Avian Vacuolar Myelinopathy Linked to Exotic Aquatic Plants and a Novel Cyanobacterial Species

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ABSTRACT: Invasions of exotic species have created environmental havoc through competition and displacement of native plants and animals. The introduction of hydrilla (*Hydrilla verticillata*) into the United States in the 1960s has been detrimental to navigation, power generation, water intake, and water quality (McCann et al., 1996). Our field surveys and feeding studies have now implicated exotic hydrilla and associated epiphytic cyanobacterial species as a link to avian vacuolar myelinopathy (AVM), an emerging avian disease affecting herbivorous waterbirds and their avian predators. AVM, first reported in 1994, has caused the death of at least 100 bald eagles (*Haliaeetus leucocephalus*) and thousands of American coots (*Fulica americana*) at 11 sites from Texas to North Carolina (Thomas et al., 1998; Rocke et al., 2002). Our working hypothesis is that the agent of this disease is an uncharacterized neurotoxin produced by a novel cyanobacterial epiphyte of the order Stigonematales. This undescribed species covers up to 95% of the surface area of leaves in reservoirs where bird deaths have occurred from the disease. In addition, this species is rare or not found on hydrilla collected at sites where AVM disease has not been diagnosed. Laboratory feeding trials and a sentinel bird study using naturally occurring blooms of cyanobacteria on hydrilla leaves and farm-raised mallard ducks (*Anas platyrhynchos*) induced the disease experimentally. Since 1994 AVM has been diagnosed in additional sites from Texas to North Carolina. Specific site characteristics that produce the disjunct distribution of AVM are unknown, but it is probable that the incidence of this disease will increase with the introduction of hydrilla and associated cyanobacterial species into additional ponds, lakes, and reservoirs. © 2005 Wiley Periodicals, Inc. Environ Toxicol 20: 348–353, 2005.

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INTRODUCTION

Hydrilla has been introduced into the United States from India and southern Asia as a plant used in freshwater aquariums. This invasive plant has infested freshwater lakes and rivers from California to Maine (McCann et al., 1996).

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Other recently introduced invasive aquatics include *Egeria densa* (Brazilian elodea) and *Myriophyllum spicatum* (Eurasian water milfoil). These invasive weeds have disrupted ecosystems by excluding native vegetation and altering nutrient chemistry, oxygen dynamics, and advective flow (Pesacreta, 1988; Aulbach-Smith and deKozlowski, 1996; McCann et al., 1996). In addition to having a direct impact, invasive weeds are hosts to a diverse and abundant community of epiphytic algae. This epiphytic assemblage is often dominated by cyanobacteria (blue-green algae), prokaryotes with numerous toxin-producing species.

Cyanotoxins are secondary metabolites of cyanobacteria that can cause cell and tissue damage in a variety of organisms (Carmichael, 1992). Identification of novel cyanotoxins reported in the last decade has expanded knowledge of the overall prevalence of cyanobacteria and their toxins (Carmichael, 1992; Falconer, 1993; Chorus and Bartram, 1999). Our working hypothesis is that a novel neurotoxin produced by an undescribed species of the order Stigonematales is the agent for an emerging wildlife disease—avian vacuolar myelinopathy (AVM).

AVM is the cause of the largest undiagnosed eagle mortality in U.S. history, a 1994 die-off of 30%–65% of all eagles wintering at DeGray Lake, Arkansas (Thomas et al., 1998). AVM was first recognized at DeGray Lake and has been the confirmed or suspected cause of death of at least 100 bald eagles in southeastern U.S. reservoirs. However, because the band recovery rate for bald eagles is less than 10%, observed mortality is likely to be a small fraction of the actual number killed by AVM. The disease also has afflicted untold numbers of waterbirds, including thousands of American coots (*Fulica americana*), a preferred prey of eagles. The cause of this disease is still unknown.

AVM is characterized by widespread bilaterally symmetrical vacuolation of the white matter of the brain and spinal cord of affected animals (Thomas et al., 1998). Affected birds have difficulty flying and are uncoordinated on land and in water. The tissue of afflicted birds has been analyzed for a variety of disease-causing agents, including bacteria, viruses, and parasites. To date, all such analyses have been negative (Thomas et al., 1998; Rocke et al., 2002). Experimental evidence also indicates that AVM is not contagious between birds. Farm-raised mallards that were cohoused with AVM-positive coots collected from an active disease site did not contract the disease (Larsen et al., 2002; Larsen et al., 2003). It is believed that a neurotoxin is the most probable cause of the brain lesions associated with AVM. Chemicals known to create similar lesions include triethyltin, bromethalin, and extracts from toxic plants that grow in Africa and Australia (*Stypandra imbricata* and *Heliochrysum argyrosphaerum*). None of these lesion-producing chemicals have been detected in water, soil, plants, or tissue where AVM has been diagnosed. Although extensive tests have been conducted for a wide range of toxins including organic metals, pharmaceuticals, and plant toxins, none of these have been detected at concentrations known to cause detrimental effects in AVM-diseased birds (Thomas et al., 1998; Rocke et al., 2002; Dodder et al., 2003).

Sick or dead eagles are generally found from October to March, with a peak in eagle deaths occurring from mid-November through December. AVM-related coot mortality also occurs during these months; for example, Arkansas collections established that 81% of coots tested were AVM positive during November–December 1996 (Thomas et al., 1998). Other birds affected by AVM include Canada geese (*Branta canadensis*), great horned owls (*Bubo virginianas*), killdeer (*Charadrius vociferus*), mallards (*Anas platyrhynchos*), and ring-necked ducks (*Aythya collaris*). AVM also is suspected in the deaths of buffleheads (*Bucephala albeola*), northern shovelers (*Anas clypeata*), American widgeons (*Anas americana*), and other waterfowl (Rocke et al., 2002; Augspurger et al., 2003).

The disease has been linked through the food chain from plants to waterfowl to predators. Eagles contract AVM by preying on afflicted coots and other waterfowl unable to effectively escape. Feeding studies confirmed this link when the disease was induced in nonreleasable red-tailed hawks (*Buteo jamaicensis*) that were fed tissue from AVM-positive coots (Fischer et al., 2003). Mallard feeding trials established that waterfowl contract the disease by consuming aquatic plants and their associated biota from AVM-positive reservoirs during the AVM season (Birrenkott et al., 2004). These feeding studies used hydrilla colonized by an abundant, novel Stigonematales species growing on leaves found exclusively in AVM-positive locations.

This undescribed Stigonematales species was noted during exotic plant surveys in AVM-positive reservoirs. Our working hypothesis for AVM transmission is that birds consume a neurotoxin associated with this epiphytic cyanobacterium. Hydrilla (*Hydrilla verticillata*), Brazilian elodea (*Egeria densa*), and Eurasian water milfoil (*Myriophyllum spicatum*) are the most abundant species available as substrates for epiphytes in AVM-positive reservoirs.

MATERIALS AND METHODS

Reservoir Surveys

Reservoir surveys were conducted from March 2001 to March 2004. Sampling was conducted from a boat and included standard water quality measurements, dissolved oxygen, temperature, and pH. Water, sediment, and plants were collected from replicate (3–20) sites in each pond or reservoir. Fresh samples were screened for all algal species, and relative abundance (cells ml^{-1}) was noted. Subsamples from each site were frozen for later analyses. Representative leaves were removed from stems, mounted on glass slides using permount, and viewed using light and

Fig. 1. Map of the sites where AVM has been confirmed (closed circles, numbered— see Table II) and additional sites (open circles, letters—see Table I) sampled for invasive aquatic plants and epiphytic cyanobacteria. Alphanumeric symbols on individual sites are referenced in Tables I and II.

epifluorescence microscopy. Surface aerial coverage was determined using image analysis. Cell counts were conducted on intact leaves, as the epiphytic Stigonematales species of interest was not readily removed by agitation or sonification. During the sentinel trial at Davis Pond, hydrilla samples were collected weekly and screened for harmful algae and to follow the abundance of target Stigonematalan colonies over the course of the trial.

Mallard Duck Sentinel Trial

The trial was initiated on October 29, 2003, and continued for 42 days. Twenty farm-raised male mallard ducks (at least 18 weeks old) were received from Whistling Wings (Hanover, IL, USA). These birds had their entire primary and secondary feathers clipped on their right wing and were banded on the right leg prior to release. Birds were monitored daily to document the number and behavior of sentinel ducks and to record the presence of wild ducks. Sentinel mallards were consistently observed on daily visits. Dysfunctional (symptomatic) mallards were captured and euthanized when observed. Ducks remaining on the pond at the end of the trial were corralled with a net and euthanized by CO_2. Brains were extracted on site and put in 10% buffered formalin. Histological sections of brain tissues were examined by light microscopy for the presence of AVM lesions (Thomas et al., 1998).

RESULTS AND DISCUSSION

Aquatic plants were screened to evaluate epiphytic algal communities from all known AVM locations and additional control reservoirs over the AVM season (Fig. 1). In August and September water temperatures at these sites typically are 25°C–30°C, with healthy-appearing hydrilla on the surface. During October–November coots and waterfowl migrate in from the northern United States and Canada, and water temperatures drop to 15°C–20°C. This latter period coincides with the highest incidence of AVM disease among waterfowl. By December most hydrilla have been stripped by coots and are senescing as water temperatures drop below 10°C. The highest numbers of eagle deaths occur during this time.

In reservoirs in which bird deaths from AVM have not been diagnosed, epiphytic assemblages were diverse and abundant, but the suspect Stigonematales species was either rare or not present (Table I). At these sites diatoms (Bacillariophyceae) typically dominated winter assemblages on hydrilla leaves, whereas green algae (Chlorophyta) and cyanobacteria were most abundant in late summer and autumn. The percentage of the assemblages made up of diatoms increased from 30%, in August–September, to 40%, in December–January. Cyanobacteria decreased over the same period from ∼40% in August–September to ∼12% in the latter period. The occurrence of chlorophyta also decreased,

TABLE I. Percentage of coverage of suspect *Stigonematales* species on three different invasive aquatic plants in reservoirs where AVM has not been documented

Map Letter	Additional Reservoirs	Size (ha)	*Hydrilla verticillata*	*Egeria densa*	*Myriophyllum spicatum*
a	Back River Reservoir, SC	1,124	0		
b	Potato Creek Embayment, SC	655	0		
c	Lake Keowee, SC	7,487	0–20		
d	Lake Russell, SC/GA	10,704	0–20	0–10	
e	Stevens Creek Reservoir, SC	324		0	
f	Mtn. Island Reservoir, NC	1,828	0		
g	Harris Lake, NC	1,675	0		
h	Lake Norman, NC	13,142	0–5		
i	Nickajack Reservoir, TN	4,197	0		
j	Lake Seminole, FL	15,176	0		
k	Smith Reservoir, GA	325	0–2		
l	Lake Horton, GA	320	0		
m	Lake Worth, GA	567	0		

TABLE II. Percentage of coverage of suspect Stigonematales species on three invasive aquatic plants in confirmed AVM reservoirs

Map Number	Year AVM Confirmed	AVM Reservoirs	Size (ha)	*Hydrilla verticillata*	*Egeria densa*	*Myriophyllum spicatum*
1	1994	DeGray,* AR	5,585	25–95	25–75	
2	1996	Quachita,* AR	16,212	25–95		10–25
3	1997	Hamilton, AR	2,938			10–25
4	1998	Thurmond,* SC/GA	28,328	25–95		
5	1998	Juliette,* GA	1,416	25–50	10–25	10–25
6	1998	Woodlake,* NC	457	25–50		
7	1998	Par Pond, SC	1,068			10–25
8	1998	L Lake, SC	418			10–25
9	1999	Murray, SC	20,558	0–25		
10	1999	Sam Rayburn, TX	46,337	0–10		
11	2003	Davis Pond, SC	2	25–95		
12	2003	Emerald Lake, GA	4	25–75		

* High (>20) eagle and waterfowl mortality reported from these sites.

from 32%, in August, to 10%, in March. Fifty taxa were noted in screening the aquatic plants for epiphytic algae. Hydrilla supported the highest density and variety of epiphytic algae, whereas bladderwort (*Utricularia sp.*) supported the least. Illinois pondweed (*Potamogeton illionoiensis*) supported all epiphytic groups.

The unique species found in surveys of AVM-positive reservoirs was an unknown Stigonematales species (Table II). In reservoirs where eagle and waterfowl deaths were most prevalent, this species was dominant, with less diversity of other groups (primarily diatoms and Chlorophytes). The suspect Stigonematales species grew on native species in these sites including Illinois pondweed, bladderwort, lemon bacopa (*Bacopa caroliniana*), fragrant water lily (*Nymphaea odorata*), watershield (*Brasenia schreberi*), and coontail (*Ceratophyllum demersum*). However, the Stigonematales species was denser and more prevalent on hydrilla and egeria leaves, and these plants made up orders of magnitude more biomass (and substrate for epiphytic growth) than native aquatic plants.

The abundant epiphytic Stigonematalan species has morphological characteristics consistent with *Hapalosiphon*, *Fischerella*, and *Thalpophila* (Komarek et al., 2003; Fig. 2). The species exhibits uniseriate basal trichomes, true T-branching, and intercalary heterocysts. The species ranges from initial filaments of 3–5 cells with no branches to large, mature thallus colonies with thousands of individual cells in a matrix of branches. The sheath surrounding the filaments becomes fused once thallus colonies are formed. The thallus form can be visualized macroscopically in the field as conspicuous dark blue-green spots primarily on the underside of leaves [Fig. 2(A)].

In an effort to corroborate the morphological distinction of this species as a new representative of the order Stigonematales, 16S rRNA sequences were determined from environmental isolates using denaturing gradient gel electrophoresis (data not shown). The 16S rRNA sequences were aligned with 40 additional cyanobacterial sequences. On the basis of these alignments and morphological characterizations of culture and field material, it was determined that this species represents a new cyanobacterium (Habrun, 2004). This data will be used to determine phylogeny, formally describe the species, and develop molecular probes for detection from environmental samples.

Environmental survey samples collected in the fall of 2003 enabled an experimental test of the hypothesized association of the novel Stigonematales species with AVM disease. In October–November 2003 a sentinel mallard study was conducted in Davis Pond, a 1.6-ha pond in upstate South Carolina (33° 42.44 N, 82° 08.20 W). Hydrilla was

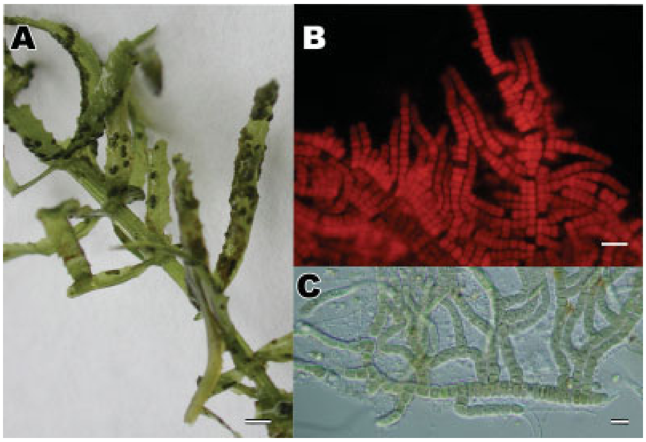

Fig. 2. Macro- and microscopic images of the unknown Stigonematales species suspected of producing the AVM agent. (A) Colonies visible to the unaided eye are growing on stems and the undersides of hydrilla leaves (scale bar, 1 mm); (B) Epifluorescent image of colonies (using Rhodamine Red filter set) showing phycobiliprotein fluorescence (scale bar, 10 μm); (C) Differential interference contrast image of the colonies (scale bar, 10 μm).

documented in Davis Pond for the first time in the summer of 2003, and it expanded to cover the entire bottom contour of the pond by October 2003. The hydrilla from this pond was densely colonized by the suspect epiphytic Stigonematalan species (ranging from 50% to 95% coverage of the hydrilla surface area coverage). We hypothesized that three elements are needed to produce AVM: an abundance of preferred aquatic vegetation (e.g., hydrilla), an abundance of the suspect Stigonematalan species growing on the available substrate, and herbivorous waterfowl. Mallard ducks, which readily eat hydrilla, were chosen as the AVM sentinel. Of 20 mallards released in the pond, five developed clinical symptoms of AVM and were recovered during the 6-week trial. Ten additional mallards were recovered at the end of the trial, for a total of 15 ducks retrieved, all of which were positive for AVM based on brain tissue biopsies.

CONCLUSIONS

Documentation of AVM in a small, isolated farm pond raises concern about the increased potential for this disease to affect bald eagles and other avian species. Prior to this study AVM had been documented in only 10 reservoirs (in Arkansas, Georgia, North Carolina, South Carolina, and Texas), all greater than 450 ha in size. Only 19 reservoirs greater than 450 ha exist in South Carolina, but an additional 1598 lakes are larger than 4 ha, and there are thousands of smaller lakes. It is therefore important to determine the potential of this disease in ponds because mortality may go undetected in these small, isolated systems. AVM has already adversely affected local breeding populations of eagles and has the potential to produce a regional impact if the disease continues to spread with new invasions of exotic aquatic macrophytes and cyanobacterial epiphytes.

Future AVM studies will follow divergent paths in basic and applied research to continue to advance knowledge of the disease and potential ways to eradicate it. Basic analytical research will focus on the genetic origin and physiology of this novel Stigonematales species in culture. The dominance of the suspect Stigonematales species epiphytic on aquatic plants has been established at the AVM sites and during the laboratory hydrilla feeding trials; however, the definitive test of our working hypothesis will be to conduct algae-only feeding trials. These studies are underway, and if this species is indeed the neurotoxin producer, the research needs to progress to examining environmental factors that promote its growth and toxin production, as well as elucidate the chemical structure of the toxin.

A second compelling line of research will be to apply our knowledge of the disease occurrence to investigate mitigating strategies for reducing bird deaths at affected sites. Sentinel trials using triploid grass carp (*Ctenopharyngodon idella*) capable of eliminating hydrilla have been initiated. Success of this mitigation strategy, however, depends on the sensitivity of grass carp to AVM and their capacity to transfer the disease through the food chain. These questions are important in understanding the benefits and/or potential problems with using grass carp as a biological control agent of nuisance aquatic vegetation and as a potential way of controlling AVM transmission from epiphytic cyanobacteria to waterfowl and birds of prey. This and other methods of eradicating hydrilla in AVM-positive sites will have the added benefit of reducing the biomass of the suspected source of the disease neurotoxin, the epiphytic Stigonematales species.

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