungal cultures were performed on Sabouraud dextrose agar plates with chloramphenicol and tetracycline (Hardy Diagnostics, Santa Maria, California, USA). Fungal isolates were identified morphologically by features of their hyphae and spores.

Parasites were identified to phylum during necropsies. Representative helminths and insects were archived in hot buffered formalin or 70 % ethanol. Identifications to genus were based on external morphology of live helminths at a dissecting microscope, tissue location in the host, and histological features.

Portions of ventral skin, digits, heart, liver, lung, spleen, mesonephros, stomach, intestine, pancreas, urinary bladder and gonads were fixed in 10 % buffered neutral formalin, processed routinely, sectioned at 5 microns, and stained with hematoxylin and eosin. Portions of liver, ventral skin, muscle, lung and mesonephros were placed in 1.8 ml cryovials and archived at –70°C at the National Wildlife Health Center (Madison, Wisconsin, USA).

RESULTS AND DISCUSSION

We found oral chytridiomycosis in 4 of 5 *R. catesbeiana* from Warm Springs NFH (Fig. 1). This pathogenic fungus was not detected in histological examinations in any other species from any other hatcheries. Although tadpoles of several species from all four hatcheries had macroscopic changes in their jaw sheaths and toothrows (i.e., loss of black pigment or depigmentation) suggestive of amphibian chytridiomycosis, the pathogenic chytrid fungus was not detected histologically. Oral chytrid infections were not detected in 15 other tadpoles of 3 species (*Bufo fowleri*, *Hyla cinerea*, and *Rana clamitans*) from the same pond at Warm Springs NFH. The size of the chytrid infected bullfrog tadpoles (6.0 - 7.7 g body mass) suggests they had over-wintered in the pond during the winter of 2004-2005, whereas the tadpoles of the other 3 species probably resulted from eggs deposited in the spring 2005.

We found a previously unreported microsporidian infection of amphibians in 4 tadpoles from Welaka NFH. The infections occurred in the brain, spinal cords, spinal ganglia and renal glomeruli of 3 of 5 *Hyla gratiosa* and 1 of 48 *Rana sphenocephala*. Tadpoles of *H. cinerea* and *Hyla squirella* from the same pond had no evidence of microsporidia. The size of the microsporidial cysts and their tropism for neurons suggests they may belong to the genera *Glugea* or *Spraguea*, the latter previously reported only from marine fish (*Lophius* spp.). The microsporidia in these tadpoles may be a new species or perhaps were transmitted from fish in the pond. Whether this is an endemic or epizootic disease of fish and amphibians, or whether the infection is limited to certain species of amphibians, is unknown.

A variety of internal helminthic parasites and external ectoparasites were found in the tadpoles from all four fish hatcheries. Internal parasites consisted mostly of the common tadpole pinworm *Gyrinicola batrachiensis* and multiple species of encysted immature trematodes (metacercariae); the significance of metacercariae in amphibians is usually negligible. Protozoan ectoparasites of the innocuous genera *Epistylis* and *Trichodina* were found in the chambers of the mouth, pharynx and gills and on the ventral skin. About 10% of *R. sphenocephala* from Welaka NFH had *Gyrodactylus* sp. (a monogean trematode) on the skin of their bodies; these parasites were observed on submerged live anesthetized larvae only under a dissecting microscope.

Metacercariae of the parasite, *Ribeiroia*, and a new undescribed microsporidian parasite of the brain, spinal cord and ganglia are infectious and may cause morbidity, mortality or malformations in amphibians. These diseases could have adverse impacts on free-living amphibian populations should infected hatchery animals be released into naïve amphibian populations. In addition, the unidentified metacercariae in the thyroids of *R. catesbeiana* from Harris Neck may be significant because an infection of larval thyroids could result in hypothyroidism and impaired metamorphosis.

An unidentified myxozoan parasite, *Myxidium* sp., was found in at least one amphibian from all four hatcheries and Harris Neck. Infestations of the gall bladders were observed histologically in 36 tadpoles of 7 species (*Bufo terrestris, H. cinerea, H. gratiosa, H. squirella, R. catesbeiana, R. clamitans, R. sphenocephala*), but there was no histological evidence of any myxozoans in the brains and skulls of amphibian larvae. Two common and geographically widespread myxozoan parasites are found in larval and post-metamorphic amphibians: *Myxidium* spp. in the gall bladder and *Sphaerospora ohlmacheri* in the mesonephroi. The taxonomy of *Myxidium* is currently undergoing revision (Jirku et al. 2006), and other species may be identified from amphibians. Illness and death have not been reported from infestations by these myxozoa, but the impact on amphibians of the initial unidentified infective stage of these organisms is unknown. The widespread presence of *Myxidium* sp. and the absence of *S. ohlmacheri* suggest these species may use different final invertebrate hosts, and that only the final host for *Myxidium* spp. was present at the hatcheries and refuge.

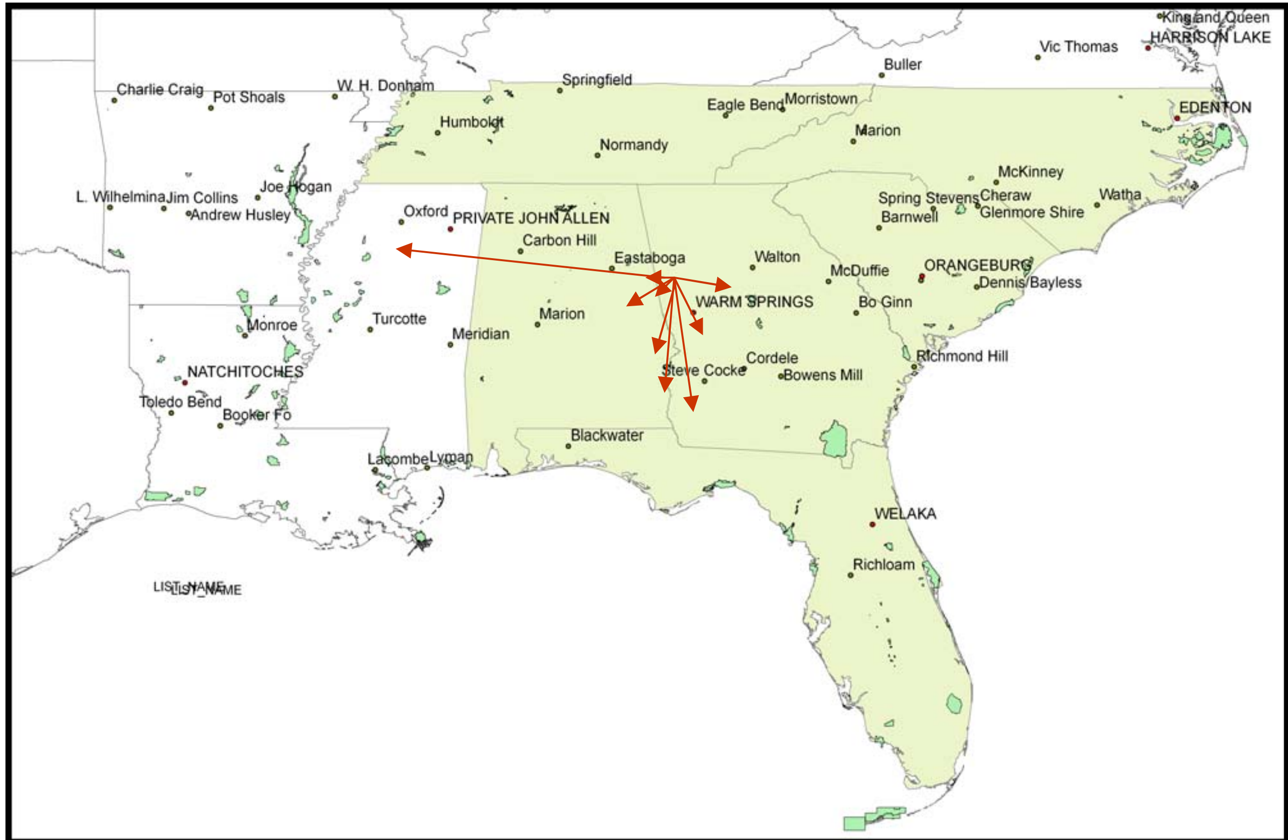

FIGURE 2. Locations where fishes (mostly striped bass *Morone saxatilis*) were moved from Warm Springs National Fish Hatchery to recipient sites, 2002-2005. The total number of fishes stocked was > 1.4 million in 54 shipments.

We found no evidence of viruses in either cultures or histological sections. No pathogenic bacteria were isolated in cultures of the internal organs. The bacterium *Aeromonas hydrophila* was isolated from the intestines of 10 of 21 tadpoles; this bacterium was isolated from at least one tadpole from each of the 4 fish hatcheries. No significant protozoan or mesomycetozoan infections were detected in any larval amphibians.

The only serious and lethal amphibian disease we found in amphibians from the four hatcheries and refuge was oral chytridiomycosis. The pond from which these bullfrog tadpoles were collected, including its amphibian and fish fauna and water, should be considered contaminated with this disease agent. Although two other serious and lethal infectious diseases of amphibians, ranaviruses and a *Perkinsus*-like organism, were not found during this study, this does not mean that these ponds will remain free of infectious diseases in the future.

When fish are stocked, a host of other aquatic invertebrates and vertebrates, including tadpoles and salamander larvae, may be included in the shipments. We have observed large numbers of anurans breeding in the large outdoor fish rearing ponds; these populations may produce tens of thousands of tadpoles annually. Fish shipments generally are not screened for amphibian larvae, and we know of no hatcheries screening for amphibian diseases. Where attempts are made to remove non-target vertebrates and invertebrates from shipments, the water containing these organisms is discharged into surrounding wetlands as fish rearing ponds are drained. Releasing or discharging large numbers of amphibian larvae of unknown health status into streams and wetlands throughout a region (Fig. 2) could spread parasites and pathogens quickly having serious consequences to resident amphibian populations. BD may remain viable and infectious for 7 days in contaminated water, thus providing opportunities for disease transmission without direct contact with infected amphibians (Johnson and Speare 2003).

The spread of amphibian disease agents has been linked to the spread of nonindigenous species, particularly *R. catesbeiana* (Mazzoni et al. 2003; Hanselmann et al. 2004; Garner et al. 2006), a species widely cultivated and transported from farms in the southeastern United States throughout the world**.** Even where bullfrogs and other anurans occur naturally, moving infected larvae and water throughout an area during fish stocking could spread highly pathogenic amphibian disease agents. Improved monitoring and screening of these diseases at fish hatcheries might help to reduce this threat.

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