An Extract of *Hydrilla verticillata* and Associated Epiphytes Induces Avian Vacuolar Myelinopathy in Laboratory Mallards

Faith E. Wiley,[1,2]* Michael J. Twiner,[2] Tod A. Leighfield,[2] Susan B. Wilde,[3,4] Frances M. Van Dolah,[2] John R. Fischer,[5] William W. Bowerman[1]

[1]Department of Forestry and Natural Resources, Clemson University, 261 Lehotsky Hall Clemson, South Carolina 29634, USA

[2]Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, NOAA/National Ocean Service, 219 Fort Johnson Road, Charleston, South Carolina 29412, USA

[3]Belle W. Baruch Institute for Marine and Coastal Sciences, University of South Carolina, Charleston, SC 29412

[4]South Carolina Department of Natural Resources, Marine Resources Research Institute, Charleston, SC 29412, USA

[5]Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602, USA

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ABSTRACT: Avian vacuolar myelinopathy (AVM) is a neurological disease affecting bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), waterfowl, and other birds in the southeastern United States. The cause of the disease is unknown, but is thought to be a naturally produced toxin. AVM is associated with aquatic macrophytes, most frequently hydrilla (*Hydrilla verticillata*), and researchers have linked the disease to an epiphytic cyanobacterial species associated with the macrophytes. The goal of this study was to develop an extraction protocol for separating the putative toxin from a hydrilla-cyanobacterial matrix. Hydrilla samples were collected from an AVM-affected reservoir (J. Strom Thurmond Lake, SC) and confirmed to contain the etiologic agent by mallard (*Anas platyrhynchos*) bioassay. These samples were then extracted using a solvent series of increasing polarity: hexanes, acetone, and methanol. Control hydrilla samples from a reference reservoir with no history of AVM (Lake Marion, SC) were extracted in parallel. Resulting extracts were administered to mallards by oral gavage. Our findings indicate that the methanol extracts of hydrilla collected from the AVM-affected site induced the disease in laboratory mallards. This study provides the first data documenting for an "extractable" AVM-inducing agent. © 2008 Wiley Periodicals, Inc. Environ Toxicol 24: 362–368, 2009.

Keywords: avian vacuolar myelinopathy; AVM; bald eagle; *Haliaeetus leucocephalus*; cyanobacteria; algal toxin; mallard; American coot

Correspondence to: F. E. Wiley; e-mail: fwiley@aug.edu

*Current address: Department of Biology, Augusta State University, 2500 Walton Way, Augusta, Georgia 30904-2200, USA.

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INTRODUCTION

Avian vacuolar myelinopathy (AVM) is a neurological disease affecting bald eagles (*Haliaeetus leucocephalus*), American coots (*Fulica americana*), and other birds in the southeastern United States. First documented in bald eagles in 1994, AVM is responsible for the deaths of several

species of birds in five southeastern states but the cause of the disease has not yet been determined (Thomas et al., 1998; Fischer et al., 2002; Rocke et al., 2002).

AVM is characterized and diagnosed by spongy degeneration of the white matter of the central nervous system due to intramyelinic edema (Thomas et al., 1998). Affected birds may display clinical signs of neurological impairment, including difficulty in flying, swimming, and/or walking. However, not all birds with AVM lesions display clinical signs, thus pathological evaluation of brain tissue is necessary for diagnosis. The pathology of AVM is consistent with several known chemicals, including triethyltin, hexachlorophene, and bromethalin (van Gemert and Killeen, 1998), but no significant concentrations of these or other known toxins have been detected in tissues of affected birds or surrounding environmental samples (Thomas et al., 1998; Dodder et al., 2003). There has also been no evidence linking AVM to infectious agents and the disease was not found to be contagious (Thomas et al., 1998; Larsen et al., 2003). Although the etiology of the disease remains unknown, recent investigations have suggested that the causative agent for AVM is a cyanobacterial toxin (Birrenkott et al., 2004; Wilde et al., 2005; Wiley et al., 2007).

Previous investigations have established that AVM is site-specific and seasonal (Rocke et al., 2002), associated with a number of southeastern reservoirs with epizootics occurring during late fall to early winter. The only route of exposure identified thus far has been through a contaminated food source. Coots and waterfowl contract the disease by feeding on aquatic vegetation (Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005), and bald eagles and other avian predators contract the disease by consuming affected prey (Fischer et al., 2003). All AVM-affected reservoirs contain extensive nonendemic aquatic macrophytes, but the same or similar macrophytes are also abundantly present in other nearby aquatic systems where AVM is not observed. Therefore, it has been hypothesized that the causative agent is something associated with the macrophytes, rather than the macrophytes themselves (Birrenkott et al., 2004; Lewis-Weis et al., 2004).

The macrophyte hydrilla (*Hydrilla verticillata*) and associated materials that induced AVM in a laboratory controlled, mallard (*Anas platyrhynchos*) feeding study (Birrenkott et al., 2004) were shown to contain large quantities of an epiphytic cyanobacterial species. Colonies of this species, a previously uncharacterized member of the order Stigonematales, covered 50–90% of the leaf surface of the hydrilla. Surveys of all known AVM reservoirs and similar reservoirs where AVM does not occur, revealed a strong positive correlation between the presence of this cyanobacterial species and AVM (Wilde et al., 2005). Researchers have thus postulated that this cyanobacterial species may produce a toxin that causes AVM in birds such as eagles and coots.

Cyanobacteria are ubiquitous in fresh, marine, and brackish waters, as well as terrestrial environments all over the globe. Numerous genera are known to produce toxins, which can have hepatotoxic, neurotoxic, or dermatotoxic effects in human and wildlife populations (Codd, 1995). The list of known toxins continues to grow, as new and emerging toxins are recognized due to advances in analytical techniques and an increasing awareness of the potential for cyanotoxin poisoning events (Codd, 1995; Codd et al., 2001; Cox et al., 2005).

To date, laboratory investigations of AVM have been limited to feeding studies utilizing whole tissues or vegetation samples without any quantitative indication of the presence of the AVM toxin. The aim of the present study was therefore to develop a method to extract the causative agent of AVM from aquatic vegetation as the first step toward its purification and characterization. Our successful isolation of a partially purified toxic extract further provides the tools for *in vitro* and *in vivo* toxicology studies including investigations determining the mechanism of action.

MATERIALS AND METHODS

Vegetation Collection

Hydrilla was collected from J. Strom Thurmond Lake (JSTL), SC, a site of previous and current AVM epizootics (Fischer et al., 2002, 2003; Lewis-Weis et al., 2004; Fischer et al., 2006). This vegetation contained abundant quantities of an epiphytic cyanobacterium that has been suggested as a possible source of the disease agent (Wilde et al., 2005), with leaf surface area coverage ranging from 51 to 100%. Control hydrilla was collected from Lake Marion, SC, a reservoir with no known history of avian vacuolar myelinopathy (AVM) occurrence. The suspect cyanobacterium was absent from all hydrilla collections made from Lake Marion, as confirmed by microscopic analysis of representative leaves.

Samples were collected weekly, for 4 weeks, from mid-November to mid-December 2003, a period when active surveillance confirmed AVM in 7% to 22% of wild coots at JSTL (Fischer et al., 2006). Collections were made with a rake, from the surface to a depth of $\sim$1.5 m, and samples were stored and transported in covered plastic containers (500 gallon, Rubbermaid). Excess water was drained and samples were stored at -20°C, except as described later for the mallard vegetation bioassay.

Mallard Bioassay I: Vegetation

To confirm the presence or absence of the AVM causative agent, half of the vegetation collected was fed *ad libitum* to farm-reared mallard ducks in the laboratory. Detailed results of this study are reported elsewhere (Wiley et al., 2007). Briefly, six ducks were fed vegetation from JSTL and six additional ducks ingested vegetation from Lake Marion.

Three ducks in each group received fresh vegetation that had not been frozen, but kept at room temperature. The remaining three ducks received vegetation that had been frozen at −20°C and thawed before feeding. All six ducks receiving JSTL vegetation developed microscopic white matter lesions consistent with AVM, confirming the presence of the AVM agent. No ducks receiving Lake Marion vegetation developed AVM lesions, confirming the absence of the AVM agent. This trial also confirmed that freezing does not affect stability of the AVM causative agent.

Toxin Extraction

The remaining half of the collected vegetation was used for a solvent extraction procedure. Frozen vegetation was lyophilized (Virtis Company, Gardener, NY) and blended (Waring Commercial Blender, Waring, Torrington, CT) at medium speed for 1.0 min into a coarse powder in preparation for extraction. Each weekly sample of vegetation was lyophilized and extracted separately, and dry weight of each sample was recorded before extraction. Dry weights of weekly samples ranged from 418.48 to 1219.88 g, with an average of 837.83 g. Dried, powdered vegetation was loaded evenly into 2 L separatory funnels (200–300 g per funnel; three to four batches) and sequentially extracted with hexanes, acetone, and methanol (Fisher Chemical, HPLC grade, Fisher Scientific, Hampton, NH). Hexanes were added to the vegetation until saturated (1–1.5 L of solvent per funnel), and the sample was mechanically extracted by shaking for 5 × 2-min periods over 1 h. The extract was then allowed to drain, and the sample was rinsed with a final volume of solvent (0.5 L). Drained hexanes were pooled. This process was then sequentially repeated with acetone, then with methanol. The collected extracts were filtered through 70 mm Whatman qualitative grade filter paper (Whatman, Inc., Florham Park, NJ) to remove particulates, and solvent was removed by a rotary evaporator (Buchi Rotavapor, Buchi, Flawil, Switzerland) with a water bath set no higher than 28°C. When dry, the extracts were resuspended in the appropriate volume of the original solvent to a concentration of 10 g vegetation/mL solvent, based on the initial dry weight of the vegetation. Ten percent of this volume was archived for future *in vitro* experiments, and the remaining 90% was transferred to a glass storage jar for use in the *in vivo* mallard bioassay. All extracts were stored at −20°C.

Mallard Bioassay II: Extracts

To determine which extract(s) contained the AVM causative agent, they were evaluated by a mallard bioassay. Eighteen adult male mallards (average body weight: 1017 g) were obtained from Frost Waterfowl Trust (Georgetown, SC) and housed in floor pens according to experimental group at Godley-Snell Research Center, Clemson University. Birds were weighed on arrival and randomly assigned to one of six experimental groups (three birds per group): JSTL hexanes, JSTL acetone, JSTL methanol, Lake Marion hexanes, Lake Marion acetone, and Lake Marion methanol. Commercial feed and water were provided *ad libitum*, except as described for gavage. Birds were allowed to acclimate for 3 days before initiation of the treatment period.

Before dosing, extracts were dried under a N_2 stream and resuspended in nontoxic vehicles. Hexanes extracts were resuspended in corn oil (MP Biomedicals, Irvine, CA), acetone extracts were resuspended in a 50:40:10 (v:v:v) mixture of corn oil:deionized water:propylene glycol (EMD Chemicals, Gibbstown, NJ), and methanol extracts were resuspended in a 90:10 (v:v) mixture of deionized water:propylene glycol. Vehicles used for resuspension were based on the polarities of each extract. Additional control (non-AVM) extracts produced from vegetation not used in the mallard bioassays were used to determine the appropriate vehicle mixtures that would adequately resuspend the dried extracts. Each weekly vegetative extract was resuspended and used to dose the birds separately over the course of 1 week (4 weeks in total), so as to mimic the conditions in the mallard vegetation bioassay. This was done to avoid a possible dilution of the toxin in the case that weekly collections may have contained differing toxin concentrations. Extracts were resuspended in the appropriate volume of vehicle to deliver the entire extract in three equivalent doses for more than 1 week. Week one extracts were resuspended in 45 mL of vehicle to provide a 5 mL dose volume per duck/per dosing. Dose volume was reduced to 4 mL following the first week in an attempt to reduce regurgitation that occurred with some birds, as explained in the Results section. Therefore, the remaining extracts were resuspended in 36 mL, to provide a 4 mL dose volume per bird/per dosing.

Mallards were orally gavaged with the resuspended extracts three times a week for 4 weeks. Administration of the extracts was immediately followed by administration of a rinse with the respective vehicle to help wash residual extract out of the feeding tube. Initial rinse volumes were 3 mL, but were reduced to 2 mL following the first week of dosing because of the observed regurgitation. Therefore, total dose volume including extract and rinse was 8 mL during the first week and 6 mL for the remainder of the study. Food was removed approximately 4 h prior to treatment and provided immediately posttreatment. Birds were observed for at least 30 min posttreatment and any regurgitation was noted.

All birds were observed twice daily during the duration of the study and weights were recorded twice weekly. Birds were euthanized 3 days following the final dose administration, 28 days after the treatment was initiated. Birds were sacrificed by CO_2 asphyxiation with a Euthanex CO_2/O_2 blending system (Euthanex E-23000, Euthanex Corporation, Palmer, PA), using a 70:30 CO_2:O_2 mixture until birds lost

TABLE I. Occurrence of regurgitation in mallards after administration of an extract of *Hydrilla verticillata* and associated epiphytes collected from Lake Marion, South Carolina or J. Strom Thurmond Lake, South Carolina

Extraction/ Administration Solvent	Lake Marion		J. Strom Thurmond Lake	
	n Doses	*n* Doses Regurgitated[a]	*n* Doses	*n* Doses Regurgitated[a]
Hexanes	12	0	12	1
Acetone	12	1	12	3
Methanol	12	4	12	12[b]

[a] Indicates the number of times at least one bird in group regurgitated.
[b] Significant ($P < 0.05$) difference between occurrence of regurgitation in JSTL Methanol and all other groups.

consciousness, followed by 100% CO_2 to complete euthanasia. Whole brains were removed and placed in 10% neutral buffered formalin in preparation for AVM diagnosis. Whole blood and other tissues, including liver, kidney, pectoral muscle, gizzard, intestines, and adipose tissue, were also collected and archived at −20°C. Brain histopathology was conducted at the Southeastern Cooperative Wildlife Disease Study by methods previously described (Fischer et al., 2003). Briefly, formalin-fixed brain samples were embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin, and examined under a light microscope with particular attention devoted to the optic lobe, which is a predilection site for AVM lesions. Low numbers of small vacuoles in portions of the white matter of the optic lobe were regarded as mild lesions, whereas moderate lesions comprised numerous vacuoles widely distributed throughout all white matter of the optic lobe and sometimes within white matter of the cerebrum.

RESULTS

No clinical signs of neurological disease, including ataxia, incoordination, or lethargy, were observed during the trial. All animals maintained a fairly constant body weight throughout the study. On day 15 of the treatment period, a bird in the JSTL methanol (AVM-positive methanol) group broke a wing during removal from the pen for treatment. This bird was immediately euthanized and necropsied.

Following gavage, several birds were observed regurgitating the administered dose, usually within 5 min of administration. This occurred in the JSTL and Lake Marion methanol groups following the first two treatments, with at least one bird in both groups regurgitating. Since the birds were housed in groups, we were unable to determine with confidence how many birds regurgitated or which bird regurgitated unless it was observed directly. In an attempt to prevent regurgitation, the total dose volume was reduced to 6 mL, as described in the Methods section. Following the next two dose administrations, at least one bird in both methanol groups again regurgitated. However, for all successive treatments, no regurgitation was observed in the Lake Marion methanol group. At least one bird, and often all birds, in the JSTL methanol group continued to regurgitate after each treatment. In addition, at least one bird in the JSTL acetone group regurgitated three times during the study, and at least one bird in the JSTL hexane and Lake Marion acetone groups regurgitated once. The occurrences of regurgitation by experimental group are shown in Table I. There was a significant difference in the occurrence of regurgitation between the JSTL methanol group and all other groups ($P < 0.001$ for all, Fisher's exact test).

The results of the brain analysis are shown in Table II. All three birds in the JSTL methanol group developed definitive lesions of moderate severity consistent with AVM (Fig. 1). One bird in the JSTL acetone group developed possible mild lesions. All other birds had no apparent lesions.

TABLE II. Presence of AVM-type brain lesions in mallards after administration of an extract of *Hydrilla verticillata* and associated epiphytes collected from Lake Marion, South Carolina or J. Strom Thurmond Lake, South Carolina

Extraction/ Administration Solvent	Lake Marion		J. Strom Thurmond Lake	
	n	Birds with Lesions	*n*	Birds with Lesions
Hexanes	3	0	3	0
Acetone	3	0	3	1[a]
Methanol	3	0	3	3

[a] Possible mild lesions. Results inconclusive.

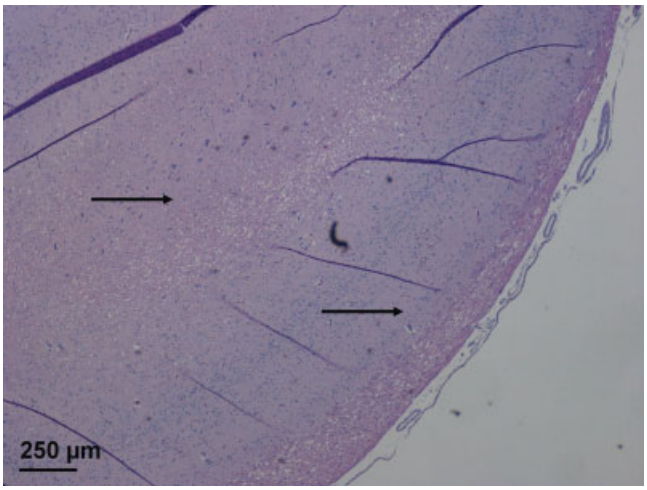

Fig. 1. Light photomicrograph image of the optic lobe of a mallard given JSTL methanol extract. Definitive lesions of moderate severity are seen in the white matter tracts (arrows) and the bird was diagnosed as having avian vacuolar myelinopathy (Hematoxylin–eosin stain). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

This study describes the induction of numerous clear vacuoles consistent with those of AVM within the white matter of the brain in mallards gavaged with a methanolic fraction extracted from hydrilla and its associated epiphytic flora. The presence of the toxic activity in the methanol fraction of the solvent series suggests a polar compound. One bird administered the acetone extract also exhibited mild lesions. Given that the polarity of acetone and methanol do not differ greatly (Snyder, 1978; Marcus, 1998; Fitzpatrick and Dean, 2002), it is possible that the toxic activity was also present in the acetone extract at lower concentration. Both solvents have similar octanol/water partition coefficients ($K_{o/w}$ acetone = −0.24; methanol = −0.70) (Marcus, 1998). Methanol, however, has a much greater hydrogen bonding ability than acetone (Marcus, 1998; Fitzpatrick and Dean, 2002), another factor that can play a role in extraction efficiency. Further fractionation of the crude extract will be necessary to provide a more precise description of the physical properties of the toxic agent(s).

The AVM inducing fraction retained activity at −20°C for at least 1 year and at room temperature for at least several days. The highest temperature to which the extracts were exposed was 28°C, so it is not known whether or not the activity is stable at higher temperatures. The lesion-inducing activity does not appear to be volatile, as it survived extensive lyophilization and vacuum concentration. However, since there is currently no method available to quantify its concentration, we cannot gauge the efficiency of the extraction procedure or rates of degradation or loss.

Although clinical signs of neurotoxicity were not observed in the lesioned mallards exposed to the extracts, previous field, and laboratory data have shown that lesioned birds collected from AVM positive lakes and birds fed hydrilla from those lakes do not always display signs of disease (Rocke et al., 2002, 2005; Fischer et al., 2003; Birrenkott et al., 2004; Lewis-Weis et al., 2004). It is not yet known why birds with severe lesions can appear clinically normal whereas birds displaying signs of neurological impairment may have mild or moderate lesions (Larsen et al., 2002; Rocke et al., 2002; Fischer et al., 2003). Toxin concentration, exposure time, route of administration, and individual susceptibility or health status of the bird may play a role. It is also possible that the lesions are unrelated to clinical signs, and that a second, unidentified pathology exists.

The results of this study revealed an emetic response in association with the AVM-inducing fraction. Although birds in several other dose groups, including controls, also regurgitated during the study, they did not do so consistently. Regurgitation as a method of rejection has not been noted previously in other AVM research that used *ad libitum* feeding of either vegetation (i.e., *Hydrilla verticillata*) containing the AVM agent or tissues from coots with AVM (Fischer et al., 2003; Birrenkott et al., 2004; Lewis-Weis et al., 2004; Rocke et al., 2005). However, this is the first study to administer a concentrated extract. Mallards are prone to a regurgitation response in toxicity testing, which may be caused by the test substance itself, the volume administered, the vehicle or dosing technique, or a combination of these factors (OECD, 2002). During the first week of dose administration, regurgitation occurred in both the Lake Marion and JSTL methanol groups, suggesting that the vehicle or some other component of the methanol extracts other than that of the AVM activity was responsible for the emetic response. However, following the mid-experiment reduction in dosing volume regurgitation eventually stopped in the Lake Marion methanol group, whereas the frequency of regurgitation for the JSTL methanol birds remained high. Many known toxins and toxicants are known to have an emetic effect, but given the complex matrix remaining in the methanol fraction, it is unclear from the current study if the AVM inducing agent, or some other component in the extract, is responsible for the increased frequency of regurgitation observed in the affected birds.

The occurrence of regurgitation was a concern during the trial because the animals that regurgitated did not receive the entire administered dose. However, we believe that the occurrence of regurgitation has not significantly impacted the results, due to the fact that the frequency of regurgitation was low in all groups except the JSTL methanol group. All birds in the JSTL methanol group developed definitive lesions consistent with AVM despite the high

occurrence of regurgitation. These results suggest that the toxic activity was sufficient in the JSTL methanol extract to produce the disease despite the loss of extract during regurgitation. However, future studies should take into consideration that regurgitation may occur when administering similar extracts to mallards. Researchers may want to consider the use of a test subject less prone to regurgitate.

The time of disease onset in the affected birds in this study is not precisely known. At least one bird developed lesions within 2 weeks of treatment initiation, as the mallard euthanized due to a broken wing at day 15 of the treatment period was diagnosed with AVM. Previous studies have shown that clinical signs and lesions can develop as early as 5 days postexposure to the causative agent (Rocke et al., 2002; Wiley et al., 2007). It is not known whether or not disease onset occurs with a single acute exposure of a threshold concentration or if it can develop after chronic exposure to lower concentrations.

The results of the current study provide further evidence that the etiological agent of AVM is associated with aquatic vegetation such as hydrilla. Given that the birds receiving Lake Marion control hydrilla extracts did not develop AVM lesions, the agent is most likely not the vegetation itself, but rather something associated with the vegetation. Our working hypothesis is that AVM is caused by an algal or cyanobacterial toxin. The seasonal nature of AVM epornitics supports this hypothesis, as algal and cyanobacterial communities are successional with seasonal changes. Wilde et al. (2005) conducted algal surveys at all known sites of AVM epornitics, as well as additional control reservoirs, to identify algal or cyanobacterial species that were either unique to AVM sites or of unusual abundance during the AVM season. A previously uncharacterized cyanobacterium of the order Stigonematales fit the temporal and spatial epidemiological evidence of disease occurrence. This epiphytic cyanobacterium was associated with lake vegetation, particularly hydrilla, was observed consistently at all known AVM sites, and was the dominant epiphyte present at most AVM sites during the late fall to early winter, when AVM is commonly observed (Wilde et al., 2005). At sites where AVM positive birds are diagnosed most frequently, the Stigonematales species covered up to 95% of the leaf surface area of the vegetation during these months. In addition, at control sites where AVM diagnosed birds are noticeably absent, the Stigonematales cyanobacterium was not dominant ($<$10% of the leaf surface area) and only infrequently observed.

The vegetation used in the present study was selected based on the recent AVM histories of each study site in addition to the presence or absence of the Stigonematales species. JSTL hydrilla producing AVM was extensively covered by the Stigonematales species, with 50–100% of the leaf surface area covered with colonies. In contrast, Lake Marion hydrilla, while containing numerous algal and cyanobacterial epiphytes, contained no colonies of the Stigonematales species, based on microscopic evaluation as well as molecular level analysis using real-time PCR (Wiley et al., 2007; Williams et al., 2007). Although the most obvious difference in the JSTL and Lake Marion vegetation is the presence or absence of the Stigonematales species, there could be other unknown factors involved in disease onset. To confirm the link between the Stigonematales cyanobacteria and AVM, the disease must be demonstrated from direct exposure of birds to pure cultures of the cyanobacteria species.

Although documented species susceptibility is currently confined to birds, the list of avian species continues to grow and additional research on mammalian susceptibility is needed. To date, AVM has not been found in mammals in the wild despite active surveillance at AVM sites during documented outbreaks in birds; although there was an anecdotal report of neurological signs in a beaver (*Castor canadensis*) at JSTL in 2001 (Fischer et al., 2006). Previous feeding trials have investigated mammalian susceptibility in mice and swine; yet, none of these animals developed lesions (Lewis-Weis et al., 2004; Rocke et al., 2005). The authors note, however, that the quantities consumed or duration of exposure may not have been adequate to induce disease.

The successful extraction of an AVM inducing fraction from hydrilla provides the first step toward its purification and characterization. In addition fractionation will allow for more refined *in vivo* studies including testing of species such as mice that will not readily consume vegetation, and will facilitate *in vitro* studies to gain further insight into its mechanism of action. To further characterize the toxic agent, additional fractionation of the methanolic extract is needed. At present, the extract contains a large mixture of compounds, including pigments and other plant components. Future research will be directed at the further fractionation of the crude extract, paired with animal bioassays to screen for toxic activity in the resulting fractions. Attempts to develop an *in vitro* bioassay to screen for AVM-associated toxic activity are also in progress. An *in vitro* assay would allow rapid, high-throughput screening of potentially toxic fractions and would thus be an extremely useful tool in the isolation of the toxic activity. Once a sufficiently purified toxic extract is obtained, it can then be evaluated analytically to determine the physical and chemical properties, and ultimately its structural identity.

While the overall bald eagle population continues to increase in the southeastern United States, local populations of resident eagles have been significantly impacted by AVM (Birrenkott, 2003). AVM lesions continue to be seen in wintering American coots where active monitoring is conducted (Fischer et al., 2006). In addition, recent data indicate that AVM has the potential to spread to new water bodies with the invasion of hydrilla and other exotic aquatic macrophytes and their associated epiphytes (Wilde et al., 2005), creating the potential to further impact populations

of eagles, coots, and waterfowl. The current study provides a critical tool needed to identify and characterize the etiologic agent, to better understand the potential impacts to wildlife populations, and to develop management strategies in an effort to curb those impacts.

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