INVESTIGATION OF THE LINK BETWEEN AVIAN VACUOLAR MYELINOPATHY AND A NOVEL SPECIES OF CYANOBACTERIA THROUGH LABORATORY FEEDING TRIALS

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ABSTRACT: Avian vacuolar myelinopathy (AVM) is a neurologic disease affecting Bald Eagles (*Haliaeetus leucocephalus*), American Coots (*Fulica americana*), and other birds in the southeastern United States. The cause of the disease has not yet been determined, although it is generally thought to be a natural toxin. Previous studies have linked AVM to aquatic vegetation, and the current working hypothesis is that a species of cyanobacteria growing epiphytically on that vegetation is producing a toxin that causes AVM. Surveys of epiphytic communities have identified a novel species of cyanobacteria in the order Stigonematales as the most likely suspect. The purpose of this study was to further examine the relationship between the suspect Stigonematales species and induction of AVM, by using animal feeding trials. Adult Mallards and domestic chickens were fed aquatic vegetation from two study sites containing the suspect cyanobacterial epiphyte, as well as a control site that did not contain the Stigonematales species. Two trials were conducted. The first trial used vegetation collected during mid-October 2003, and the second trial used vegetation collected during November and December 2003. Neither treatment nor control birds in the first trial developed AVM lesions. Ten of 12 treatment Mallards in the second trial were diagnosed with AVM, and control birds were not affected. This study provides further evidence that the novel Stigonematales species may be involved with AVM induction, or at the least it is a good predictor of AVM toxin presence in a system. The results also demonstrate the seasonal nature of AVM events.

Key words: Avian vacuolar myelinopathy, Bald Eagle, chicken, cyanobacteria, hydrilla, mallard, neurologic disease, toxin.

INTRODUCTION

Avian vacuolar myelinopathy (AVM) is a neurologic disease affecting Bald Eagles (*Haliaeetus leucocephalus*), American Coots (*Fulica americana*), and other birds in the southeastern United States. Avian vacuolar myelinopathy was first observed in 1994, but the cause of AVM remains elusive despite extensive investigation. The pathology of the disease and epizootiologic studies suggest a toxin (Thomas et al., 1998; Rocke et al., 2002).

AVM is characterized by microscopic vacuolization of the white matter of the central nervous system, resulting from a separation of the myelin lamellae at the intraperiod line (Thomas et al., 1998). This pathology is consistent with intramyelinic edema, a condition that can be caused by several known compounds, including triethyltin, hexachlorophene, and bromethalin (van Gemert and Killeen, 1998). No significant concentrations of any of these compounds or various other environmental contaminants have been detected in tissues or environmental samples from AVM mortality events. In addition, there has been no evidence to link AVM to infectious agents (Thomas et al., 1998; Larsen et al., 2003).

Epidemiologic investigations and sentinel studies have established that AVM is site-specific, seasonal in occurrence, and can have a rapid onset (Rocke et al., 2002). Avian vacuolar myelinopathy generally

occurs during late fall and early winter and the onset of disease can occur as quickly as 5 days after exposure to the causative agent. In addition to Bald Eagles and American Coots, several other avian species have been diagnosed with AVM, including Mallards (*Anas platyrhynchos*), Ring-necked Ducks (*Aythya collaris*), Buffleheads (*Bucephala albeola*), Canada Geese (*Branta canadensis*), Great-horned Owls (*Bubo virginianus*), and a Killdeer (*Charadrius vociferus*) (Fischer et al., 2002; Augspurger et al., 2003). Birds with AVM may display clinical signs, including difficulty or inability to walk, swim, and fly, and general ataxia. Not all birds with AVM lesions display these signs; thus, microscopic evaluation of brain tissue is necessary for diagnosis.

Recent studies have established that AVM is acquired through ingestion and transferred through the food chain. Transfer of AVM from water birds to raptors was demonstrated when captive Red-tailed Hawks (*Buteo jamaicensis*) developed AVM after ingesting AVM-affected coot tissue in the laboratory (Fischer et al., 2003). AVM was experimentally induced in both Mallards and domestic chickens (*Gallus domesticus*) through ingestion of aquatic vegetation (*Hydrilla verticillata* and associated materials) from AVM-affected reservoirs (Birrenkott et al., 2004; Lewis-Weis et al., 2004).

Researchers are now examining a possible link between AVM and a species of cyanobacteria associated with the aquatic vegetation. Surveys of epiphytic algal communities revealed the presence of a novel species of cyanobacteria at all confirmed AVM sites (Wilde et al., 2005). This species, a previously undescribed member of the order Stigonematales, was the dominant epiphyte during the AVM season in those water bodies where AVM has been observed most frequently, and it was the dominant epiphyte associated with hydrilla that induced AVM in Mallards in the laboratory (Birrenkott et al., 2004). One hypothesis is that the etiologic agent

of AVM is a neurotoxin produced by this cyanobacterium. The purpose of this study was to further examine the relationship between the stigonematalan species and AVM induction, by using animal feeding trials. In two separate trials, we attempted to induce AVM in animal models by feeding aquatic vegetation containing large quantities of the stigonematalan species. Control animals received vegetation of the same type that did not contain the stigonematalan species. In addition, we attempted to examine the time period necessary to induce AVM in the laboratory, and we were able to further explore the temporality of AVM occurrence.

MATERIALS AND METHODS

Study area

Aquatic vegetation (*Hydrilla verticillata* and associated materials) was collected from three sites in South Carolina, USA. Hydrilla containing the stigonematalan species was collected from J. Strom Thurmond Lake (JSTL) (34°40′40″N, 82°13′0″W) and Davis Pond (33°42′23″N, 82°08′15″W). J. Strom Thurmond Lake is a 28,700-ha reservoir located on the Savannah River on the border of South Carolina and Georgia, USA, and it is a site of previous AVM epornitics. For this study, hydrilla was collected from the Parksville Recreation Area, located along the southeastern region of the lake. The stigonematalan species was the most prevalent epiphyte associated with the hydrilla. Davis Pond is a 1.6-ha private pond located 6.8 km east of JSTL. The pond had been colonized by hydrilla during summer 2003, and no AVM morbidity or mortality had been observed previously at this site. At the time of the study, the stigonematalan species was determined to be the most prevalent epiphytic species at this site.

Control hydrilla was collected from Lake Marion, South Carolina (33°32′18″N, 80°16′05″W), a 44,500-ha reservoir with no known AVM morbidity or mortality. Hydrilla was collected from the Potato Creek Embayment area. This site was chosen because it contained an abundance of hydrilla and associated epiphytes, but it contained no observable colonies of the suspect stigonematalan cyanobacteria. American Coots also were collected from this site, and they were analyzed for AVM lesions to ensure that the disease was not present.

Trial I

Mallards and domestic chickens served as animal models. Sixteen adult male Mallards were purchased from Whistling Wings (Hanover, Illinois, USA), and 16 adult female chickens were obtained from the Clemson University Morgan Poultry Center (Clemson, South Carolina, USA). All birds were obtained 14 October 2003, and they were housed in Godley-Snell Research Center at Clemson University. Both species were randomly divided into two experimental groups, treatment and control, with eight birds in each group. Animals were housed according to experimental group. Treatment birds were fed hydrilla from JSTL, and control birds were fed hydrilla from Lake Marion.

Hydrilla was collected from Lake Marion 14 October 2003 and from JSTL 7 October 2003. Hydrilla was collected with a rake from depths of up to 1.5 m, and it was transported in covered plastic containers. A subsample from each site was examined microscopically to determine the associated epiphytes. Three random entire leaves were mounted on a glass slide, and the average surface area covered by the cyanobacterial epiphytes was measured using epifluorescence microscopy. Surface area coverage was classified into four categories: present (1–25%), common (26–50%), abundant (51–75%), or dominant (76–100%). Real-time polymerase chain reaction (PCR) also was performed to genetically confirm the presence and abundance of the stigonematalan species (Williams et al., 2007). All collected vegetation was frozen at -20 C. Half of the hydrilla was distributed into gallon-size plastic bags before freezing for use in the feeding trials, and the remaining half was stored at -20 C for potential future toxin analysis. Administration of hydrilla began 15 October 2003. All hydrilla was thawed before feeding. For Mallards, hydrilla was given twice daily and placed in swim tanks for birds to consume ad libitum. Each group of Mallards ($n=8$) received approximately 1,000–1,700 g wet weight (ww), although this measurement includes a substantial amount of water, so the actual mass of hydrilla would be much less. Chickens received hydrilla twice daily as well, placed in feeding trays. Each group of chickens ($n=8$) received approximately 500–1,200 g ww daily. All birds were given progressively less hydrilla as the number of birds per experimental group was reduced each week, as described below. Both species received supplemental commercial feed for 1 hr twice daily, and water was available ad libitum. Behavior was observed twice daily, and weights were recorded twice weekly.

Following the first week of feeding, one-fourth of the birds (two treatment and two control of both species) were euthanized each week, with the last group of birds receiving hydrilla for 4 wk. Birds were euthanized by CO_2, and whole brains were removed. Brains were placed in 10% neutral buffered formalin, and they were transported to the Southeastern Cooperative Wildlife Disease Study (SCWDS, Athens, Georgia, USA) for analysis.

Trial II

Trial II was conducted 18 November–17 December 2003. Eighteen adult male Mallards were received from Whistling Wings 18 November. Mallards were housed at Godley-Snell Research Center according to experimental group.

The study sites for this trial included JSTL, Davis Pond, and Lake Marion. Hydrilla was collected weekly at each site during the study, beginning 18 November. Subsamples of hydrilla were examined for epiphytes as described for trial I. Quantitative real-time PCR was performed on select samples. Collection methods were identical to those described for trial I, with the exception that half of the hydrilla used in the feeding trials was kept at room temperature, whereas the other half was frozen and subsequently thawed, before feeding. This procedure was done to determine whether freezing the hydrilla had an impact on viability of the etiologic agent. Therefore, birds were randomly divided into six experimental groups, with three birds per group: 1) JSTL, frozen hydrilla; 2) JSTL, fresh hydrilla (kept at room temperature); 3) Davis Pond, frozen hydrilla; 4) Davis Pond, fresh hydrilla; 5) Lake Marion, frozen hydrilla; and 6) Lake Marion, fresh hydrilla. Feeding methods were the same as described for trial I, with birds receiving hydrilla twice daily. Each group of birds ($n=3$) received approximately 500–1,200 g ww daily. Behavior was observed twice daily, and weights were recorded twice weekly. Because of the smaller experimental groups ($n=3$) in trial II, all birds were euthanized after 4 wk of feeding. However, two birds were euthanized before this date after developing signs of neurologic impairment. Methods of euthanasia and brain analysis were the same as described for trial I.

TABLE 1.   Percentage of leaf surface area coverage of cyanobacterial epiphytes on collected vegetation (*H. verticillata*). Coverage was based on microscopic analysis of three random entire leaves and classified as either present (1–25%), common (26–50%), abundant (51–75%), or dominant (76–100%).

Cyanobacterial genus	Trial I[a]		Trial II[b]		
	Lake Marion	JSTL[c]	Lake Marion	JSTL	Davis Pond
Anabaena	Present	Present		Present	Present
Aphanothece	Present	Present	Present	Present	
Gloeotrichia	Abundant		Common		
Gomphosphaeria			Present	Present	
Leptolyngbya	Common		Common	Present	Present
Microcoleus			Present		
Oscillatoria				Present	Present
Phormidium			Present		
Pseudanabaena		Present	Present		Present
Spirulina			Present		
Stigonematales sp.		Abundant		Dominant	Abundant
Trichormus	Common	Present	Common		Present

[a] Vegetation collected 7 October 2003 for JSTL and 14 October 2003 for Lake Marion.

[b] Vegetation collected 18 November 2003. See Table 2 for *Stigonematales* coverage on additional collection dates.

[c] J. Strom Thurmond Lake.

RESULTS

Trial I

In trial I, no birds developed any clinical signs of AVM, and all brains were negative for AVM lesions. Mallard body weights remained relatively constant over the course of the study, with an average increase of 6.1% (±8.0% [SD]), and no individual weight loss exceeding 3.0%. Mallards readily ate the vegetation, consuming almost all of the offered material. The chickens consumed much less material than the ducks, only consuming one third to one half of the material offered, and they had an average weight loss of 5.4% (±5.6%) from the beginning of the trial to euthanasia. No individual weight loss exceeded 20%.

Cyanobacterial epiphytes associated with the vegetation collected for trial I, and their relative abundance, are shown in Tables 1 and 2. Several genera were present on both Lake Marion and JSTL material, with the stigonematalan species dominant on JSTL vegetation and absent from Lake Marion vegetation. Real-time PCR confirmed the absence of stigonematalan colonies on Lake Marion material, and they provided an estimate of 577.96 cells/ml for JSTL material.

Trial II

In trial II, two Mallards developed signs of neurologic impairment consistent with AVM. On the afternoon of 23 November, 5 days after initiation of the study, a member of the JSTL fresh group was observed leaning backwards with a slightly unsteady gait. The bird was still eating and drinking readily. On the morning of 24 November, the bird displayed more severe ataxia, including falling onto its side and backwards, and inability to walk properly. At this time, the bird was euthanized, and the brain was placed in formalin.

On 1 December, 13 days after initiation of the study, another member of the JSTL fresh group developed clinical signs consistent with AVM. This bird was observed tilting forward and generally seemed off balance and lethargic. The bird was euthanized, and the brain was placed in formalin.

All other birds in trial II remained clinically normal during the study. All weights remained relatively constant, with

TABLE 2.   Percentage of leaf surface area coverage of the *Stigonematales* species on collected vegetation (*H. verticillata*). Coverage was based on microscopic analysis of three random entire leaves and classified as either present (1–25%), common (26–50%), abundant (51–75%), dominant (76–100%), or not detected (ND). Quantitative real-time polymerase chain reaction performed on select samples is listed in parentheses below coverage.

Trial	Lake Marion	JSTL[a]	Davis Pond
I			
7 October	—[b]	Abundant (577.96 cells/ml)	—
14 October	ND	—	—
II			
18 November	ND	Dominant	Abundant (162.59 cells/ml)
25 November	ND	Abundant	Abundant
2 December	ND	Abundant (345.46 cells/ml)	Common
9 December	ND	Abundant	Common

[a] J. Strom Thurmond Lake.

[b] No collection at this site.

an average weight gain of 6.8% (±11.5%) over the study and no individual weight loss greater than 8.0%. The birds readily consumed the majority of the vegetation offered. Microscopic examination of brain tissue revealed the presence of AVM lesions in 10 Mallards, including the two Mallards displaying clinical signs. All six of the birds receiving JSTL hydrilla developed AVM, and four of the six birds receiving hydrilla from Davis Pond developed the disease (two of three birds in both the Davis Fresh and Davis frozen groups). No AVM lesions were observed in any bird receiving hydrilla from Lake Marion.

Cyanobacterial epiphytes associated with vegetation collected for trial II are shown in Tables 1 and 2. The stigonematalan species was not observed on Lake Marion material, although several other cyanobacterial genera were present. Leaf coverage of the stigonematalan species on JSTL hydrilla was classified as abundant to dominant (51–100% leaf coverage), and results from quantitative real-time PCR on the 2 December 2003 collection provided an estimate of 345.46 cells/ml. Leaf coverage for Davis Pond was classified as common to abundant (26–75% leaf coverage), and quantitative real-time PCR results for the 18 November 2003 collection resulted in an estimate or 162.59 cell/ml.

DISCUSSION

No birds in trial I contracted AVM, apparently due to the absence or insufficient concentration of the causative agent in the collected vegetation. At the time there was concern about whether freezing the material would affect the viability of the AVM agent, but results from trial II have demonstrated that freezing did not have an effect; this finding is consistent with previous studies (Fischer et al., 2003; Lewis-Weis et al., 2004). Despite being housed in larger groups, ducks in trial I had access to approximately the same amount of hydrilla as those in trial II, and all birds seemed to eat readily. The chickens, however, consumed much less hydrilla than the Mallards, and they were therefore not used in trial II. Chickens have been shown to be susceptible to AVM, and they were an acceptable animal model for vegetation feeding trials in another study (Lewis-Weis et al., 2004).

Ten of 12 treatment ducks in trial II were diagnosed with AVM lesions, indicating that at least some vegetative samples from both JSTL and Davis Pond collected during November and December contained the causative agent in sufficient quantity to produce lesions. The hydrilla collected from JSTL during

the week of November 18 contained the etiologic agent, because the bird that displayed clinical signs at 5 days had only consumed material from this collection.

Hydrilla was collected from the same site at JSTL for both trials; however, only that material collected during the second trial, collected November 18–December 9, induced AVM, whereas material collected in October did not. The lack of AVM induction with earlier collections is consistent with other observations of AVM seasonality. Previous studies have reported the occurrence of AVM from November through March or April, with the highest prevalence in November and December (Fischer et al., 2002; Rocke et al., 2002). On JSTL specifically, the majority of diagnostic accessions submitted to SCWDS since 1998 were found from November to January, although active surveillance of coots on JSTL from 1998 to 2004 revealed the presence of lesions from October to March (Fischer et al., 2006). At the time of our collections in 2003, active surveillance of coots revealed no prevalence of lesions in October, but lesions were present in 22% and 7% of coots collected in November and December, respectively (Fischer et al., 2006). This marked seasonality supports the idea that AVM is the result of a naturally produced toxin.

Factors that initiate toxin production in cyanobacteria and other algal species are not precisely known (Haider et al., 2003). Toxins are usually associated with bloom formation, which can occur in response to environmental factors such as a change in nutrient concentrations, temperature, or light. In AVM reservoirs, the suspect stigonematalan species experiences a bloom beginning late summer to early fall, occurring in abundance until the vegetation senesces during the winter, and it loses a suitable substrate (Wilde, unpubl. data). Observations of cyanobacterial epiphytes and PCR confirmed the presence of the stigonematalan species during both the October and November

collections. Comparable quantities were present for all except for higher leaf coverage (76–100%) for the November 18 JSTL collection compared with the remaining November collections and October collection (51–75%).

That the October material did not produce AVM does not imply that the stigonematalan species is not involved, only that it may not have been actively producing toxin at the time. Although changes in algal toxin concentrations can often be explained by changes in algal abundance, there are many instances where there is no correlation between numbers of cyanobacteria and toxin production (Kardinaal and Visser, 2005). The same environmental factors that influence bloom formation (light, temperature, pH, nutrient ratios, and concentrations) also can influence toxin dynamics. In addition, toxic and nontoxic strains of cyanobacteria can occur within the same species, further complicating the relationship between cyanobacterial biomass and toxin presence and concentration (Kardinaal and Visser, 2005).

It was our intention in trial I to study the time of lesion formation by feeding our test animals vegetation from a single collection over a 1-mo period and euthanizing them at different time points, but no birds developed visible AVM lesions. However, clinical signs of AVM were observed in one bird 5 days after initial exposure to the hydrilla in trial II. This quick time of onset has been observed previously with sentinel birds released onto an AVM-affected reservoir (Rocke et al., 2002). It is unclear when the other birds that contracted AVM in the study may have developed lesions.

Of the 10 birds in the second feeding trial to have confirmed lesions consistent with AVM, two birds were observed with clinical signs of the disease. This frequency is consistent with the previous laboratory trial that successfully reproduced AVM in Mallards, in which one of six ducks with confirmed lesions displayed

clinical signs (Birrenkott et al., 2004). It is not clear why some birds display clinical signs and others do not. It may be related to the amount of the etiologic agent that the bird ingests, the duration of exposure, variation in individual susceptibility and health of the bird, or some other factor. Lewis-Weis et al. (2004) reported a higher prevalence of clinical signs in chickens affected with rickets than in birds of good nutritional status. The Mallards developing clinical signs in this study did not have any obvious signs of impaired health compared with the other birds, but they could have had undetected conditions making them more susceptible. Thus far, there seems to be no correlation between presence of clinical signs and severity of brain lesions. Birds with severe lesions can seem clinically normal, whereas those with signs of neurologic impairment can have mild-to-moderate lesions (Rocke et al., 2002; Fischer et al., 2003). Regardless of the reason, many birds that develop AVM lesions do not develop clinical signs, suggesting that the disease may go undiagnosed unless active surveillance is conducted.

Although not conclusive, this study provides further evidence that AVM induction may be linked to a newly discovered cyanobacterial species. At the least, this novel stigonematalan species seems to be a good indicator of AVM disease potential, because material collected from two separate study sites based on the presence of this species induced AVM. We were able to correctly predict the presence of the AVM causative agent in Davis Pond due to the existence of two factors: the abundant submerged aquatic vegetation and the associated abundant stigonematalan cyanobacteria. However, because the control and treatment hydrilla were collected from different study sites, factors other than presence or absence of the stigonematalan species may be involved, and further study is needed. Vegetation collected from the same reservoir, some containing the stigonematalan species and some without, would provide more convincing evidence. However, no reservoirs provided such a situation at the time of our collections. In addition, because of the nature of the epiphytic growth of cyanobacteria on the vegetative material, it is impractical, and perhaps not possible, to separate the cyanobacterial colonies from the vegetation to test it separately.

The stigonematalan species is currently being cultured in the laboratory, and future experiments include the testing of this material by Mallard bioassay. In addition, experiments are underway involving attempts to extract the putative AVM toxin from collected vegetation samples. Much research is still needed to determine the cause of AVM as well as to provide more information on the pathology, range of susceptible species, implications for wildlife populations, and management strategies.

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