TROPHIC TRANSFER OF A NOVEL CYANOTOXIN IN FISHES

by

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(Under the Direction of Robert B. Bringolf and Susan B. Wilde)

ABSTRACT

Aetokthonotoxin (AETX) is a formally undescribed neurotoxin produced by the cyanobacterium *Aetokthonos hydrillicola* (*Ah*). Animals that consume *Ah*-colonized vegetation may develop vacuolar myelinopathy (VM), a neurological condition causing motor impairment and death. We quantified AETX concentrations in the gastrointestinal tracts and fillets of secondary and tertiary consumer fishes from an *Ah*-inhabited reservoir. We also created a laboratory food chain involving *Ah*, an omnivorous fish, and a carnivorous fish to determine the trophic transferability of AETX in fishes. Finally, we used a swim chamber to quantify sublethal effects of AETX on fish swimming performance. Although AETX bioaccumulated in fishes, evidence of AETX biomagnification was not found. Fish did not develop VM, nor did AETX affect fish swimming performance. AETX accumulation in primary consumers should be quantified to determine the ecological risk of AETX in various food chains. Mammalian bioassays are also recommended to help determine the risk of AETX consumption in humans.

INDEX WORDS: *Aetokthonos hydrillicola*; aetokthonotoxin; vacuolar myelinopathy; AVM; J. Strom Thurmond Reservoir; Clarks Hill Reservoir; swimming performance

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The cyanobacterium *Aetokthonos hydrillicola* (*Ah*) inhabits reservoirs throughout the southeastern U.S.A. where it grows epiphytically on the invasive aquatic macrophyte, hydrilla (*Hydrilla verticillata*) (Wilde *et al.* 2005). Reservoir managers and wildlife biologists have recently become concerned about *Ah* because it produces a formally undescribed neurotoxin known as aetokthonotoxin (AETX). This novel cyanotoxin has been associated with vacuolar myelinopathy (VM), a neurologic condition in birds, fishes, reptiles, and amphibians that consume *Ah*-colonized hydrilla (referred to hereafter as *Ah*/hydrilla) (Thomas *et al.* 1998, Rocke *et al.* 2002, Birrenkott *et al.* 2004, Haynie *et al*. 2013, Mercurio *et al.* 2014, Wilde *et al*. 2014, Maerz *et al.* 2018). VM has primarily been reported in American coots (*Fulica americana*), which forage frequently on hydrilla, and in bald eagles (*Haliaeetus leucocephalus*) that consume affected coots. Thousands of American coots and hundreds of bald eagles have died from apparent AETX exposure and VM across the southern and southeastern U.S.A. since 1994 (Thomas *et al.* 1998, Rocke *et al.* 2002, Dodder *et al.* 2003, Wilde *et al.* 2014). VM has also been documented in mallard ducks (*Anas platyrhynchos*), buffleheads (*Bucephala albeola*), and ring-necked ducks (*Aythya collaris*, Augspurger *et al.* 2003) as well as Canada geese (*Branta canadensis*), great horned owls (*Bubo virginianus*), and killdeer (*Charadrius vociferus*) (Fischer *et al.* 2006).

The spread of *Ah* and VM is a concern because of the invasive nature of hydrilla. Hydrilla can tolerate a wide range of pH, has few natural predators, and reproduces quickly via sexual and asexual methods. These traits allow hydrilla, and therefore possibly *Ah* and VM, to spread readily to new locations (Jacono *et al*. 2019). For example, hydrilla was first discovered

in the U.S.A. in Florida in 1960 (Blackburn and Weldon 1969) and has since been documented in over 30 states as of April 2018 (Jacono *et al*. 2019). Although *Ah* is present on hydrilla throughout the year in *Ah*-colonized reservoirs, it is suspected to produce AETX only during the autumn and winter (Rocke *et al.* 2005, Wilde *et al.* 2005) because clinical symptoms of VM, including motor impairment, seizures, ataxia, and death (Larsen et al. 2002), have only been documented in wildlife between October and March.

Experimental feeding of *Ah*/hydrilla has induced VM in sentinel and laboratory specimens including mallard ducks (Rocke *et al.* 2002, Birrenkott *et al.* 2004, Rocke *et al.* 2005, Wiley *et al.* 2008, Haynie *et al.* 2013), chickens (*Gallus domesticus*, Lewis-Weis *et al.* 2004, Mercurio *et al.* 2014, Dodd *et al.* 2016), and red-tailed hawks (*Buteo jamaicensis*, Fischer *et al.* 2003). Similar laboratory feeding trials have also induced VM in non-avian species including painted turtles (*Chrysemys picta*, Mercurio *et al.* 2014), water snakes (*Nerodia* sp., unpublished data from Martin *et al.* 2018), and grass carp (*Ctenopharyngodon idella*, Haynie *et al.* 2013). During all of these studies, VM was diagnosed via histological analyses with characteristic vacuolation patterns in the brain's optic lobe as described by Thomas *et al.* (1998). Although vacuolation patterns in the brains of these laboratory specimens have been consistent among taxa, the severity of vacuolation and of observed clinical symptoms, namely various forms of motor impairment, differed greatly among and within species studied.

Despite recent efforts to describe effects of *Ah*/hydrilla and AETX in various taxa, the physiological and ecological effects of Ah/hydrilla and AETX exposure remain poorly understood in fishes. Many fishes in southeastern U.S. reservoirs feed and live within hydrilla beds, so they may be exposed either directly or indirectly to AETX at least periodically. The mechanism by which AETX transfers to secondary consumers (i.e., predators that prey on VM-affected animals) also remains unclear. For example, whether AETX bioaccumulates in the tissues of secondary consumers or transfers passively from the gastrointestinal (GI) tracts of primary

consumers to those of secondary consumers without being assimilated into muscle tissues has yet to be determined.

To further understand the extent and risks of AETX exposure in fishes, we investigated the natural occurrence of AETX in wild fishes of multiple trophic guilds (primary consumers, secondary consumers, and tertiary consumers) from an *Ah*-colonized reservoir. Fishes evaluated included spottail shiners (*Notropis hudsonius*), bluegill (*Lepomis macrochirus*), redear sunfish (*L. microlophus*), warmouth (*L. gulosus*), and largemouth bass (*Micropterus salmoides*). AETX concentrations were measured in the GI tracts and lateral musculature (fillets) of each species except spottail shiners, for which whole body concentrations were determined due to their small size. Understanding which fishes may accumulate AETX will help determine the ecological risks of AETX consumption for various trophic guilds of fishes and will help identify ecologically relevant model fish species for future laboratory studies. Identifying fish species and specific tissues therein that may accumulate AETX will also help identify any potential health concerns for piscivorous animals, including people, that consume fishes from *Ah*-colonized waters.

The potential for trophic transfer of AETX between fishes, as well as the potential for AETX to biomagnify in predatory fishes, remain unknown. Therefore, during a controlled laboratory study, we quantified dietary trophic transfer of AETX from *Ah*/hydrilla to an omnivorous fish (tilapia, *Oreochromis aureus* x *O. niloticus*) and then to a carnivorous fish (largemouth bass). Finally, we quantified the physiological effects of dietary AETX exposure on motor function in largemouth bass. Burst swimming speed (U_{burst}) is an ecologically relevant endpoint for measuring sublethal effects of environmental stressors in fish because swimming performance represents a fish's ability to capture prey and evade predators (i.e., Darwinian fitness) (Sprague 1971, Reidy *et al*. 1995, Hammer 1995, Reidy *et al*. 2000). If fishes exposed to AETX exhibit similar clinical symptoms of VM as birds, then exposed fish should demonstrate impaired motor function (i.e. U_{burst}), a form of reduced fitness. Largemouth bass were chosen for

these studies because of their high trophic status and because they are among the most popular sport fishes in the United States (USDI 2018). Furthermore, anglers commonly harvest this species from reservoirs throughout the southeastern U.S. (e.g. Lake Tohopekaliga, Myers *et al*. 2008 and J. Strom Thurmond Reservoir, W. E. Houck and D. M. Rankin, South Carolina Department of Natural Resources, unpublished data) where VM outbreaks have been documented. Results from our studies will help increase understanding of how AETX moves through aquatic food webs and how AETX may affect fishes. Furthermore, comparing those effects in fishes with the effects of AETX exposure in other taxa and in other trophic guilds will help identify which species may have the greatest risk of being affected by future VM outbreaks. Finally, our studies will help identify whether fishes may be vectors for transmitting AETX and VM to piscivorous animals and humans.

Literature Review

Biological and ecological characteristics of VM

The first recorded VM outbreak affected bald eagles and American coots from DeGray Lake and Lakes Ouachita and Hamilton in southwestern Arkansas, U.S.A. during the winter of 1994-1995 (Thomas *et al*. 1998). Beginning shortly after arrival from autumn migration in November 1994, bald eagles were observed overflying their perches and colliding with rock walls. These signs of motor impairment were observed again in bald eagles at the same locations during the winter of 1996-1997. During this second outbreak, eagles were also observed preying upon American coots which also displayed signs of motor impairment. Twenty-nine bald eagles died from VM during the 1994-1995 outbreak, and 26 more bald eagles died from VM during the 1996-1997 outbreak. Approximately 5% of the coot population displayed clinical symptoms including various forms of motor impairment during the 1996-1997 outbreak; however, no American coot deaths were reported.

Soon after the 1996-1997 VM outbreak, Thomas *et al.* (1998) described the diagnostic vacuolation patterns in the brains from these birds. Five eagle carcasses from the 1994-1995 outbreak and 21 eagle carcasses and 77 coot carcasses from the 1996-1997 outbreak underwent thorough post mortem examinations in an attempt to diagnose the cause of the disease. All toxicological screenings, bacterial cultures, parasitological investigations, and virus isolation tests returned negative results. However, microscopic examination of all eagles and 81% of coots revealed "bilaterally symmetrical vacuolation of the white matter of the central nervous system," including the spinal cord and all areas of the brain, most noticeably in the optic tectum (Thomas *et al.* 1998). The authors further describe the lesions as being "characterized by diffuse spongiform myelinopathy, compatible with intramyelinic edema." The vacuolation patterns described therein are still cited as the main reference for diagnosing VM in affected animals. Thomas *et al.* (1998) is therefore the main reference for diagnosing histological evidence of VM in the studies presented here.

Most VM outbreaks following those in 1996-1997 have been reported in reservoirs in the southeastern U.S.A. The earliest of these reports included American coots from Georgia and North Carolina and bald eagles from Georgia, North Carolina, and South Carolina (Fischer *et al*. 2002). This study also reported symptoms of VM in wild mallard ducks, Canada geese, ring-necked ducks, buffleheads, great horned owls, and killdeer (described in greater detail by Rocke *et al.* (2002), Augspurger *et al.* (2003), and Fischer *et al.* (2006)) (Appendix A). These studies reported lesions similar to those present in the coots and eagles (Thomas *et al.* 1998), suggesting that VM affects a much wider range of species across a broader geographic distribution than was first reported.

Shortly following these diagnostic studies, clinical symptoms of VM were formally described in wild coots using physical, neurological, and hematological examinations (Larsen *et al*. 2002). The authors examined 26 symptomatic coots from a reservoir from which VM has been reported and 12 asymptomatic coots from a reservoir from which VM has never been

reported. Their findings most commonly identified ataxia, decreased withdrawal reflexes, absence of pupillary light responses, and head tremors among the most common clinical symptoms of VM-affected coots. Coots from the reservoir that lacked historical VM outbreaks did not display these symptoms. VM was confirmed in all 26 symptomatic coots via histological examination of intramyelinic edema as described by Thomas *et al.* (1998). Larsen *et al.* (2002) is therefore an important reference for diagnosing cases of VM in birds from both wild and laboratory studies. This study provided key information to help determine when to euthanize subjects during laboratory exposures to minimize suffering of the test animals. The clinical symptoms described by Larsen et al. (2002) also allow researchers to diagnose the presence of VM in living animals without having to euthanize them to observe brain tissue. Finally, and perhaps most importantly, the clinical symptoms describing motor impairment in birds (e.g. ataxia, imbalance, uncoordinated movements) are readily adaptable for non-avian species such as fishes, the focus of the present studies.

In 2002, wild American coots and farm-raised mallard ducks were introduced to a VM-affected lake (Lake Surf, North Carolina, U.S.A.) and developed diagnostic symptoms of VM (as described by Larsen *et al.* 2002) in as few as five days (Rocke *et al.* 2002). This study provided experimental support for the hypotheses that VM occurrence is site-specific, seasonal, and likely caused by a natural chemical as opposed to one of anthropogenic origin. This experimental induction of VM in sentinel birds spearheaded the investigation for the causative agent behind VM.

The search for the causative agent for VM in birds

To identify the cause of VM, Larsen *et al.* (2003) attempted to induce VM in waterfowl via feeding trials. The authors fed water, sediment, and hydrilla material from a VM-affected reservoir to mallard ducks. VM was not induced in any mallards despite the hydrilla being collected in December, when VM outbreaks are known to occur (e.g. Thomas *et al.* 1998, Rocke

et al. 2002). Three factors may help explain why mallards in the Larsen *et al.* (2003) study failed to develop VM. First, the exposure period (seven days) may have been too brief to elicit VM symptoms. Second, the amount of food fed to each bird (10 grams per day) may have been too small to elicit VM symptoms. Third, the AETX concentration in the hydrilla samples may have been relatively low as compared with *Ah*/hydrilla samples from other years. Similarly, the hydrilla may have lacked *Ah* altogether. Because Rocke *et al.* (2002) showed that sentinel birds feeding *ad libitum* on hydrilla could develop VM in as few as five days, it remains most likely that Larsen *et al.* (2003) either did not feed their mallards enough *Ah*/hydrilla to induce VM or the *Ah* may have produced less AETX during that year as compared with most other years. However, the identity of AETX remained unknown at that time, so methods did not exist to quantify AETX in *Ah*/hydrilla samples. In response to the surprising lack of VM induction reported by Larsen *et al.* (2003), precautions were made in our studies to feed laboratory specimens *Ah*/hydrilla with relatively high AETX concentrations as compared with *Ah*/hydrilla samples from all available years. Additionally, lab specimens were fed *Ah*/hydrilla *ad libitum* to allow animals to forage on *Ah*/hydrilla at natural rates. We suspect *ad libitum* feeding also increased the likelihood of detecting AETX in fish tissues in our studies.

Dodder *et al.* (2003) used chromatographical analyses to improve upon initial investigations by Larsen *et al.* (2003) to measure concentrations of many organic and inorganic contaminants in sediments and coot tissues from four lakes with previous VM documentation and from two lakes lacking previous VM documentation. Dodder *et al*. (2003) suggested that the causative agent of VM was likely unassociated with the benthos, leading Birrenkott *et al.* (2004) to investigate the hypothesis that a biological agent may cause VM. Birrenkott *et al.* (2004) fed hydrilla from VM-affected reservoirs to mallard ducks and provided the strongest evidence at the time of their publication that hydrilla consumption may induce VM in waterfowl. Furthermore, the authors hypothesized that an unknown Stigonematalan cyanobacterial species (*Ah*, since

reclassified under the order Nostocales) associated with hydrilla may be a causative agent for VM in waterfowl.

Wilde *et al.* (2005) conducted extensive surveys of aquatic macrophyte communities and associated epiphytes in 13 reservoirs with historical VM outbreaks and 12 reservoirs lacking historical VM outbreaks. These surveys showed that *Ah* was present in all reservoirs with historical VM outbreaks and was absent in all reservoirs lacking historical VM outbreaks. The authors also conducted a feeding trial with sentinel mallard ducks at a pond in South Carolina with historical VM outbreaks and found that consumption of *Ah*/hydrilla led to VM development in all 15 ducks recaptured during the experiment. Wilde *et al.* (2005) convincingly linked *Ah* to VM, and the authors hypothesized that an associated cyanotoxin, which was undescribed at the time (since called AETX), was likely produced by *Ah* and may induce VM in birds.

Further evidence for the link between *Ah* and VM was provided by Wiley *et al.* (2007), who conducted two feeding trials with mallard ducks and chickens. The first feeding trial, conducted in October 2003, failed to induce VM in any birds. The second trial, conducted in November and December 2003, induced VM in 10 of 12 experimental mallard ducks and in zero of six control ducks. This study further supported the role of *Ah* in inducing VM in waterfowl and again highlighted the seasonality of VM. Perhaps most importantly, this study supported the hypothesis proposed by Wilde *et al*. (2005) that *Ah* alone may not be the cause of VM because the *Ah*/hydrilla samples used during the October trials failed to induce VM. The authors agreed with Wilde *et al*. (2005) that *Ah* may produce a toxin responsible for VM and further suggested that *Ah* may only produce this toxin seasonally.

Wiley *et al.* (2008) further supported the hypothesis posed by Wilde *et al*. (2005) and Wiley *et al.* (2007) that the toxic agent is not *Ah* cyanobacteria itself, but rather an associated compound. The authors ran samples of *Ah*/hydrilla (VM site) or hydrilla lacking *Ah* (reference site) through a series of solvents of increasing polarity to extract any hypothesized toxic agent from the *Ah*/hydrilla complexes. Mallard ducks were then randomly assigned to be fed the

extracts from either the VM site or the reference site. Ducks that were fed extracts from VM site *Ah*/hydrilla developed VM, but ducks that were fed extracts from reference site hydrilla did not develop VM. The protocol developed by Wiley *et al*. (2008) successfully extracted AETX from *Ah*/hydrilla, enabling researchers to eventually isolate and describe the causative agent responsible for recent VM outbreaks (S. Wilde, University of Georgia, unpublished data). We adapted the AETX extraction protocols described by Wiley *et al*. (2008) for extraction of AETX from fish tissues and *Ah*/hydrilla in our studies.

AETX susceptibility in non-avian species

As the search for the cause of VM was concluding, research shifted focus towards non-avian susceptibility to AETX. Several species of reptiles, amphibians, and fishes forage directly on hydrilla (e.g. Hardin *et al*. 1984, Fields *et al*. 2003, Adler *et al*. 2018) and may therefore be susceptible to VM. Grass carp are of great concern to reservoir managers because these fish are commonly stocked as juveniles in reservoirs throughout the southeastern U.S.A. to eat hydrilla (see Pípalová 2006 for a review) and may be consumed by larger fish or avian predators. Haynie *et al*. (2013) investigated the effects of *Ah*/hydrilla consumption in sentinel grass carp by introducing them to enclosed *Ah*/hydrilla beds and allowing them to forage *ad libitum* for five weeks. In a separate trial, the authors kept grass carp in large tanks in a laboratory and manually fed the carp *Ah*/hydrilla. In both trials, clinical symptoms of VM were not observed in the grass carp, but diagnostic lesions were discovered in carp fed toxic *Ah*/hydrilla and not in carp fed nontoxic *Ah*/hydrilla (i.e. *Ah*/hydrilla lacking AETX). Haynie *et al.* (2013) therefore showed that VM can be experimentally induced in non-avian taxa via the consumption of *Ah*/hydrilla. Tissues from grass carp from both feeding treatments were then fed to chickens which did not develop clinical or diagnostic symptoms of VM. The authors therefore suggested that the risk of VM to predatory birds via consumption of VM-affected fishes may be low. Given the lack of clinical symptoms in grass carp during this study, and the paucity of

additional studies regarding fish and *Ah*/hydrilla, whether consumption of toxic *Ah*/hydrilla by fishes is likely to affect their fitness remains unknown. Our studies into the occurrence of VM in wild fishes, accompanied by physiological investigations into the effects of AETX on fish fitness, are therefore necessary to provide critical novel information about the risk of VM to fishes and animals that consume them.

One year after Haynie *et al.* (2013) reported inducing VM in grass carp, Mercurio *et al*. (2014) reported induction of VM in painted turtles. Turtles fed toxic *Ah*/hydrilla developed similar diagnostic and clinical symptoms of VM as has been documented in most previous studies. However, the turtles developed symptoms much more slowly (>80 days) than most birds (as few as five days, Rocke *et al.* 2002). Given the absence of clinical symptoms in grass carp (Haynie *et al*. 2013) and the length of time required to elicit clinical symptoms in turtles (Mercurio et al. 2014), it appears that an animal's metabolic rate may affect the duration of exposure to AETX necessary to elicit clinical symptoms of VM. If metabolic rate correlates inversely with the onset of clinical symptoms of VM, then fishes and other ectotherms may require longer feeding trials before clinical symptoms are observed. It therefore remains undetermined whether Haynie et al. (2013) did not observe clinical symptoms of VM in grass carp because these fish are not susceptible to VM or because the duration of AETX exposure was too brief. Still, the broadly expanding list of taxa which may be susceptible to VM suggests that AETX may have a relatively general mode of action, likely putting an even greater diversity of taxa at risk for VM. This growing list of taxa that are susceptible to VM further justifies the need to describe the potential risks of AETX consumption in fishes and consumers thereof, including humans.

Maerz *et al.* (2018) further highlight the ecological complexity of AETX by demonstrating various sensitivities to *Ah*/hydrilla among tadpoles of different frog species. Tadpoles of some frog species expressed lower survival rates upon consumption of toxic *Ah*/hydrilla as compared with conspecifics that consumed nontoxic *Ah*/hydrilla; however, some frog species did not show clinical symptoms of VM after consuming toxic *Ah*/hydrilla. The results from this study further

show the potentially diverse and unpredictable effects that AETX could have in *Ah*-colonized reservoirs. Furthermore, as the list of taxa that are affected by AETX increases, so too should concern regarding the spread of *Ah*/hydrilla into ecosystems previously unoccupied by *Ah*.

Effects of AETX in secondary consumers

Fischer *et al.* (2003) provided experimental support for the hypothesis that AETX may accumulate in predatory birds after eating American coots with VM. The authors induced VM in 5/5 red-tailed hawks that were fed tissues from VM-affected American coots and failed to induce VM in the one hawk fed tissues from healthy American coots. Despite the lack of clinical signs of VM in all six hawks and the small sample size, the diagnostic evidence suggests that predatory birds can develop VM from eating tissues from VM-affected coots. This results from this study support the hypothesis that AETX may bioaccumulate in carnivorous species preying on animals which have consumed toxic *Ah*/hydrilla.

More evidence for trophic transfer of AETX to secondary consumers emerged when VM was induced in chickens that consumed hydrilla and tissues from wild coots collected during VM outbreaks (Lewis-Weis *et al*. 2004). This study also showed that secondary consumers may only be at risk for VM when they consume their prey's GI tract. For example, chickens never developed VM after consuming muscle tissue from VM-affected coots, but all chickens consuming GI tracts from the same coots developed VM. Results from this study therefore suggest that AETX may not accumulate in coot muscle tissue, given that neither hawks (Fischer *et al*. 2003) nor chickens (Lewis-Weis *et al*. 2004) developed VM after consuming muscle tissues from VM-affected coots. These results suggest that predators which consume their prey whole, for example most wildlife including raptors and predatory fishes, may be at greater risk for developing VM than other consumers like humans which usually avoid eating the viscera of their prey.

Dodd *et al.* (2016) suggested that invertebrate consumers of *Ah*/hydrilla can act as vectors for inducing VM in secondary consumers. During this study, apple snails (*Pomacea maculata*) were fed *Ah*/hydrilla before being fed to chickens, which served as a surrogate for the endangered Florida snail kite (*Rostrhamus sociabilis*). Although neither clinical symptoms nor diagnostic symptoms could feasibly be detected in the snails, VM was induced in the chickens that ate snails which had eaten *Ah*/hydrilla from a VM-affected reservoir. VM was not induced in chickens that ate snails which had eaten *Ah*/hydrilla from a VM-unaffected reservoir. This suggests that many types of prey species, including non-avian prey species, could act as vectors through which secondary consumers could develop VM. As clinical symptoms of VM have been detected neither in fishes (Haynie *et al.* 2013) nor in snails (Dodd *et al*. 2016), but given that the consumption of snails fed AETX did induce VM in birds, it remains imperative to continue the investigation into whether fishes can develop VM and act as vectors for piscivorous predators.

Bioaccumulation and trophic transfer of other cyanotoxins in fishes

Bioaccumulation describes the process by which xenobiotics assimilate into plant and animal tissues. Bioaccumulation occurs via passive or active diffusion of xenobiotics from the environment (e.g. the water column) and from food into the blood, skin, or other tissues of organisms or via trophic transfer of xenobiotics from prey to predator. Many compounds that bioaccumulate in aquatic organisms are anthropogenic in origin, including pharmaceuticals and pesticides (see Zenker *et al.* 2014 for a review). However, naturally occurring compounds including cyanotoxins also bioaccumulate in aquatic organisms including fishes (see Ferrão-Filho and Kozlowsky-Suzuki 2011 for a review). For example, microcystins were found in fillets of fishes occupying a variety of trophic guilds, including herbivores / detritivores (largescale suckers, *Catostomus macrocheilus*), omnivores (e.g. pumpkinseed sunfish, *Lepomis gibbosus*), and carnivores (e.g. largemouth bass and rainbow trout, *Oncorhynchus mykiss*). This survey of

microcystins in wild fishes suggests that many trophic guilds are susceptible to bioaccumulation of microcystins (Hardy *et al.* 2015). However, this accumulation may not be due to trophic transfer in most cases. Although microcystins have been shown to transfer from prey to predatory fishes in few cases (Smith and Haney 2006; Lance 2008), a meta-analysis of microcystin concentrations in aquatic organisms of various trophic guilds suggests that the potential for trophic transfer of microcystins to secondary and tertiary consumers is low (Kozlowsky-Suzuki *et al.* 2012). Furthermore, this meta-analysis suggested that microcystins likely biodilute (i.e., decrease in concentration as trophic level increases within a food web) in aquatic ecosystems. Contrary to this general trend, however, the same study reports that microcystins may biomagnify in zooplanktivorous fishes (Kozlowsky-Suzuki *et al.* 2012). The susceptibility of secondary and tertiary consumers to AETX via bioaccumulation and trophic transfer remains difficult to infer from these microcystin studies because microcystins and AETX likely have different modes of action. For example, microcystins are unhalogenated hepatotoxins (Rinehart *et al*. 1994, Dawson 1998), whereas AETX is a highly-brominated neurotoxin (S. Wilde, University of Georgia, unpublished data).

Other cyanotoxins, including anatoxins and saxitoxins, are neurotoxic (like AETX) and have been shown to affect fish survival and development. Pure anatoxin-a altered the heart rate of larval zebrafish (*Danio rerio*, Oberemm *et al.* 1999) but was effectively harmless to embryonic common carp (*Cyprinus carpio*, Osswald *et al.* 2008). Crude extracts from *Anabaena* sp. (strain 37) cultures which produce anatoxin-a have been shown to impair development and increase mortality rates in common carp embryos (Osswald *et al.* 2008). Although Oberemm *et al.* (1999) also showed that pure saxitoxin impaired development and hatching rate of zebrafish embryos, the authors agreed with Osswald *et al*. (2008) that the effects of pure cyanotoxins on aquatic organisms may differ from those of crude aqueous extracts of cyanotoxins. In many cases, the synergistic actions among toxins in crude cyanobacterial extracts may increase toxicity as compared with those of the pure toxins individually (Ferrão-Filho and Kozlowsky-Suzuki 2011).

Cyanotoxins have also been shown to affect fish behavior and motor function including swimming performance (see Ferrão-Filho and Kozlowsky-Suzuki 2011 for a brief review). For example, Lefebvre *et al*. (2005) documented inhibited swimming behavior in larval Pacific herring (*Clupea harengus pallasi*) after exposure to saxitoxins. In contrast, Ferrão-Filho *et al*. (2008) showed that exposure to a saxitoxin-producing cyanobacterial strain led to increased swimming activity in zebrafish. Anatoxins have also been shown to affect fish behavior; exposure to an anatoxin-producing strain of *Anabaena* sp. increased opercular movement (a metric of respiration rate and stress) and abnormal swimming behavior in common carp (Osswald *et al*. 2007). Given the physiological and behavioral effects of neurotoxic cyanobacterial strains in fishes, exposure to AETX from *Ah*/hydrilla may lead to altered swimming behavior and therefore decreased fitness in fishes.

Swimming performance as a metric of fish fitness

A fish's ability to swim greatly affects its fitness (Reidy *et al*. 1995, Reidy *et al*. 2000, Plaut 2001). For example, swimming is either the primary or the only means for most fishes to avoid detection or capture by predators. Similarly, a fish's ability to chase or ambush prey, to find mates and spawning grounds, and to migrate also depends greatly on its swimming ability (Reidy *et al*. 1995, Drucker 1996, Reidy *et al*. 2000). If behavioral effects of VM are similar in fishes as they are in birds (e.g. motor imbalance, ataxia, and seizures), then fishes afflicted with VM may suffer decreased fitness resulting from impaired swimming ability. Swimming performance tests may therefore provide researchers with methods for detecting sublethal effects of AETX exposure on fish fitness, including a fish's ability to capture food, evade predators, reproduce, and migrate.

Quantifying the relationship between a fish's swimming performance and its potential success at accomplishing ecological tasks (e.g. feeding, avoiding predators, spawning, and migrating) has proven to be difficult. Most studies of fish swimming performance occur under

laboratory conditions which fail to resemble natural conditions (Plaut 2001). For example, fishes used during swimming performance experiments are usually kept in tanks or experimental ponds, are fed either by hand or by automation, and are swam in cylindrical chambers like those described by Brett (1964) which inhibit voluntary movement. Under these conditions, fish cannot be expected to swim, feed, or otherwise behave in similar ways as they would in the wild. To measure fish swimming performance under natural conditions, swimming performance must be measured *in situ*. However, measuring fish swimming performance in the field is difficult because researchers typically cannot make direct visual observations of the fish. Although the development of biotelemetry has allowed scientists to track voluntary fish swimming velocities in the field (e.g. Hanson *et al*. 2007, Hasler *et al*. 2009), the behaviors associated with these velocities and the fish's success at these behaviors, for example food capture rate and mating success, remain in question with biotelemetry alone. As Plaut (2001) describes when discussing the ecological relevance of studying fish swimming performance, "the ideal way to establish such relevancy would be to mark fish with known pre-measured swimming abilities (measured as critical swimming speed of each individual), release them into their natural habitat, and follow their reproductive success, or more practically, to recapture them, and then correlate their survival with their individually pre-measured critical swimming speeds." As Plaut noted, such a study has never been published.

Three types of swimming performance tests have been developed to date: sustained, prolonged, and burst (Beamish 1978). Sustained swimming tests measure aerobic swimming which can be continued indefinitely and represent migratory and routine swimming behaviors (Brett 1964, Beamish 1978, Reidy *et al*. 1995, Reidy *et al*. 2000). Prolonged swimming tests measure primarily aerobic swimming which can be continued for lengthy but finite periods of time and represent somewhat urgent behaviors, for example foraging or navigating through swift waters. Prolonged swimming is difficult to differentiate from sustained swimming based solely on swimming velocity, but unlike sustained swimming, prolonged swimming results in both

physiological (muscular fatigue) and behavioral endpoints (Nelson 1990, Plaut 2001). Finally, burst swimming tests measure anaerobic swimming capacity which can only be continued for fewer than approximately 15 seconds at a time. Burst swimming represents a fish's ability to capture prey and escape predators (Blaxter 1969, Reidy *et al*. 1995, Reidy *et al*. 2000). Because predator-prey interactions are the most ecologically relevant for investigating the possibility of AETX trophic transfer between taxa, and because sustained and prolonged swimming tests require vast amounts of time (Brett 1964, Beamish 1978), we selected burst swimming speed as the most appropriate endpoint for fish swimming performance in our study.

As with sustained and prolonged swimming tests, burst swimming protocols may be either increased velocity tests or fixed velocity tests. Increased velocity tests involve gradual and indefinite step-wise increases of the water velocity until the fish fatigues. The water velocity at the time of fatigue may therefore differ between test subjects. Fixed velocity tests involve gradual and finite step-wise increases in the water velocity until a fixed, relatively slow water velocity is reached. The time is then recorded from the moment the fixed velocity is reached until the test subject reaches exhaustion. The time necessary to complete these swimming performance tests is difficult to predict and is usually greater than increased velocity tests. Increased velocity tests are also thought to be more ecologically relevant than fixed velocity tests because increased velocity tests measure a maximum swimming velocity as would be required to capture prey or evade predators, whereas the fixed velocity chosen for fixed velocity tests often lack biological and ecological context (Hammer 1995). Finally, increased velocity tests are more effective than fixed velocity tests at minimizing variability among individual swimming performances (Hammer 1995), which Kolok (1992) and Marras *et al*. (2009) show can be large enough to mask the effects of experimental treatments. Using increased velocity tests therefore likely maximizes the power to detect possible effects of AETX on fish swimming performance as compared with fixed velocity tests. We therefore selected an increased velocity burst swimming protocol to quantify the effects of AETX on largemouth bass motor function.

Swimming performance as an endpoint for measuring the effects of xenobiotics in fishes

Swimming performance has been noted as one of best ways to quantify sublethal effects in fishes from various stressors (Cairns 1966), including toxins (Sprague 1971). Swimming performance tests have become a choice for bioassessment because most swimming performance tests are relatively inexpensive to conduct, use portable equipment, require relatively little time especially for prolonged and burst swimming endpoints, assess cumulative physiological and ecological effects on a test subject, and are nonlethal (Hammer 1995). Swimming performance tests have consequently been used to quantify sublethal effects of heavy metals (e.g. Beaumont *et al*. 1995, Taylor *et al*. 2000, Rajotte and Couture 2002, McKenzie *et al*. 2007), various pesticides (Kumaraguru and Beamish 1983, Cripe *et al*. 1984, Beggel *et al*. 2010), crude oil (Hicken *et al*. 2011, Mager *et al*. 2014), and other pollutants and environmental factors on fishes (see Hammer (1995) and Plaut (2001) for reviews). A great variety of swimming performance test protocols has accompanied the variation in xenobiotics which these protocols were developed to assess. Swimming performance tests have involved many species, measured many different endpoints, and employed several types of swim chambers. A universally-preferred swimming protocol appears not to exist, as most studies either adapt precedent protocols to accommodate their experimental needs or develop new protocols altogether.

Swimming behavior has also been used to assess the effects of cyanotoxins in fishes. Microcystin-LR has been shown to induce nonlinear responses in voluntary swimming behavior of zebrafish (*Danio rerio*) and sunbleak (*Leucaspius delineatus*), increasing swimming activity at low concentrations and decreasing activity at higher concentrations (Baganz *et al*. 2004). Microcystin-LR had a similar effect on zebrafish when exposed to the bacterium *Microcystis aeruginosa*, which caused zebrafish to swim shorter distances and spend more time immobile in their tanks (Kist *et al*. 2011). Beta-methylamino-L-alanine (BMAA), a neurotoxin produced by

many families of cyanobacteria, induced weaker swimming and increased fatigue in adult zebrafish (Powers *et al*. 2017). However, all of these studies used motion-tracking cameras to quantify voluntary swimming velocities while the subjects were confined in tanks. The potential effects of cyanotoxin exposure on swimming behaviors representative of open-water foraging and predator evasion (Hammer 1995, Reidy *et al*. 2000, Plaut 2001) remain unknown. The results of our burst swimming performance tests will clarify the possible effects of cyanotoxin exposure on swimming performances of an economically and recreationally important fish.

Understanding how AETX accumulation and VM induction differ among trophic levels and among trophic guilds of fishes will help determine which species and which feeding behaviors may put animals at risk for AETX exposure. Comparing differences in AETX accumulation among trophic levels will also help determine whether AETX biomagnifies through fish food chains. Conducting burst swimming performance tests is expected to provide a relatively simple and inexpensive method for quantifying sublethal effects of AETX in fishes. Investigations of AETX dynamics in wild fishes and controlled laboratory dietary studies with fish will be synthesized to determine ecological risks of AETX at the food web level rather than at the food chain level as previous laboratory feeding trials have addressed. Potential risks of AETX exposure to humans via fish consumption are also discussed, and results presented herein provide baseline data that future studies can use to help evaluate specific health concerns for consuming fish harvested from *Ah*-inhabited reservoirs as well as for determining whether fish consumption advisories are necessary for these reservoirs.

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CHAPTER 2

A SURVEY OF AETOKTHONOTOXIN IN FISHES FROM J. STROM THURMOND RESERVOIR, GEORGIA/SOUTH CAROLINA, U.S.A.[1]

[1] Pelletier, A. R., S. B. Wilde, A. C. Camus, T. J. Phillips, and R. B. Bringolf. To be submitted to *Environmental Toxicology*.

Abstract

Aetokthonotoxin (AETX) is a novel cyanotoxin associated with the cyanobacterium *Aetokthonos hydrillicola* (*Ah*). Waterfowl foraging on *Ah*-colonized hydrilla (*Hydrilla verticillata*) have developed vacuolar myelinopathy (VM), a neurological condition which causes motor impairment, seizures, ataxia, and death. Bald eagles (*Haliaeetus leucocephalus*) have expressed similar symptoms after consuming American coots (*Fulica americana*) afflicted with VM. Recent laboratory feeding experiments suggest that fishes, reptiles, and amphibians may also develop VM after consuming *Ah*-colonized hydrilla. However, VM has never been investigated in wild fishes despite many fishes frequently foraging and finding refuge within hydrilla beds. We quantified AETX concentrations in the gastrointestinal tracts and axial musculature (fillets) of five fish species occupying different trophic guilds and trophic levels from J. Strom Thurmond Reservoir, a water body with a well-documented history of VM outbreaks in waterfowl. Although histological evidence of VM (e.g., brain lesions) was not detected in any fishes, AETX was detected in all species, ranging from 49.3 ± 1.5 (mean ± sd) ppb dry weight in warmouth (*Lepomis gulosus*) muscle to 6,629.2 ± 7,192.4 ppb dry weight in bluegill (*L. macrochirus*) gastrointestinal tracts. Mean gastrointestinal tract AETX concentrations were approximately 70 times greater in secondary consumers (bluegill and redear sunfish, *L. microlophus*) than in tertiary consumers (warmouth and largemouth bass, *Micropterus salmoides*). Muscle AETX concentrations (and whole body concentrations for spottail shiners, *Notropis hudsonius*) were similar among all five species. The discovery of AETX in muscle of omnivorous and carnivorous fishes suggests that AETX likely bioaccumulates in fishes via multi-step trophic transfer; however, AETX was not observed to biomagnify in fishes. The physiological effects of AETX on fishes and piscivorous animals, including humans, should therefore be investigated.

INDEX WORDS: *Aetokthonos hydrillicola*; vacuolar myelinopathy; Clarks Hill Reservoir; cyanobacteria; hydrilla

Introduction

The cyanobacterium *Aetokthonos hydrillicola* (*Ah*) produces a recently discovered neurotoxin referred to hereafter as aetokthonotoxin (AETX). Animals that consume this cyanotoxin have developed vacuolar myelinopathy (VM), a neurological condition that causes motor impairment, ataxia, seizures, and death.[1-4] VM was first documented during the winters of 1994-1995 and 1996-1997 in American coots (*Fulica americana*) and bald eagles (*Haliaeetus leucocephalus*) in Arkansas[1] and has since been reported throughout the southeastern U.S.A. (Appendix A).[5-8] To date, all VM outbreaks have been reported during autumn and winter, possibly because *Ah* may only produce AETX then.[3,9]

Birds develop VM after consuming aquatic macrophytes that are colonized by epiphytic *Ah*.[3-4] The spread of *Ah*, and therefore VM, to new locations concerns natural resource managers due to the invasive nature of *Ah*'s primary host plant, hydrilla (*Hydrilla verticillata*). Hydrilla can tolerate wide ranges of pH, has few predators, grows rapidly, and can reproduce sexually and asexually. These traits allow hydrilla to outcompete native plants for light and micronutrients and to rapidly invade new environments.[10] For example, hydrilla was first reported in the U.S.A. in Florida in 1960[11] and as of April 2018 has been documented in over 30 U.S. states.[10] The invasiveness of hydrilla increases the potential for *Ah* to spread to new ecosystems, putting an expanding list of species at risk for AETX exposure and VM development.

Eight bird species which either consume hydrilla or which prefer hydrilla beds as habitat[12-16] have been documented with VM.[1,5-8] However, many non-avian species may also be at least indirectly exposed to *Ah* given their associations with hydrilla. For example, hydrilla beds provide habitat for many aquatic macroinvertebrates[17-18] and fishes.[17,19-21] Hydrilla beds provide suitable cover habitat for economically important game fishes including lepomid sunfishes (*Lepomis* spp.) and black basses (*Micropterus* spp.), especially during early life stages which are critical for growth and development.[17,20,22] Some turtle species will also eat

hydrilla when its abundance is high.[23-24] Grass carp (*Ctenopharyngodon idella*) forage actively on hydrilla and have been introduced in many aquatic ecosystems to reduce hydrilla.[25] The use of hydrilla as food and as habitat by many species highlights the need to investigate VM occurrence in non-avian taxa.

Although field investigations have not yet been conducted, laboratory feeding trials confirm that non-avian animals may develop VM upon consumption of *Ah*-colonized hydrilla (referred to hereafter as *Ah*/hydrilla). Fish, reptiles, and amphibians that were fed *Ah*/hydrilla collected during autumn and winter sometimes developed VM, and conspecifics that were fed either hydrilla lacking *Ah* or *Ah*/hydrilla collected from spring and summer never developed VM.[26-28] VM was discovered in birds only after they were observed colliding with walls and overflying perches,[1] behaviors which people can readily observe and report. Analogous behaviors in fishes, reptiles, and amphibians are likely more difficult for people to observe in the wild. We therefore suspect that wild non-avian animals may be affected by VM outbreaks that may go unnoticed.

We quantified the concentrations of AETX in wild fishes from an *Ah*/hydrilla reservoir with historical VM outbreaks. AETX was quantified in a primary consumer, spottail shiners (*Notropis hudsonius*), as well as secondary consumers bluegill (*Lepomis macrochirus*) and redear sunfish (*L. microlophus*), and tertiary consumers warmouth (*L. gulosus*) and largemouth bass (*Micropterus salmoides*). We hypothesized that AETX may be detected in some spottail shiners and in the GI tracts of few bluegill because spottail shiners occasionally feed on algae[29] and bluegill occasionally feed on submerged aquatic vegetation[30-31]; therefore, these species may potentially consume *Ah* or *Ah*/hydrilla directly. We also expected to find AETX in the GI tracts of all redear sunfish because they almost exclusively eat snails,[31] and red-tailed hawks consuming snails fed *Ah*/hydrilla have developed VM.[32] We expected to detect AETX in fewer GI tracts of tertiary consumers as compared with secondary consumer GI tracts, and for tertiary consumer GI tracts to contain less AETX than secondary consumer GI tracts. Finally, we did not

expect to detect AETX in the muscle tissue of any fishes because VM has not been induced in laboratory specimens that have consumed muscle tissue from VM-affected animals.[33] A better understanding of trends in AETX accumulation among wild fish species will help describe how AETX cycles through ecosystems, as well as identify which trophic guilds may be at risk for effects of AETX exposure. Furthermore, identifying fish species and tissues that may accumulate AETX will help identify potential health risks for piscivorous consumers, including humans.

Methods

Study location

All *Ah*/hydrilla and fishes were collected on 20 November 2017 from J. Strom Thurmond Reservoir (JSTR), a 28,800 ha impoundment of the Savannah River on the border between Georgia and South Carolina, U.S.A. *Ah*/hydrilla samples and fishes were collected from JSTR because more VM cases in American coots and bald eagles have been documented at JSTR than at any other reservoir globally.[1,3,9] Also known as Clarks Hill Reservoir, JSTR is the second-largest lake by surface area in South Carolina and the largest lake by surface area in Georgia. Fish and *Ah*/hydrilla samples were collected along the shoreline of Bussey Point Recreation Area, located about 30 km northwest of Augusta, Georgia (33.699°N, 82.257°W, Figure 2.1). JSTR supports many popular recreational and harvest fisheries including those for temperate basses (*Morone* spp.),[34] lepomid sunfishes, and black basses.[35]

Fish and Ah/hydrilla collections

All fishes were collected under guidelines provided by Georgia Department of Natural Resources scientific collecting permit number 1000552262 and University of Georgia Animal Use Protocol #A2018 02-001-A1. Fishes were collected via shoreline boat electrofishing along hydrilla beds. Harvested specimens were euthanized within one hour of capture via ice bath to

avoid potential analytical complications with use of a chemical anesthetic treatment. Approximately six gallons of *Ah*/hydrilla were also collected with a throw rake where electrofishing occurred. *Ah*/hydrilla samples were transported to the laboratory in coolers and were stored frozen until ready for processing. The prevalence of *Ah* colonies within the *Ah*/hydrilla sample was confirmed via epifluorescence microscopy with a rhodamine B longpass fluorescence filter set. The autofluorescence of *Ah* phycocyanins enables visual inspection of these colonies when observed through a red barrier filter. Percent surface coverage of *Ah* colonies was estimated on 10 haphazardly selected hydrilla leaves from the *Ah*/hydrilla sample, as discussed in previous studies.[3,36]

A total of 30 fish were collected for AETX analysis, including 10 spottail shiners, 10 largemouth bass, four redear sunfish, four bluegill, and two warmouth. Fifteen of the fish (five spottail shiners, five largemouth bass, two redear sunfish, two warmouth, and one bluegill) were decapitated and heads were fixed immediately in 10% neutrally-buffered formalin for later histological analysis of VM. The heads and bodies of decapitated fish were tagged with uniquely-numbered and matching zip tie tags. The bodies of all 30 fish were stored on ice until dissection of tissues at the University of Georgia Aquatic Biology and Ecotoxicology Lab. The GI tracts and axial musculature (fillets) of all centrarchids were dissected and stored individually in hermetically-sealed bags at -20°C until AETX extraction and quantification. Whole spottail shiners were stored individually in hermetically-sealed bags at -20°C. Spottail shiners were not dissected because individual GI tracts and axial musculature were too small for accurate AETX extraction and quantification.

Extraction and quantification of AETX

Ah/hydrilla. Frozen *Ah*/hydrilla was thawed at room temperature, oven dried at 40 ˚C for 48 hours, and ground to a coarse powder in a high-speed blender. AETX was extracted from each of three 1.00 g subsamples of dried hydrilla with 50 mL of high performance liquid

chromatography (HPLC)-grade methanol following established methods.[37] Extracts were then gravity-filtered through Whatman #41 ashless filter paper, and methanol was removed via nitrogen evaporation. The resulting residues were resuspended in HPLC-grade methanol and transferred to 2.0 mL microcentrifuge tubes before being re-evaporated and reconstituted in 0.5 mL of 1:1 acetonitrile:deionized (DI) water. The resulting solutions were centrifuged at 4500 rpm for 5 minutes, and the supernatants were removed and refrigerated before being analyzed with HPLC to determine the concentration of AETX in each sample. HPLC analyses were conducted with an Agilent Infinity 1260 HPLC with gradient elution over a reverse phase C18 column and an UV detector. Ten serial dilutions of pure AETX (range = 7.8 to 500 ppb AETX) dissolved in 1:1 acetonitrile:DI water were used to develop a standard curve which was used to calculate AETX concentrations from *Ah*/hydrilla and fish tissue samples. Blank samples containing HPLC-grade methanol were analyzed after every fifth fish or *Ah*/hydrilla sample to monitor the shifting of the integration peak associated with AETX due to dirtying of the C18 column.

Fish tissues. The AETX extraction method was adapted from protocols described previously for hydrilla.[26,37] Ten whole spottail shiners, as well as the GI tracts and axial muscle of 10 largemouth bass, four redear sunfish, four bluegill, and two warmouth, were lyophilized for at least 72 h before being weighed to the nearest 0.1 mg. Lyophilized tissues were ground into a fine powder with a mortar and pestle. A 2:1 solvent mixture of chloroform and methanol was added to extract AETX from each sample of powdered fish tissue. Solvent was added to each sample at a ratio of 25 mL solvent per 1.0 g fish tissue, with samples weighing fewer than 2.0 g dry weight (d.w.) receiving 50 mL solvent to ensure complete AETX extraction. The resulting tissue/solvent mixtures were sonicated at room temperature for 20 minutes to encourage dissolution of AETX into the solvent mixture. Solvent was separated from fish tissues via gravity filtration through Whatman #41 ashless filter paper, and chloroform and methanol were removed via nitrogen evaporation. The remaining residues were reconstituted in 6 mL of 10% propylene glycol in DI water and vacuum-filtered through solid phase extraction (SPE) using Oasis HLB 6

cc 500mg columns. The adsorbed compounds were eluted from the SPE column with 12 mL methanol which was then removed via nitrogen evaporation. The remaining residues were reconstituted in 0.5 mL 1:1 acetonitrile:DI water before being vortexed for up to two minutes and sonicated at room temperature for 10 minutes. Sonicated mixtures were transferred to 2.0 mL glass vials for quantification of AETX concentrations via HPLC / mass spectrometry (HPLC/MS).

Histological analyses of vacuolar myelinopathy

Whole brains from five spottail shiners, five largemouth bass, two warmouth, two redear sunfish, and one bluegill were removed via dissection, placed in individual histology cassettes, and stored in 10% neutrally-buffered formalin until further processing. The forebrain (telencephalon), optic (mesencephalon), thalamic and hypothalamic (diencephalon), cerebellum (metencephalon), and medulla (myelencephalon) regions of each brain were evaluated via microscopic observation, and the corresponding tissue sections were placed back into their respective cassettes. The trimmed brains were processed routinely by being embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Brain sections were analyzed by a veterinary fish pathologist at the University of Georgia's College of Veterinary Medicine for the presence of intramyelinic edema, characteristic of VM as described previously.[1] An Olympus BX51 microscope and DP71 camera system (Olympus Corporation, Center Valley, PA, USA) was used to evaluate prepared slides with the identities of individual fish blinded to the pathologist. The primary feature indicative of VM lesions used during evaluation was the presence of bilaterally symmetrical spongy degeneration of white matter areas of the central nervous system. Additional criteria included vacuoles of variable size that occurred individually or in clusters, were round to oval in shape, and were limited by smooth rounded borders.[1,26,38] The presence of clear spaces alone, particularly those limited by irregular or jagged borders, within the neuroparenchyma were interpreted as not compatible with VM.[1]

Statistical analyses

All statistical analyses were conducted in R statistical software version 3.5.1.[39] Results from all statistical analyses were considered statistically significant at $\alpha = 0.05$. All AETX concentration data were log-transformed to meet the assumptions of equal variances and normality as required for parametric testing. Data were confirmed for homoscedasticity via Bartlett's tests[40] and for normal distribution of residuals via Shapiro-Wilk's tests.[41] Among centrarchid species, one-way analyses of variance (ANOVA) was used to compare mean GI tract as well as muscle AETX concentrations. Differences in AETX concentrations among species were further investigated via Tukey's HSD post-hoc analyses. The strength of relationship between GI tract AETX concentrations and muscle AETX concentrations in these species was then investigated via simple linear regression. This linear regression model was performed using data only from the 10 uniquely tagged centrarchid fishes (five largemouth bass, two redear sunfish, two warmouth, and one bluegill) because the remaining fish either were too small to separate GI tracts from muscle samples (i.e., spottail shiners) or lacked identification numbers necessary to pair GI tracts with muscle samples from the same individuals (i.e., the untagged centrarchid fishes).

To determine how AETX concentrations may differ among trophic levels, centrarchid fishes were classified as either secondary consumers (bluegill and redear sunfish) or tertiary consumers (warmouth and largemouth bass).[31] Although specific diets can differ greatly between bluegill (planktivores) and redear sunfish (molluscivores),[31] their mean trophic positions within the food web are likely similar because they both feed mainly on primary consumers (e.g., zooplankton for bluegill and molluscs for redear sunfish). In contrast, warmouth and largemouth bass feed mainly on other fish and crawfish[31] and occupy higher trophic positions than bluegill and redear sunfish. A Welch's two sample T-test was used to compare mean muscle AETX concentrations between secondary and tertiary consumers. A similar Welch's two sample T-test was performed to compare mean GI tract AETX concentrations between secondary and tertiary

consumers. A Welch's two sample T-test was also used to determine whether mean GI tract AETX concentrations differed from mean muscle AETX concentrations within each trophic level. Spottail shiners were removed from comparisons of AETX concentrations between trophic levels to avoid grouping whole-body AETX concentrations with tissue-level AETX concentrations.

Results

All tissues from all species analyzed contained measurable levels of AETX (Table 2.1). The greatest mean AETX concentration (± 1 sd) was observed in the *Ah*/hydrilla samples (29,967 ± 20,434 ppb d.w.), and the lowest mean AETX concentration was observed in warmouth muscle (49.3 ± 1.5 ppb).

GI Tract AETX Concentrations

Mean GI tract AETX concentrations (± 1 sd d.w.) were 6,629.2 ± 7,192.4 ppb in bluegill, 3,154.0 ± 2,936.1 ppb in redear sunfish, 178.8 ± 275.1 ppb in largemouth bass, and 99.5 ± 97.8 ppb in warmouth (Table 2.1). GI tract AETX concentrations differed among species (one-way ANOVA, $F_{19} = 8.655$, $p = 0.001$, Figure 2.2). Tukey's HSD post-hoc analyses revealed that mean GI tract AETX concentrations in redear sunfish (n = 4, $p_{adj} = 0.010$) and bluegill (n = 4, $p_{adj} = 0.003$) were greater than those in largemouth bass (n = 10). Mean GI tract AETX concentrations in secondary consumers (n = 8) were approximately 70 times greater than in tertiary consumers (n = 12, Welch's two sample T-test, $t_{16.74} = 6.019$, $p < 0.001$, Figure 2.3).

Muscle and Whole Body AETX Concentrations

<u>Centrarchid musculature.</u> Mean muscle AETX concentrations (± 1 sd d.w.) were 351.2 ± 275.1 ppb in bluegill, 142.4 ± 146.6 ppb in redear sunfish, 101.6 ± 170.3 ppb in largemouth bass, and 49.3 ± 1.5 ppb in warmouth (Table 2.1). Muscle AETX concentrations were similar

among all centrarchid species (one-way ANOVA, $F_{19} = 1.474$, $p = 0.259$) and between secondary (n = 8) and tertiary consumers (n = 12, Welch's two sample T-test, $t_{13.606} = 1.449$, $p = 0.170$). Within secondary consumers, AETX concentrations in muscle were approximately 27 times lower than those in GI tracts (n = 8, Welch's two sample T-test, $t_{10.31} = 4.126$, $p = 0.002$, Table 2.1). However, AETX concentrations did not differ between muscle and GI tracts in tertiary consumers (n = 12, Welch's two sample T-test, $t_{21.26} = 0.380$, $p = 0.708$). For centrarchid species, $\log_{10}$ GI tract AETX concentrations did not accurately predict $\log_{10}$ muscle AETX concentrations (n = 10, $t_9 = 1.270$, $F_9 = 1.613$, $R^2 = 0.168$, $p = 0.240$) (Figure 2.4).

Spottail shiner whole body AETX concentrations. Whole body AETX concentrations in spottail shiners (n = 10) did not differ from muscle AETX concentrations in any species of centrarchid fish (one-way ANOVA, $F_{29} = 1.58$, $p = 0.210$). However, whole body AETX concentrations in spottail shiners differed from GI tract AETX concentrations among centrarchids (one-way ANOVA, $F_{29} = 10.02$, $p < 0.001$). Spottail shiner AETX concentrations were similar to AETX concentrations in tertiary consumer GI tracts (Tukey's post-hoc analyses, $p_{adj} = 0.92$ when compared with largemouth bass (n = 10) and $p_{adj} = 1.00$ when compared with warmouth (n = 2)) but were lower than those in secondary consumer GI tracts (Tukey's post-hoc analyses, $p_{adj} = 0.009$ when compared with redear sunfish (n = 4) and $p_{adj} = 0.001$ when compared with bluegill (n = 4)).

Vacuolar myelinopathy

Visual observation by a certified veterinary fish pathologist did not reveal microscopic differences among brains from any fish in any region of the brain. Indistinguishable vacuolated spaces were widespread among all fishes, particularly in the optic tectum, thalamus, hypothalamus, and medulla. However, these vacuoles were interpreted as likely post mortem or processing artifacts as described previously.[42-43] Although distributed symmetrically, vacuolated

spaces did not possess additional features typical of VM lesions, particularly round-to-oval shape and smooth rounded borders.[1]

Discussion

The discovery of AETX in the muscle tissue of omnivorous and carnivorous fishes suggests that AETX likely bioaccumulates in fishes via multi-step trophic transfer. However, the similarity of muscle AETX concentrations among secondary and tertiary consumers suggests that AETX may not biomagnify in fish muscle. Results from previous laboratory feeding trials further support this notion, as VM has only ever been induced in laboratory subjects either after consuming *Ah*/hydrilla directly or after consuming GI tissue from VM-afflicted animals. Red-tailed hawks (*Buteo jamaicensis*) and chickens (*Gallus domesticus*) remained healthy after consuming muscle tissue from VM-afflicted coots.[33,38] Piscivorous animals like eagles and carnivorous fishes may therefore accumulate more AETX by feeding lower on the food chain, possibly due to incidental exposure to undigested stomach contents in prey GI tracts, as suggested by decreasing AETX concentrations from *Ah*/hydrilla to secondary consumer GI tracts to tertiary consumer GI tracts (Figure 2.3). The much greater frequency of VM outbreaks in primary consumers (mainly waterfowl[1,5-8]) compared with secondary or tertiary consumers (bald eagles[1,5,8]) further supports the claim that AETX in GI tracts may result mostly from undigested *Ah*/hydrilla. However, AETX should be quantified separately from GI tracts and associated gut contents to help determine whether AETX bioaccumulates in fish GI tissue, as well as other tissues such as brain and blood.

Previous diet studies suggest that AETX in spottail shiners, bluegill, and redear sunfish most likely occurred via consumption of zooplankton and aquatic macroinvertebrates.[29-31,44-46] Similarly, based on observations of stomach contents and existing knowledge of diet preferences,[31] we suspect AETX accumulation in tertiary consumers (warmouth and largemouth bass) most likely occurred via consumption of finfish and crawfish. However, AETX

concentrations from these fishes cannot be attributed to specific diet patterns because stomach contents were not analyzed from these fishes, nor were stable isotopes analyzed to quantify trophic status.

Unlike most piscivorous animals, humans rarely consume the guts of harvested fish and may therefore experience similar risk of AETX consumption regardless of which species they consume. The specific risk for humans eating fish from JSTR remains unknown because toxicological responses to AETX exposure have not been tested in mammals. However, centrarchids are the most commonly targeted and harvested fish species in Georgia.[47-48] Although largemouth bass harvest rates have declined over the last 50 years in Georgia and throughout the U.S.A.,[49-51] the catch rate is high enough that a low harvest rate may still equate to many pounds of fish being harvested. With an estimated moisture content in centrarchid fillets of 80%,[52] human consumers may be exposed to an average of 15.9 ± 12.5 (mean ± 1 sd) µg AETX per 8 oz serving of bluegill fillet, 6.5 ± 6.6 µg AETX per 8 oz serving of redear sunfish fillet, 4.6 ± 7.7 µg AETX per 8 oz serving of largemouth bass fillet, and 2.2 ± 0.1 µg AETX per 8 oz serving of warmouth fillet. Bioassays are therefore recommended to help determine the dose of AETX which may affect the health of humans and other mammals. Such bioassays, when coupled with an estimated mass of AETX in fish muscle as presented here, would be useful in determining whether fish consumption advisories are needed for fishes from *Ah*-inhabited waters.

Although VM or other evidence of detrimental AETX effects have been documented in birds,[3,32-33,37-38] reptiles,[27] amphibians,[28] and fishes[26] in previous laboratory studies, evidence of AETX in wild animals had until now only been documented in birds.[1,5-8] These results, though limited to a single sample date in one reservoir, suggest that AETX may travel through aquatic ecosystems through complex ecological pathways. To help better describe these exposure pathways, quantitative diet analyses should be conducted on fishes from JSTR to determine common prey items for fishes therein. AETX concentrations should then be quantified from

these common prey species within JSTR to determine which prey transfer AETX to fishes and other secondary and tertiary consumers. Bluegill are likely ideal study specimens for such a diet study because they exhibit trophic polymorphism (i.e., diet varies greatly among distinct subgroups within populations).[30,53-55] Comparing AETX in the GI tracts and muscle of trophically polymorphic subgroups of bluegill may help control for extraneous variability by comparing how different diets affect AETX accumulation in the same species within the same reservoir. Diet studies like the one proposed above should be conducted at multiple *Ah*-inhabited reservoirs because diets among fishes, especially spottail shiners[29,44-46] and bluegill,[30-31,53-55] can vary greatly geographically and temporally. Surveying AETX concentrations in animals among seasons and from more reservoirs will also help determine spatial and temporal dynamics of AETX in the environment. Given the great fishing pressure that occurs at reservoirs throughout the southern and southeastern U.S.A. where *Ah* has most strongly established, attention should also be focused on understanding mammalian susceptibility to AETX accumulation and VM development to assess potential risk of AETX to humans. Although we have quantified the concentrations of AETX in some popular sport fishes, the consequences for humans consuming these fishes remain unknown without understanding dose-response relationships of AETX exposure in mammals.

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Tables

Table 2.1: Aetokthonotoxin (AETX) concentrations in *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla), spottail shiners (*Notropis hudsonius*), redear sunfish (*Lepomis microlophus*), bluegill (*L. macrochirus*), warmouth (*L. gulosus*), and largemouth bass (*Micropterus salmoides*). All samples were collected on 20 November 2017 from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. Samples from which AETX was not detected are reported as containing 0.0 ppb AETX. *Gastrointestinal tract.

	Total	Sample	Samples	AETX Concentration (ppb dry weight)			
Species	Samples	Type	w/ AETX	Min	Mean	Max	Std. Dev.
Ah/hydrilla	3	Whole	3	7,687.0	29,966.8	47,835.1	20,434.3
Spottail shiner	10	Whole	10	10.6	93.0	270.8	72.8
Bluegill	4	GI tract*	4	1,854.0	6,629.2	17,269.3	7,192.4
	4	Muscle	4	183.1	351.2	759.0	275.1
Redear	4	GI tract	4	835.3	3,154.0	7,067.4	2,936.1
sunfish	4	Muscle	3	0.0	142.4	341.6	146.6
Largemouth	10	GI tract	8	0.0	178.8	782.2	275.1
bass	10	Muscle	9	0.0	101.6	552.6	170.3
Warmouth	2	GI tract	2	30.4	99.5	168.6	97.8
	2	Muscle	2	48.2	49.3	50.3	1.5

Figures

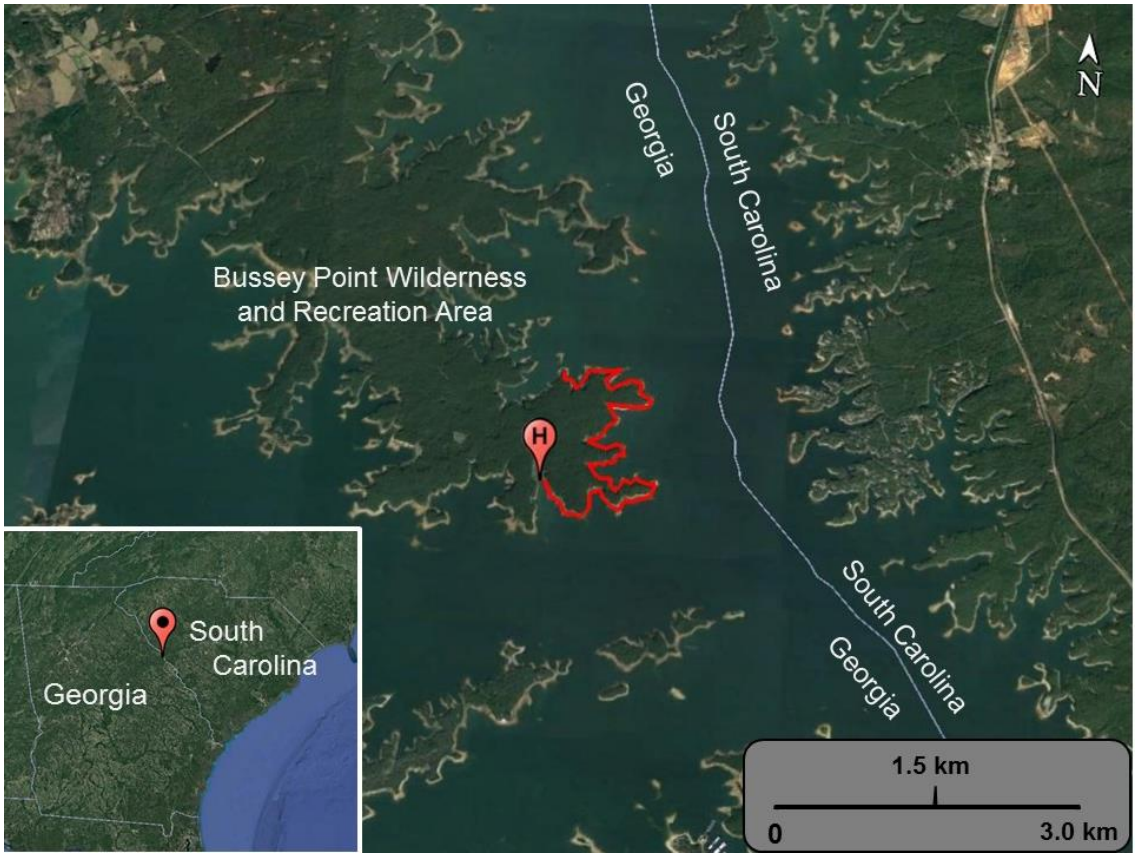

Figure 2.1: Location from which *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla) and fish samples were collected. All samples were collected on 20 November 2017 from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. The pin marks the location of *Ah*/hydrilla collection, and the adjacent path (red) indicates shorelines along which fishes were collected via boat electrofishing.

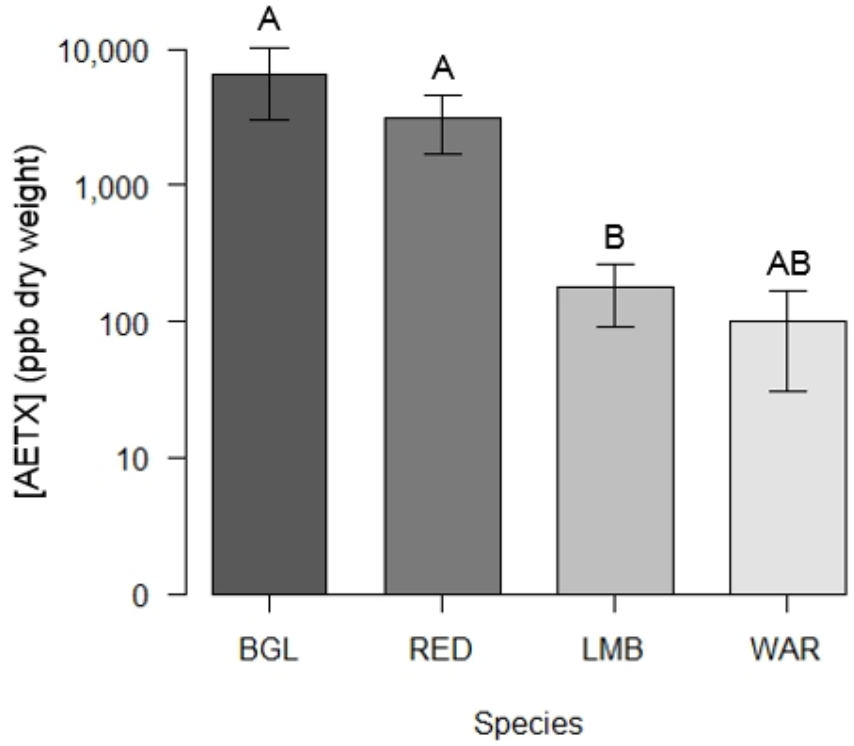

Figure 2.2: Aetokthonotoxin concentrations ([AETX]) in the gastrointestinal tracts of four centrarchid species: bluegill (*Lepomis macrochirus*, BGL, n = 4), redear sunfish (*L. microlophus*, RED, n = 4), largemouth bass (*Micropterus salmoides*, LMB, n = 10), and warmouth (*L. gulosus*, WAR, n = 2). Letters indicate statistically significant differences in $\log_{10}$ [AETX] at $\alpha = 0.05$. All fishes were captured on 20 November 2017 via shoreline boat electrofishing at J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. Error bars represent 1 se.

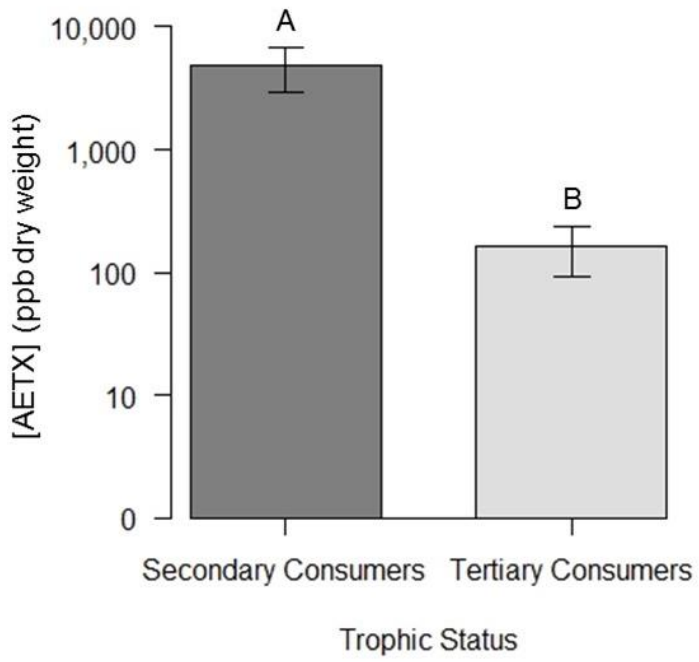

Figure 2.3: Aetokthonotoxin concentrations ([AETX]) in the gastrointestinal tracts of secondary consumers (bluegill, *Lepomis macrochirus*, n = 4 and redear sunfish, *Lepomis microlophus*, n = 4) and tertiary consumers (warmouth, *Lepomis gulosus*, n = 2 and largemouth bass, *Micropterus salmoides*, n = 10). Mean $\log_{10}$ AETX concentrations in gastrointestinal tracts are greater in secondary consumers than in tertiary consumers (Welch's two sample T-test, $t_{16.74} = 6.019$, $p < 0.001$). All fishes were captured on 20 November 2017 via shoreline boat electrofishing at J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. Error bars represent 1 se.

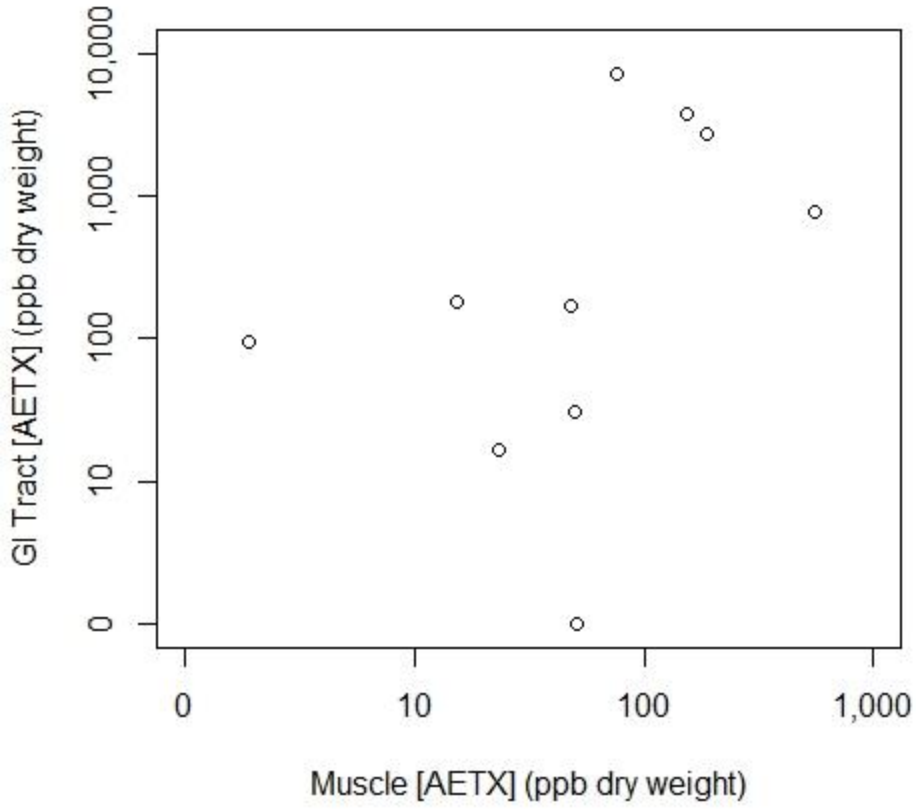

Figure 2.4: Relationship between aetokthonotoxin concentrations ([AETX]) in the axial musculature (i.e. fillets) and the gastrointestinal (GI) tracts of ten centrarchid fishes collected via shoreline boat electrofishing on 20 November 2017 from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. Fishes include five largemouth bass (*Micropterus salmoides*), two redear sunfish (*Lepomis microlophus*), two warmouth (*L. gulosus*), and one bluegill (*L. macrochirus*). Log_{10}-transformed muscle and GI tract AETX concentrations did not correlate well with each other (n = 10, $t_9 = 1.270$, $F_9 = 1.613$, $R^2 = 0.168$, $p = 0.240$).

CHAPTER 3

TROPHIC TRANSFERABILITY AND PHYSIOLOGICAL EFFECTS OF AETOKTHONOTOXIN IN FISHES[2]

[2] Pelletier, A. R., S. B. Wilde, A. C. Camus, T. J. Phillips, and R. B. Bringolf. To be submitted to *Journal of Experimental Biology*.

Abstract

The cyanobacterium *Aetokthonos hydrillicola* (*Ah*) grows epiphytically on the invasive aquatic macrophyte hydrilla (*Hydrilla verticillata*) in the southeastern U.S.A. Waterfowl consuming *Ah*-colonized hydrilla (*Ah*/hydrilla) have developed vacuolar myelinopathy (VM), a neurological condition associated with motor imbalance, ataxia, and death. VM has been linked to a novel cyanotoxin called aetokthonotoxin (AETX), produced by *Ah*. Although VM has only been documented in wild birds, laboratory feeding trials suggest that non-avian animals consuming AETX can also develop VM; however, the effects of AETX in fishes remain poorly understood. Here, we investigated whether fish (tilapia, *Oreochromis aureus* x *O. niloticus*) fed *Ah*/hydrilla can accumulate AETX and whether AETX may be passed from these tilapia to secondary consumers (largemouth bass, *Micropterus salmoides*) through consumption. We also quantified sublethal effects of AETX consumption on burst swimming performance in the largemouth bass. An experimental food chain involved feeding *Ah*/hydrilla collected in November 2016 (suspected to contain AETX) to juvenile tilapia for five days before feeding these tilapia to largemouth bass for up to 15 days. A similar but separate food chain that instead included *Ah*/hydrilla collected in September 2017 (suspected to lack AETX) also was created. Mean AETX concentrations in *Ah*/hydrilla, largemouth bass gastrointestinal tracts, and largemouth bass muscle samples were similar between September and November food chains, but AETX concentrations in whole tilapia were greater in the November food chain than in the September food chain. Mean AETX concentrations decreased from *Ah*/hydrilla to tilapia and from tilapia to mostly undetectable concentrations in largemouth bass muscle in both food chains, suggesting that AETX may biodilute in fishes. Largemouth bass burst swimming speeds did not differ with duration of feeding or between September and November food chains. Histologic evidence of VM was not detected in any largemouth bass brains. Fishes may accumulate AETX, however the sublethal effects of AETX in fishes remain unknown.

INDEX WORDS: *Aetokthonos hydrillicola*; vacuolar myelinopathy; J. Strom Thurmond Reservoir; Clarks Hill Reservoir; swimming performance; cyanobacteria

Introduction

The cyanobacterium *Aetokthonos hydrillicola* (*Ah*) grows epiphytically on the invasive aquatic macrophyte hydrilla (*Hydrilla verticillata*) in reservoirs throughout the southern and southeastern U.S.A. (Wilde *et al.*, 2005). Reservoir managers and wildlife biologists have become concerned with *Ah* because this cyanobacterium produces a previously undescribed neurotoxin referred to hereafter as aetokthonotoxin (AETX). This novel cyanotoxin is suspected to induce a neurological condition called vacuolar myelinopathy (VM) in birds, reptiles, and amphibians consuming *Ah*-colonized hydrilla (referred to hereafter as *Ah*/hydrilla) (Thomas *et al.*, 1998; Rocke *et al.*, 2002; Birrenkott *et al.*, 2004; Mercurio *et al.*, 2014; Wilde *et al*., 2014; Maerz *et al.*, 2018). Documented VM outbreaks have affected eight bird species, namely waterfowl (Thomas *et al*., 1998; Rocke *et al*., 2002; Augspurger *et al*., 2003; Fischer *et al*., 2006) which either forage on hydrilla or use hydrilla beds as habitat (Montalbano *et al*., 1979; Hardin *et al*., 1984; Esler, 1990; Balkcom, 2011). Bald eagles (*Haliaeetus leucocephalus*) also develop VM after consuming other birds, primarily American coots (*Fulica americana*), that have VM (Thomas *et al*., 1998; Fischer *et al*., 2002; Fischer *et al*., 2006). Although percent surface coverage of *Ah* on hydrilla increases throughout the growing season, VM outbreaks have only been documented between October and March (Rocke *et al*., 2005; Wilde *et al*., 2005; Wilde *et al*., 2014). Clinical symptoms of VM include motor impairment, seizures, ataxia, and death (Larsen *et al*., 2002).

Experimental feeding of *Ah*/hydrilla has led to VM development in avian species including mallard ducks (Rocke *et al.*, 2002; Birrenkott *et al.*, 2004; Rocke *et al.*, 2005; Wiley *et al.*, 2007; Haynie *et al.*, 2013), chickens (*Gallus domesticus*, Lewis-Weis *et al.*, 2004; Mercurio *et al.*, 2014; Dodd *et al.*, 2016), and red-tailed hawks (*Buteo jamaicensis*, Fischer *et al.*, 2003). Laboratory feeding trials have also induced VM in non-avian species including painted turtles (*Chrysemys picta*, Mercurio *et al.*, 2014), grass carp (*Ctenopharyngodon idella*, Haynie *et al.*, 2013) and tadpoles (Maerz *et al*., 2018). Vacuolation patterns in the optic lobes of these animals

were similar to lesions described by Thomas *et al.* (1998) in birds. Water snakes (*Nerodia* sp.) and salamanders (*Ambystoma talpoideum*) consuming prey items (tilapia, fathead minnows, tadpoles, and snails) that were fed *Ah*/hydrilla also developed vacuolar lesions characteristic of VM (unpublished data from Martin *et al.*, 2018).

Despite recent efforts to describe effects of *Ah*/hydrilla consumption in various taxa, the physiological and ecological effects of AETX remain poorly understood in fishes. Fishes may be exposed at least indirectly to AETX because many fishes in southern and southeastern U.S. reservoirs forage and take shelter within hydrilla beds (Moxley and Langford, 1982; Chick and McIvor, 1997; Valley and Bremigan, 2002; Tate *et al*., 2003; Troutman *et al*., 2007). Furthermore, the mechanism by which AETX transfers to secondary consumers (i.e., predators that prey upon VM-afflicted animals) remains unknown. For example, whether the toxin transfers passively to secondary consumers via their consumption of undigested *Ah*/hydrilla from prey stomachs, or whether AETX instead bioaccumulates in the muscle tissue of secondary consumers following dietary exposure remains unknown.

We investigated the potential for trophic transfer of AETX directly from *Ah*/hydrilla to an omnivorous fish (tilapia, *Oreochromis aureus* x *O. niloticus*) and from this omnivorous fish to a carnivorous fish (largemouth bass, *Micropterus salmoides*). We also investigated the potential for bioaccumulation of AETX in the GI tracts and axial musculature (i.e., fillets) of these fishes. Finally, we investigated the physiological effects of dietary AETX exposure on swimming performance in largemouth bass fed tilapia that consumed *Ah*/hydrilla. Burst swimming speed (U_{burst}) is an ecologically relevant endpoint for Darwinian fitness because U_{burst} represents a fish's ability to capture prey and evade predators (Reidy *et al*., 1995; Drucker, 1996; Reidy *et al*., 2000). If fishes exposed to AETX exhibit similar clinical symptoms of VM as do birds (i.e., motor impairment as described by Larsen *et al*., 2002), then the spread of *Ah*/hydrilla to new reservoirs may increase fish mortality in reservoirs throughout the southeastern U.S.A. Results of these studies will help reservoir managers and wildlife biologists understand the potential

risks of AETX exposure in fishes. These studies will also help characterize potential health risks for humans and other animals that consume fishes from *Ah*-colonized reservoirs.

Methods

Fish care

Care of all laboratory specimens was conducted in accordance with University of Georgia Animal Use Protocol #A2018 02-001-A1. Tilapia were reared in-house at the University of Georgia's Aquatic Biology and Ecotoxicology Lab (ABEL) in Athens, Georgia, U.S.A. Tilapia fry were housed in two recirculating aquaculture systems, each with 16 76-L tanks. Water in all tanks was dechlorinated and maintained between 22 and 28 ^{o}C. Tilapia were fed brine shrimp nauplii (*Artemia* sp.) (Brine Shrimp Direct, Inc., Ogden, Utah, U.S.A.) *ad libitum* until the tilapia grew to approximately 0.5 g wet weight. Tilapia were then transitioned to a diet of 1 mm crumbled fish pellets (Zeigler Bros, Inc.; Gardners, Pennsylvania, U.S.A.) until they grew to 1.0 - 2.0 g wet weight, when exposure to the experimental *Ah*/hydrilla diets (see below) began.

Largemouth bass (LMB) were obtained from American Sport Fish hatchery (Pike Road, Alabama, U.S.A.). LMB were chosen for these studies because recreational fishermen commonly harvest this species in reservoirs throughout the southeastern U.S.A. (USDI 2018), including those with historical VM outbreaks (e.g., Lake Tohopekaliga, Myers *et al*., 2008 and J. Strom Thurmond Reservoir (JSTR), Houck and Rankin, 2018). LMB (n = 65) were transported to ABEL and introduced to a 970-L holding tank for 30 days. At that time, 36 LMB were removed from the holding tank and weighed to the nearest 0.1 g before being randomly assigned and introduced to individual 76-L tanks. All 36 LMB were 186-206 mm total length (TL) and weighed 88.4-116.7 grams. All LMB in 76-L tanks were fed the same 3-mm Zeigler fish pellet diet until 7 days prior to the start of the experiment when they were transitioned to feeding on juvenile tilapia (~1.5% of body mass per day) in preparation for the feeding trials.

Ah/hydrilla collection and preparation

Ah/hydrilla was collected from JSTR, a 28,800-hectare impoundment of the Savannah River on the border between Georgia and South Carolina, U.S.A. Also known as Clarks Hill Reservoir, JSTR is the second-largest lake by surface area in South Carolina and the largest lake by surface area in Georgia. Georgia and South Carolina manage popular recreational and harvest fisheries for striped bass (*Morone saxatilis*) and hybrid striped bass (*Morone saxatilis* x *M. chrysops*), largemouth bass, crappie (*Pomoxis* spp.), and lepomid sunfishes (*Lepomis* spp.) (W. E. Houck and D. M. Rankin, South Carolina Department of Natural Resources, unpublished data). *Ah*/hydrilla samples were collected from JSTR because more VM cases in American coots and bald eagles have been documented at JSTR than at any other reservoir globally (Thomas *et al*., 1998; Rocke *et al*., 2005; Wilde *et al*., 2005).

Previous data suggest that *Ah*/hydrilla collected before mid-October does not induce VM when fed to laboratory animals, but *Ah*/hydrilla collected between November and February may induce VM in laboratory animals (Birrenkott *et al*., 2004; Wiley *et al*., 2007; Maerz *et al*., 2018). Therefore, *Ah*/hydrilla that was suspected to contain AETX was collected on 17 November 2016 by casting a throw rake along the shoreline around the Bussey Point Wilderness and Recreation Area in JSTR (33.706°N, 82.340°W). The same methods were used to collect *Ah*/hydrilla the following year on 20 September 2017 from a nearby location (33.699°N, 82.257°W, Figure 3.1). This September 2017 batch of *Ah*/hydrilla was suspected to lack AETX. Approximately 6 gallons of *Ah*/hydrilla was harvested during each collection. The prevalence of *Ah* colonies within November 2016 and September 2017 *Ah*/hydrilla samples was observed via epifluorescence microscopy with a rhodamine B longpass fluorescence filter set. The autofluorescence of *Ah* phycocyanins enables visual inspection of these colonies when observed through a red barrier filter. Percent surface coverage of *Ah* colonies was estimated on 10 haphazardly selected hydrilla leaves from each site as described previously (Wilde *et al*., 2005; Williams *et al*., 2007). *Ah*/hydrilla samples were stored at -20 ^{o}C before being prepared for feeding trials.

To prepare the *Ah*/hydrilla into an edible form for the tilapia, frozen *Ah*/hydrilla was thawed at 20 ^{o}C before being hand-squeezed to remove excess water. Sixty g of hand-squeezed *Ah*/hydrilla was homogenized in a commercial blender with 250 mL dechlorinated tap water and 6.0 g of 1.0-mm fish pellets to increase palatability. This mixture was blended for up to 5 min and pressed through a sieve to remove excess water and create a thick paste. Fresh batches of September and November *Ah*/hydrilla food were prepared at least once every 7 days to provide a steady supply of freshly prepared food.

Feeding Ah/hydrilla to tilapia

Ten 19-L aquaria were filled from the same water source and maintained at the same temperature as were the larger recirculating systems that housed tilapia. Each aquarium contained approximately 40 g of tilapia (about 30-50 individuals weighing 0.4-2.0 g each). Five of these tanks were randomly assigned to receive 5.0 g of September 2017 *Ah*/hydrilla food daily (henceforth 'September tilapia tanks'), and the remaining five experimental tanks were assigned to receive 5.0 g of November 2016 *Ah*/hydrilla food daily (henceforth 'November tilapia tanks'). For each of the 5 days prior to the start of the experiment, tilapia that were unexposed to *Ah*/hydrilla food (henceforth 'control tilapia') were transferred from the larger recirculating systems to each of one September tilapia tank and one November tilapia tank. Therefore, by the start of the experiment (19 January 2019), one tilapia tank from each diet treatment had received *Ah*/hydrilla food for each of 1, 2, 3, 4, and 5 days. All of the uneaten *Ah*/hydrilla food was removed from each tilapia tank daily, and 50% water changes were conducted in all tanks. After tilapia were fed *Ah*/hydrilla food for five days, 100% water changes were conducted to standardize toxin exposure between tilapia batches. Control tilapia were restocked in each tilapia tank after 100% water changes and fed *Ah*/hydrilla food for another 5 days.

Feeding tilapia to LMB

Seven days before the start of the experiment, LMB were transitioned to a diet of control tilapia to habituate the LMB to live food. At the start of the experiment (19 January 2019), LMB were randomly assigned to receive a diet of either September or November tilapia (n = 18 LMB per diet). LMB were fed 1.5% of their body mass daily in either September or November tilapia. LMB from each diet were randomly separated into three groups (n=6) that received their tilapia diets for 0-1 days, 7-8 days, or 14-15 days.

Swimming performance tests

After being fed the September or November tilapia diets for 0-1 days, 7-8 days, or 14-15 days, the LMB (n = 6) from each diet treatment were tested for swimming performance. A Brett-type recirculating swim flume (Brett, 1964; Figure 3.2) was used to measure U_{burst} of all 36 LMB. The swim flume held 19 L of water and was 80 cm wide, 65 cm tall, and 10.3 cm inner cross-sectional diameter. The water was circulated with a 0.25 hp DC motor. The fish were contained between two grates 36 cm apart in the uppermost section of the flume (i.e., the swim chamber). The swim flume was contained in a five-sided housing which was filled approximately one third of its depth with dechlorinated tap water (Figure 3.2). This water bath was aerated and allowed to equilibrate to the same temperature and dissolved oxygen (DO) concentration as the LMB tanks. The swim flume was then filled with water from this water bath immediately before all swimming performance tests. Water chemistry in the swim flume and in the LMB tank were measured simultaneously to ensure the temperatures, DO concentrations, and pH between the two environments were similar to avoid sudden changes in water chemistry that could affect swim performance (Hocutt, 1973; Otto and O'Hara-Rice, 1974; Randall and Brauner, 1991; Hasler *et al*., 2009; Dennis III *et al*., 2016). During each swimming performance test, one LMB was added to the swim chamber and the door was reattached and allowed to leak slightly (Hasler *et al*., 2009). This leaking allowed for steady replenishment of water in the swim flume

from the water bath, helping to maintain constant DO concentration and temperature within the flume.

During pilot swimming performance tests, fish were noticed resting on the downstream grate as water velocity in the chamber increased, despite the fish not reaching exhaustion. The fishes' exposure to elements outside the swim chamber were suspected to be inducing stress in the fish. To encourage fish to continue to swim upstream during the tests, the swim chamber was covered with black plastic wrap, except for the most downstream quarter which was left uncovered. A flashlight shined into the downstream end of the chamber through this opening and encouraged the fish to swim upstream in an effort to escape the light. This arrangement was similar to previously described swim flume modifications (Kolok, 1992b; Reidy *et al*., 1995) and allowed a video recorder to capture the moment of fatigue during each swim trial.

Each LMB was allowed to acclimate in the swim chamber for 90 minutes (as recommended by Kolok, 1991) with the flashlight on and the water circulating at 10 cm/s. After acclimation, video recording began and water velocity in the swim chamber was increased stepwise by 10 cm/s every 60 seconds until the LMB reached exhaustion. Exhaustion was reached when the LMB collapsed against the swim chamber's downstream grate for 10 consecutive seconds. Because fish would often collapse for fewer than 10 seconds during a test, the video recording was used to retrospectively calculate the exact duration of each collapse. This allowed for the most accurate recording of the duration of each swimming performance test, which ended at the start of the 10-second exhaustion period.

Immediately after completing the swimming performance test, each LMB was weighed to the nearest 0.1 g and measured to the nearest 1 mm TL. U_{burst} was then calculated for each LMB as:

$U_{burst} = \left[V_p + \left(\frac{t_f}{t_i}\right)V_i\right] / TL$ (Brett, 1964),

where U_{burst} = burst swimming speed (body lengths (BL)/s), V_p = penultimate swimming velocity reached before fatigue (cm/s), V_i = stepwise increase in water velocity (cm/s), t_f = time elapsed from the final stepwise increase to fatigue (s), t_i = step duration (s), and *TL* = total fish length (cm). U_{burst} is presented in BL/s to control for the effect of fish size, which has been shown to influence swimming speed (Beamish, 1970; Kolok, 1992a). Fulton's condition factor (Ricker, 1975; as cited in Nash *et al*., 2006), an index of fish body condition, was calculated for each LMB as:

$$K_{TL} = 100 * \frac{W}{TL^3},$$

where K_{TL} = Fulton's condition factor, *W* = fish wet weight (g) and *TL* = total fish length (cm).

Extraction and quantification of AETX from plant and animal tissues

Ah/hydrilla. Our AETX extraction procedure was adapted from extraction protocols described by Wiley *et al*. (2008) and Haynie *et al*. (2013). Briefly, frozen *Ah*/hydrilla was thawed, oven dried at 40 ˚C for 48 hours, and ground to a coarse powder in a high-speed blender. AETX was extracted from each of six 1.00-g samples of dried *Ah*/hydrilla (n = 3 per collection date) with 50 mL of high performance liquid chromatography (HPLC)-grade methanol following established methods (Wiley *et al.*, 2008). Extracts were then gravity-filtered through Whatman #41 ashless filter paper, and methanol was removed via nitrogen evaporation. The resulting residues were resuspended in HPLC-grade methanol and transferred to 2.0 mL microcentrifuge tubes before being re-evaporated and reconstituted in 0.5 mL of 1:1 acetonitrile:deionized (DI) water. The resulting solutions were centrifuged at 4500 rpm for 5 minutes, and the supernatants were removed and refrigerated before being analyzed via HPLC to determine the concentration

of AETX in each sample. HPLC analyses were conducted with an Agilent Infinity 1260 HPLC with gradient elution over a reverse phase C18 column and an UV detector. Ten serial dilutions of pure AETX (range = 7.8 to 500 ng AETX) dissolved in 1:1 acetonitrile:DI water were used to develop a standard curve which was used to calculate AETX concentrations from *Ah*/hydrilla and fish tissue samples. Blank samples containing HPLC-grade methanol were analyzed after every fifth fish or *Ah*/hydrilla sample to monitor the shifting of the integration peak associated with AETX associated with dirtying of the C18 column. Limit of detection for AETX was determined as 7.8 ng per sample.

Tilapia. After consuming *Ah*/hydrilla food for 5 days, tilapia were frozen in hermetically-sealed Whirl-Pak bags at -20 °C until toxin extraction. Chemical anesthetics were not used during euthanasia of tilapia to prevent potential interference of the anesthetic with AETX extraction and quantification. AETX was quantified from tilapia samples (n = 6 per treatment) representing each batch of September and November tilapia that were fed to the LMB 9-14 days after the start of the experiment. Tilapia from this period were selected for AETX quantification because they were the last six meals eaten by the 12 LMB from which AETX concentrations from the GI tracts and muscle were also quantified (see below).

Whole tilapia were lyophilized for at least 72 h before being weighed to the nearest 0.1 mg. Lyophilized tissues were ground into a fine powder with a mortar and pestle. A 2:1 solvent mixture of chloroform and methanol was added to extract AETX from each sample of powdered fish tissue. Solvent was added to each sample at a ratio of 25 mL solvent per 1.0 g fish tissue, with samples weighing fewer than 2.0 g dry weight receiving 50 mL solvent to ensure AETX recovery. The resulting tissue/solvent mixtures were sonicated at room temperature for 20 minutes. Solvent was separated from fish tissues via gravity filtration through Whatman #41 ashless filter paper, and chloroform and methanol was removed via nitrogen evaporation. The remaining residues were reconstituted in 6.0 mL of 10% propylene glycol in water and vacuum-filtered through Oasis HLB 6 cc 500mg columns via solid phase extraction (SPE). The adsorbed

compounds were eluted from the SPE column with 12 mL methanol which was then removed via nitrogen evaporation. The remaining residues were reconstituted in 0.5 mL 1:1 acetonitrile:DI water before being vortexed for up to 2 minutes and sonicated at room temperature for 10 minutes. Sonicated mixtures were transferred to 2.0 mL glass vials for quantification of AETX concentrations via HPLC/MS as described above for plant samples.

Largemouth bass. Each LMB was euthanized via decapitation immediately following its swimming performance test. Chemical anesthetics were not used during euthanasia of LMB to prevent potential interference of the anesthetic with AETX extraction and quantification. The GI tract and axial muscle samples were removed via sharp dissection from each of the 12 LMB swam after consuming tilapia diets for 14-15 days. The GI tracts and muscle samples were stored individually in hermetically-sealed Whirl-Pak bags at -20 °C. AETX concentrations in individual LMB tissues were quantified in the same manner as AETX concentrations from the tilapia samples described above.

Histological analyses of vacuolar myelinopathy

Brains from all 36 LMB were analyzed microscopically for histological evidence of VM in the forebrain (telencephalon), optic (mesencephalon), thalamic and hypothalamic (diencephalon), cerebellum (metencephalon), and medulla (myelencephalon) regions as described by Thomas *et al*. (1998). The dorsal cranial bone was removed from each LMB skull with a scalpel to expose the brains, and heads were placed into individually labelled containers of 10% neutral buffered formalin. All brains were fixed in formalin within 10 minutes of euthanasia, and most were fixed in fewer than 5 minutes after euthanasia. Following 48 hours of decalcification in Kristensen's solution, excess tissue was trimmed away to leave a thin cradle of bone to protect the lateral and ventral aspects of the brains. A series of transverse cuts were made through the forebrain, optic lobes, and cerebellum of each brain, and the corresponding tissue sections were placed into a single tissue cassette. The trimmed brains were processed

routinely, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Brain sections were analyzed by a veterinary fish pathologist at the University of Georgia's College of Veterinary Medicine for the presence of intramyelinic edema characteristic of VM as described by Thomas *et al.* (1998). An Olympus BX51 microscope and DP71 camera system (Olympus Corporation, Center Valley, Pennsylvania, USA) was used to evaluate prepared slides with the identities of individual fish hidden from the pathologist. The primary feature indicative of VM lesions used during evaluation was the presence of bilaterally symmetrical spongy degeneration of white matter areas of the central nervous system (Thomas *et al*., 1998). Additional criteria for VM included vacuoles of variable size that occurred individually or in clusters, were round to oval in shape, and were limited by smooth rounded borders (Thomas *et al*., 1998; Fisher *et al*., 2003; Haynie *et al*., 2013). The presence of clear spaces alone, particularly those limited by irregular or jagged borders, within the neuroparenchyma were deemed not to be indicative of VM (Thomas *et al*., 1998).

Statistical analyses

All statistical analyses were conducted in R statistical software version 3.5.1 (R Core Team 2018). Results from all statistical analyses were considered statistically significant at $\alpha = 0.05$. All AETX concentration data were $\log_{10}$-transformed to meet the assumptions of equal variances and normal distribution of residuals as required by parametric statistical testing. All data were confirmed to meet these assumptions via Bartlett's tests for homoscedasticity (Bartlett 1937) and Shapiro-Wilk's tests for normal distribution of residuals (Shapiro and Wilk 1965). Means of some water chemistry variables were compared via Wilcoxin rank sum tests and Kruskal-Wallis tests because either the assumption of homoscedasticity (Bartlett, 1937) or the assumption of normally distributed residuals (Shapiro and Wilk, 1965) was not met. Samples containing AETX concentrations less than the detection limit were reported as containing 0 ppb AETX both before and after $\log_{10}$-transformation of these data.

Fish care. A one-way analysis of variance (ANOVA) and Tukey's post-hoc analysis were conducted to compare mean masses of tilapia consumed daily among the 36 LMB to determine any outliers. Because feeding rates are expected to strongly influence swimming performance (Gregory and Wood, 1998), LMB that were stressed and failed to eat were removed from all analyses. After outliers were removed, Welch's two sample T-tests were used to compare mean masses of tilapia consumed daily by September and November LMB. Welch's two sample T-tests were also used to compare means of water temperature, DO concentration, pH, and total ammonia nitrogen (TAN) concentration between September and November LMB tanks as well as between September and November tilapia tanks.

AETX concentrations. Welch's two sample T-tests were used to determine whether AETX concentrations in *Ah*/hydrilla, tilapia, LMB GI tracts, and LMB muscle differed between September and November food chains. A simple linear regression model was also developed to measure the strength of relationship between AETX concentrations in the GI tracts and muscle of the 12 LMB that were swam after consuming tilapia diets for 14-15 days.

Swimming performance. We conducted a two-way ANOVA to determine the effects of diet (September versus November tilapia) and exposure time (0-1 days, 7-8 days, and 14-15 days) and the interaction of both factors on LMB U_{burst}. If the ANOVA detected differences in U_{burst} treatment combinations, then Tukey's post-hoc tests were used to determine where the differences occurred. One-way ANOVAs were also used to determine whether mean K_{TL} and water quality parameters (water temperature, pH, and DO concentration) within the swim chamber differed among the three periods when fish were swam. If means for any of these variables differed among swim periods, then simple linear regression was used to determine whether each variable was related with swimming speed, as has been shown previously (e.g., Randall and Brauner, 1991; Martinez *et al*., 2004; Hasler *et al*., 2009). Variables that were strongly related to swimming speed were added to the main two-way ANOVA model as covariates to control for their effects on LMB U_{burst}.

Finally, a simple linear regression model was used to investigate the relationship between LMB U_{burst} and AETX concentration in the GI tracts of the 12 LMB swam after 14-15 days of experimental tilapia diet. A similar linear regression model was used to investigate the relationship between U_{burst} and AETX concentration in the muscle of the same 12 LMB.

Results

Fish care

One September LMB and one November LMB that were swam on 19 January 2019 (after having been fed their experimental diet for 0 days) often failed to eat their daily ration of tilapia during the acclimation period (mean daily consumption rate = 0.60% body weight and 0.79% body weight as compared with all other LMB which ate between 1.2% and 1.5% body weight per day) and expressed other signs of stress including intermittent periods of hiding and erratic swimming. These two LMB were therefore removed from all statistical analyses. The 34 remaining LMB measured 190-215 mm TL (mean ± 1 sd = 202 ± 5.7 mm) and weighed 89-126 g wet weight (mean = 109 ± 9.4 g) at the time of swimming performance tests. Lengths (Welch's two sample T-test, $t_{30.77} = 0.000$, $p = 1.00$) and weights (Welch's two sample T-test, $t_{30.92} = 0.794$, $p = 0.43$) did not differ between September (means = 202 ± 5.2 mm TL and 107 ± 8.5 g wet weight) and November LMB (means = 202 ± 6.4 mm TL and 110 ± 10.3 g wet weight) (Table 3.1). Mean daily feeding rates were also similar among individual LMB (mean ± 1 sd = 1.41 ± 0.35% body weight, Kruskal-Wallis test, $\chi^2_{33} = 26.656$, $p = 0.77$) and between September (1.39 ± 0.40% body weight) and November LMB (1.43 ± 0.30% body weight, Wilcoxin rank sum test, W = 24210, $p = 0.99$).

Means (± 1 sd) for water temperature (September = 20.4 ± 1.5 °C, November = 20.4 ± 1.5 °C, Wilcoxin rank sum test, W = 14509, $p = 0.87$), DO concentration (September = 8.47 ± 0.31 mg/L, November = 8.45 ± 0.40 mg/L, Wilcoxin rank sum test, W = 14114, $p = 0.78$), pH (September = 7.09 ± 0.27, November = 7.09 ± 0.26, Wilcoxin rank sum test, W = 14538, p =

0.85), and TAN (September = 0.20 ± 0.12 mg/L, November = 0.21 ± 0.12 mg/L, Wilcoxin rank sum test, W = 3560, $p = 0.50$) did not vary between September and November LMB tanks (Table 3.1). Water temperature (September = 24.3 ± 1.0 °C, November = 24.3 ± 1.0 °C, Welch's two sample T-test, $t_{157.94} = 0.510$, $p = 0.61$), DO concentration (September = 7.88 ± 0.55 mg/L, November = 7.86 ± 0.58 mg/L, Welch's two sample T-test, $t_{157.56} = 0.280$, $p = 0.78$), and TAN (September = 0.78 ± 0.46 mg/L, November = 0.69 ± 0.43 mg/L, Wilcoxin rank sum test, W = 926, $p = 0.43$) also did not vary between September and November tilapia tanks. Although mean pH differed statistically between September (7.37 ± 0.21) and November tilapia tanks (7.44 ± 0.18, Welch's two sample T-test, $t_{154.07} = 2.180$, $p = 0.031$), the observed difference of 0.07 in mean pH is not expected to be biologically relevant (Table 3.1).

AETX concentrations

AETX concentrations were similar between September (mean ± 1 sd = 94,357 ± 7,112 ppb) and November *Ah*/hydrilla samples (61,206 ± 25,230 ppb, n = 3, Welch's two sample T-test, $t_{2.09} = 1.742$, $p = 0.22$) (Table 3.2). However, November tilapia (2,337 ± 1,858 ppb) had greater AETX concentrations than September tilapia (378 ± 340 ppb, Welch's two sample T-test, $t_{8.70} = 2.665$, $p = 0.027$) (Table 3.2, Figure 3.2). GI tract AETX concentrations (September = 168 ± 187 ppb, November = 340 ± 504 ppb, n = 6, Welch's two sample T-test, $t_{6.25} = 0.594$, $p = 0.574$) and muscle AETX concentrations (September = 0.6 ± 1.4 ppb, November = 3.9 ± 6.9 ppb, n = 6, Welch's two sample T-test, $t_{6.57} = 1.046$, $p = 0.333$) were similar between September and November LMB that were fed tilapia for 14-15 days. However, muscle and GI tract AETX concentrations related weakly with each other within LMB (linear regression, n = 12, $t_{11} = -1.701$, $F_{11} = 2.894$, $R^2 = 0.224$, $p = 0.120$). Within the September food chain, AETX concentrations decreased from 94,357 ± 7,112 ppb in *Ah*/hydrilla (n = 3) to 378 ± 340 ppb in tilapia (n = 6, Welch's two sample T-test, $t_{5.16} = 16.765$, $p < 0.001$) and from 168 ± 187 ppb in LMB GI tracts to 0.6 ± 1.4 ppb in LMB muscle (n = 6, Welch's two sample T-test, $t_{6.98} = 8.911$, p

< 0.001) but were similar between tilapia and LMB GI tracts (n = 6, Welch's two sample T-test, $t_{9.36} = 1.798$, $p = 0.105$). Within the November food chain, AETX concentrations decreased from 61,206 ± 25,230 ppb (mean ± sd) in *Ah*/hydrilla (n = 3) to 2,337 ± 1,858 ppb in tilapia (n = 6, Welch's two sample T-test, $t_{6.91} = 6.187$, $p < 0.001$) and from tilapia to 340 ± 504 ppb in LMB GI tracts (n = 6, Welch's two sample T-test, $t_{6.63} = 2.542$, $p = 0.040$), but were similar between LMB GI tracts and 3.9 ± 6.9 ppb in LMB muscle (n = 6, Welch's two sample T-test, $t_{6.58} = 2.228$, $p = 0.064$) (Table 3.2, Figure 3.2).

Swimming performance

Water temperatures in the swim chamber differed among all three swim periods, and were warmest (19.8 ± 0.6 °C) during the first swim period (0-1 days) (mean ± sd) and coolest (17.2 ± 0.5 °C) during the second period (7-8 days) (one-way ANOVA, $F_{33} = 19.91$, $p < 0.001$) (Table 3.3). Consequently, DO concentrations also differed among swim periods (one-way ANOVA, $F_{33} = 13.24$, $p < 0.001$), and were lower (8.64 ± 0.16 mg/L) during the first period at than the second period (9.16 ± 0.21 mg/L) (Tukey's HSD, $p_{adj} < 0.001$) and the final swim period (8.94 ± 0.30 mg/L) (Tukey's HSD, $p_{adj} = 0.013$) (Table 3.3). However, simple linear regression models suggested that neither temperature ($R^2 = 0.01$, $t_{33} = -0.374$, $p = 0.711$) nor DO concentration ($R^2 = 0.01$, $t_{33} = 0.376$, $p = 0.709$) were related with U_{burst} during the 34 swimming performance tests. Finally, neither pH (one-way ANOVA, $F_{33} = 0.603$, $p = 0.55$) nor K_{TL} (one-way ANOVA, $F_{33} = 1.332$, $p = 0.28$) differed among swim periods (Table 3.3). Therefore, the original two-way factorial ANOVA model was maintained without the addition of covariates.

LMB burst swimming speeds (n = 34) averaged 5.05 ± 1.34 BL/s (1 sd) (102 ± 27 cm/s) over the duration of the experiment. A two-way ANOVA model revealed that burst swimming speeds did not differ between September (mean ± 1 sd = 5.10 ± 0.97 BL/s) and November LMB (4.99 ± 1.67 BL/s, $F_1 = 0.059$, $p = 0.81$) or among swim periods ($F_2 = 0.033$, $p = 0.97$) (Figure 3.3). The effect of experimental diet (i.e., consuming September versus November tilapia) on

burst swimming speed did not depend on the duration of experimental feeding ($F_2 = 0.646$, $p = 0.53$, Figure 3.3). Simple linear regression models suggest that LMB U_{burst} was weakly related with GI tract AETX concentrations ($n = 12$, $t_{11} = -1.054$, $F_{11} = 1.110$, $R^2 = 0.100$, $p = 0.317$) (Figure 3.4). A similar regression model suggests that LMB U_{burst} was negatively related with muscle AETX concentrations ($n = 12$, $t_{11} = -2.358$, $F_{11} = 5.560$, $R^2 = 0.357$, $p = 0.040$) despite AETX concentrations being above the detection limit in only three of 12 fish (Figure 3.5).

Vacuolar myelinopathy

Observation by a certified veterinary fish pathologist revealed no microscopic differences between the brains of September and November LMB in any region of the brain, either within or among swim periods. Indistinguishable vacuolated spaces, interpreted as post mortem and processing artifacts (Garman, 2011; Cantile and Youssef, 2016), were widespread in both September and November LMB, particularly in the optic tectum, thalamus, hypothalamus, and medulla (Figure 3.6). While symmetrically distributed, vacuolated spaces did not possess additional features, particularly round-to-oval shape and smooth rounded borders typical of VM lesions (Thomas *et al*., 1998) (Figure 3.7).

Discussion

The detection of AETX in LMB in the present dietary studies suggests that AETX can bioaccumulate in predatory fishes via multi-step trophic transfer. However, the biodilution of AETX from *Ah*/hydrilla to tilapia to LMB muscle from both September and November food chains (Figure 3.2) and the much greater AETX concentrations in the GI tracts of LMB as compared with their muscle suggests that the bioaccumulation efficiency of AETX in fish tissues may be low. Most of the AETX detected in LMB GI tracts is therefore suspected to be attributed to residual *Ah*/hydrilla material in consumed tilapia rather than from AETX accumulation in LMB GI tissue. Similarly, although tilapia were too small to quantify AETX separately from their GI

tracts and muscle, we suspect that most of the AETX detected in tilapia can be attributed to the raw undigested *Ah*/hydrilla material in their guts rather than accumulation of AETX in tilapia tissues. This hypothesis is supported by previous feeding trials in which chickens fed tissues from VM-afflicted grass carp did not develop VM (Haynie *et al*., 2013). Because chickens are susceptible to VM (e.g., Lewis-Weis *et al*., 2004; Mercurio *et al*., 2014; Dodd *et al*., 2016), grass carp in their study may have accumulated only a small proportion of the AETX that they consumed. Comparisons of AETX concentrations detected in plant and fish tissue in the present study remain incomparable with AETX concentrations present in organisms from other studies because methods for AETX quantification were only recently developed and validated (Tabitha Phillips *et al*., unpublished data).

The observed biodilution of AETX in fish food chains suggests that the risk for AETX exposure, and therefore VM induction, may decrease with increasing trophic status. This notion is supported by the much greater number of VM occurrences documented in primary consumers (e.g., American coots and other waterfowl, Thomas *et al*., 1998; Fischer *et al*., 2002; Rocke *et al*., 2002; Augspurger *et al*., 2003; Fischer *et al*., 2006) compared with secondary consumers (e.g., bald eagles, Thomas *et al*., 1998; Fischer *et al*., 2002; 2006) in wild populations. However, the effect of species may be at least as important as is trophic position on an animal's risk for accumulating AETX. Although many species develop VM upon consumption of *Ah*/hydrilla, birds (e.g., Rocke *et al*., 2002; 2005; Haynie *et al*., 2013; Mercurio *et al*., 2014; Dodd *et al*., 2016) appear to develop VM more readily than do fishes (as seen here in LMB and by Haynie *et al*. (2013) in grass carp), turtles (Mercurio *et al*., 2014), domestic swine (Lewis-Weis *et al*., 2004) and water snakes (*Nerodia* sp., Martin, 2018; unpublished MS thesis). Therefore, the low frequency of AETX detection in LMB may not imply low risk of AETX accumulation for all secondary and tertiary consumers, as supported by evidence of VM in raptors (Thomas *et al*., 1998; Fischer *et al*., 2003; Lewis-Weis *et al*., 2004). The lack of VM induction in experimental LMB and water snakes (Martin 2018), as well as the 80+ days required to induce VM in painted

turtles (Mercurio *et al*., 2014) suggests that metabolic rate may influence the rate of either AETX accumulation or VM induction in animals. Feeding trials involving more species of ectothermic and poikilothermic species, as well as feeding trials involving a broader range of endothermic species (e.g., mammals) are necessary to better understand the effects of species and metabolic rate on AETX accumulation.

Swimming performances may have been similar between September and November LMB for several reasons. First, AETX may have affected burst swimming performance of September and November LMB similarly because AETX concentrations were similar between September and November *Ah*/hydrilla batches; however, November tilapia had greater AETX concentrations than did September tilapia, so the role of dietary transfer of AETX remains unclear (Figure 3.2). Second, variation in individual swimming performances was high, as is commonly observed in LMB and other fishes (Kolok, 1992b; Gregory and Wood, 1998; Hasler *et al*., 2009; Marras *et al*., 2009). A larger sample size may be necessary to detect changes in swimming performance given the high variability in individual fish swimming abilities. LMB burst swimming speed was negatively related with muscle AETX concentrations in this study (Figure 3.5); however, the effects of AETX accumulation on fish motor function remain unclear because AETX was detected in only three of the 12 LMB. Interestingly, two of the three LMB in which AETX was detected had the poorest swimming performance among all fish tested. However, based on previous tests with grass carp (Haynie *et al*., 2013), we would not expect AETX to affect fish motor function at the limited concentrations to which LMB were exposed in this study. Grass carp fed *Ah*/hydrilla *ad libitum* for 28 days did not display clinical symptoms of VM despite developing VM-related vacuolation in their brains (Haynie *et al*., 2013). Burst swimming speeds reported in both September and November groups in the present study were similar to those reported for healthy LMB of similar sizes (Kolok, 1991), and development of VM vacuolation without the presence of clinical symptoms has been documented previously (e.g., Fischer *et al*., 2003; Rocke *et al*., 2005; Wilde *et al*., 2005; Wiley *et al*., 2007; Dodd *et al*., 2016). Because VM

was not induced in any LMB in the present study, the amount of AETX transferred to LMB likely was insufficient to elicit clinical symptoms of motor impairment. Additional testing with greater magnitude and duration of AETX exposure to predatory fishes may allow a more conclusive determination of the dose-response relationship between AETX and fish motor function.

A repeated measures design may be more appropriate for future studies to assess the effects of AETX on fish fitness. Multiple groups of fish would be exposed to various doses of AETX and would be exposed periodically to burst swimming performance tests (e.g., once weekly on the same day each week). This experimental design would involve swimming the same individual fishes repeatedly, thus helping to control for individual variability in swimming performance (Kolok, 1992b; Gregory and Wood, 1998; Hasler *et al*., 2009; Marras *et al*., 2009) as well as the effect of training; fish have been shown to improve swimming performance after continued exposure to swimming performance tests (Kolok, 1992b; Gregory and Wood, 1998). Unexposed fish may improve their burst swimming performance over time during a repeated measures experiment, which could be investigated via regression of U_{burst} over time. A similar regression could be performed with exposed fish, and the slopes of the regression lines could be compared between exposed and unexposed fish. However, this experimental design may not allow for increased sample sizes because all fish would likely need to be swam on the same day, and the sample size would be limited based on the 90-minute acclimation time recommended for U_{burst} protocols (Kolok, 1991). Swimming performance tests are commonly used to measure the physiological effects of other environmental factors, including temperature (e.g., Kolok, 1992a; Keen and Farrell, 1994; Hasler *et al*., 2009), salinity (Nelson *et al*., 1996; Swanson, 1998; Swanson *et al*., 1998; Plaut, 2000), dissolved oxygen (Jones, 1971), dissolved carbon dioxide (Dennis III *et al*., 2016), and various toxicants (e.g., Hammer, 1995; Beaumont *et al*., 1995; Rajotte and Couture, 2002; McKenzie *et al*., 2007; Beggel *et al*., 2010; Hicken *et al*., 2011), on fishes. With greater magnitude and duration of AETX exposure, swimming

performance tests may be a cost-effective method for quantifying sublethal effects of AETX in fishes.

Although swimming performance appeared mostly unaffected by AETX consumption in this study, Fisher *et al*. (2003) concluded that correlation between clinical signs (Larsen *et al*., 2002) and vacuolar changes in the brains of coots was poor, so VM diagnosis in LMB should be based on the presence of compatible microscopic lesions rather than on clinical signs of neurologic disease (i.e., swimming performance). Discernable microscopic differences were not observed between the brains of September and November LMB. Although vacuolated spaces were widespread in brains from both groups, these vacuoles were not compatible with those previously described for VM (Thomas *et al*., 1998). The changes described in the present study are relatively common and interpreted as artifacts of collection and processing (Garman, 2011; Cantile and Youssef, 2016). The lack of diagnostic evidence of VM in September or November LMB differs from another study which induced VM-related brain vacuolation in 55% of grass carp (*Ctenopharyngodon idella*) fed *Ah*/hydrilla in a laboratory feeding trial and in 30% of grass carp that consumed *Ah*/hydrilla in a field study (Haynie *et al*., 2013). During that study, grass carp consumed *Ah*/hydrilla directly, therefore acting as primary consumers rather than secondary consumers as were LMB in the present study. The grass carp were also fed *ad libitum* for 5 weeks during both field and laboratory trials, compared with the LMB in the present study which were fed a limited ration (1.5% body mass) for no more than 15 days. Histological results from LMB in the present study are therefore more directly comparable with histological results from the chickens that consumed tissues from the aforementioned grass carp (Haynie *et al*. 2013). None of the 15 chickens that consumed tissues from VM-affected grass carp developed VM (Haynie *et al*. 2013) despite chickens from other studies developing VM while acting as secondary consumers (Lewis-Weis *et al*., 2004, Dodd *et al*., 2016). These studies therefore suggest either that predatory fishes may only be susceptible to VM following greater

magnitude or duration of exposure to AETX, or that fishes may not transfer VM to predators as efficiently as do other AETX-contaminated prey.

Lesions of VM in bald eagles that consumed tissues of afflicted coots include vacuolation at all levels of the brain and in the spinal cord, but these lesions are most common in the optic tectum. Changes are non-inflammatory and include small hemorrhages, not seen in LMB here, in the thalamus and brainstem of some affected eagles (Thomas *et al*., 1998). Damage to lipid-rich myelin sheaths that surround and insulate nerve axons, primarily in the central nervous system, characterize VM ultrastructurally. Specifically, splitting of the myelin lamellae occurs at the intraperiod line, a change consistent with intramyelinic edema (Fisher *et al*., 2003). These characteristics of VM were unobserved in all LMB.

Previous feeding trial studies involving other secondary consumers suggest that the lack of observed VM lesions in the LMB may be partially explained by the limited magnitude or duration of AETX exposure. For example, Lewis-Weis *et al*. (2004) induced VM in a red-tailed hawk that was fed a mixture of tissues from VM-afflicted American coots. This hawk was fed 6% of its own body mass daily for 28 days, a rate that about eight times as much experimental food relative to its body weight as were the LMB in the present study. Similarly, Fischer *et al.* (2003) induced VM in five out of five red-tailed hawks after they were fed tissues from VM-afflicted American coots for 28 days. The susceptibility of predatory fish to VM therefore remains in question until greater concentrations of AETX are fed to predatory fish during longer feeding trials, perhaps at least several months as has been done in previous feeding trials (e.g., Mercurio *et al*., 2014).

The similarity of AETX concentrations between the September and November *Ah*/hydrilla samples in the present study was unexpected because VM has developed more frequently in animals that consumed *Ah*/hydrilla collected during November and December compared with animals that have consumed *Ah*/hydrilla collected before mid-October (e.g. Birrenkott *et al*., 2004; Wiley *et al*., 2007; Maerz *et al*., 2018). The similarity in AETX

concentrations between September and November *Ah*/hydrilla samples used in this study may be explained in part by inter-annual variability in AETX production by *Ah*. Inter-annual variability in the severity of VM outbreaks has been recorded since the first several VM outbreaks in Arkansas, which were severe in the winters of 1994-1995 and 1996-1997 but mild during the winter of 1995-1996 (Thomas *et al*., 1998). Causes for the inter-annual variability of VM outbreaks and AETX production remain unknown. Seasonal changes may be less important than previously suspected in determining AETX production by *Ah*, because AETX concentrations in the September 2017 *Ah*/hydrilla samples did not differ from AETX concentrations in *Ah*/hydrilla samples collected from the same hydrilla bed on 20 November 2017 (Welch's two sample T-test, $p = 0.13$, see Chapter 2 herein). Seasonal increases in AETX production may precede VM outbreaks, which historically are not observed before mid-October in the southeast U.S.A., by a longer duration than suspected previously. We recommend more thorough investigations of seasonal and inter-annual changes in AETX concentrations be conducted to understand seasonal patterns and the environmental cues which stimulate *Ah* to produce AETX. Such studies may provide baseline environmental data necessary to create statistical models that could help predict the timing and rate of AETX production and therefore the severity of future VM outbreaks in wildlife. Such predictive models could also help determine the differences in AETX production among reservoirs.

Greater AETX concentrations in November tilapia compared with September tilapia suggest that the hydrilla subsamples used for AETX quantification were not representative of AETX concentrations in the larger batches of September and November *Ah*/hydrilla. Because prevalence of *Ah* colonies on hydrilla often varies greatly among leafs and among stems within hydrilla beds (A. Pelletier and S. Wilde, personal observations), subsamples taken from each batch of *Ah*/hydrilla may not have been reflective of mean AETX concentrations in larger batches. Large batches of *Ah*/hydrilla should be thoroughly homogenized before subsampling to

help make accurate inferences about AETX concentrations in *Ah*/hydrilla beds based on subsamples.

The multi-step trophic transfer and bioaccumulation of AETX in fish suggests that piscivorous animals (including humans) may be at risk of AETX exposure via fish consumption, but that risk may be low. Additional studies with known doses of AETX fed to fishes for longer durations would help better characterize the clinical and diagnostic symptoms of chronic AETX exposure in fishes. Bioassays can be useful in determining threshold concentrations of AETX necessary to elicit adverse effects in fishes and other organisms. Furthermore, mammalian bioassays warrant immediate research to determine potential human health risks of consuming fishes harvested from *Ah*-inhabited waters. Coupled with investigations of AETX concentrations in wild fish populations, mammalian bioassays of AETX may help determine the need for fish consumption advisories in reservoirs inhabited by *Ah* throughout the southeastern U.S.A. and beyond.

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Tables

Table 3.1: Water quality data for tilapia (*Oreochromis aureus* x *O*. *niloticus*) and largemouth bass (*Micropterus salmoides*) tanks. Tilapia were fed *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla) *ad libitum* daily for 5 days. Tilapia were fed *Ah*/hydrilla collected from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. on either 20 September 2017 (September tilapia, n = 5 tanks) or 17 November 2016 (November tilapia, n = 5 tanks). Largemouth bass were fed 1.5% of their own body weight in tilapia daily for 14-15 days. September largemouth bass (n = 17 tanks) were fed September tilapia, and November largemouth bass (n = 17 tanks) were fed November tilapia. Values represent means ± 1 sd.

Species	Diet Treatment	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Total Ammonia Nitrogen (mg/L)
Tilapia	Sept.	24.3 ± 1.0	7.88 ± 0.55	7.37 ± 0.21	0.78 ± 0.46
	Nov.	24.3 ± 1.0	7.86 ± 0.58	7.44 ± 0.18	0.69 ± 0.43
Largemouth	Sept.	20.4 ± 1.5	8.47 ± 0.31	7.09 ± 0.27	0.20 ± 0.12
bass	Nov.	20.4 ± 1.5	8.45 ± 0.40	7.09 ± 0.26	0.21 ± 0.12

Table 3.2: Aetokthonotoxin (AETX) concentrations in *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla), tilapia (*Oreochromis aureus* x *O. niloticus*), and the gastrointestinal (GI) tracts and axial musculature of largemouth bass (*Micropterus salmoides*). September *Ah*/hydrilla was suspected to lack AETX and was collected from J. Strom Thurmond Reservoir on 20 September 2017. November *Ah*/hydrilla was suspected to contain AETX and was collected from a nearby location within the same reservoir on 17 November 2016. September tilapia were fed September *Ah*/hydrilla, and November tilapia were fed November *Ah*/hydrilla. All tilapia were fed their assigned *Ah*/hydrilla food for 5 days. September largemouth bass were fed September tilapia, and November largemouth bass were fed November tilapia. All largemouth bass were fed their assigned tilapia for 14-15 days. Samples from which AETX was not detected are reported as containing 0 ppb AETX. *Sample size.

	Sample		n* with	Total	AETX Concentration (ppb dry weight)			
Sample	Type	Diet	AETX	n	Min	Mean	Max	St. Dev.
Ah/hydrilla	Whole	Sept.	3	3	88,168.6	94,356.8	102,126.8	7,112.3
		Nov.	3	3	32,143.9	61,205.5	77,496.3	25,229.5
Tilapia	Whole	Sept.	6	6	95.2	378.0	1,021.7	340.1
		Nov.	6	6	168.1	2,336.6	5,010.0	1,857.5
Largemouth bass	GI tracts	Sept.	6	6	33.4	168.2	504.5	187.4
		Nov.	4	6	0.0	340.0	1,292.9	504.2
	Muscle	Sept.	1	6	0.0	0.6	3.4	1.4
		Nov.	2	6	0.0	3.9	17.1	6.9

Table 3.3: Swim chamber water quality and Fulton's condition factor (K_{TL}) during largemouth bass (*Micropterus salmoides*) swimming performance tests. Largemouth bass consumed experimental diets for 0-1 days, 7-8 days, or 14-15 days before swim testing. Experimental diets consisted of juvenile tilapia (*Oreochromis aureus* x *O. niloticus*) which had consumed *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* for 5 days. Values represent means ± 1 sd. Two largemouth bass that were fed tilapia for 0 days were removed from analyses due to observed stress-related behaviors including fasting.

Feeding Duration	Sample Size	Temperature (°C)	Dissolved Oxygen (mg/L)	pH	K_{TL}
0-1 days	10	19.8 ± 0.6	8.64 ± 0.16	7.07 ± 0.14	1.31 ± 0.09
7-8 days	12	17.2 ± 0.5	9.16 ± 0.21	7.10 ± 0.10	1.29 ± 0.10
14-15 days	12	18.5 ± 1.4	8.94 ± 0.30	7.12 ± 0.05	1.35 ± 0.10

Figures

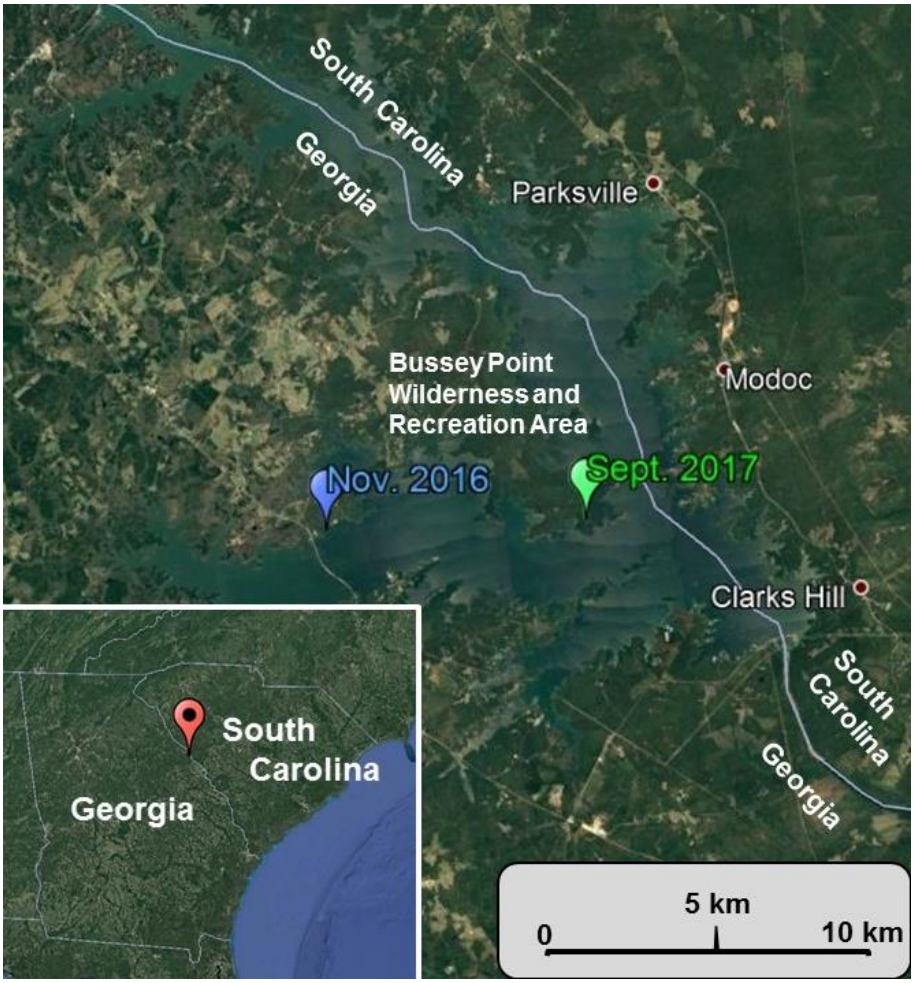

Figure 3.1: Sites from which *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla) samples were collected. All *Ah*/hydrilla samples were collected from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. *Ah*/hydrilla samples that were suspected to lack aetokthonotoxin (AETX) were collected on 20 September 2017 (green). *Ah/*hydrilla samples that were suspected to contain AETX were collected on 17 November 2016 (blue).

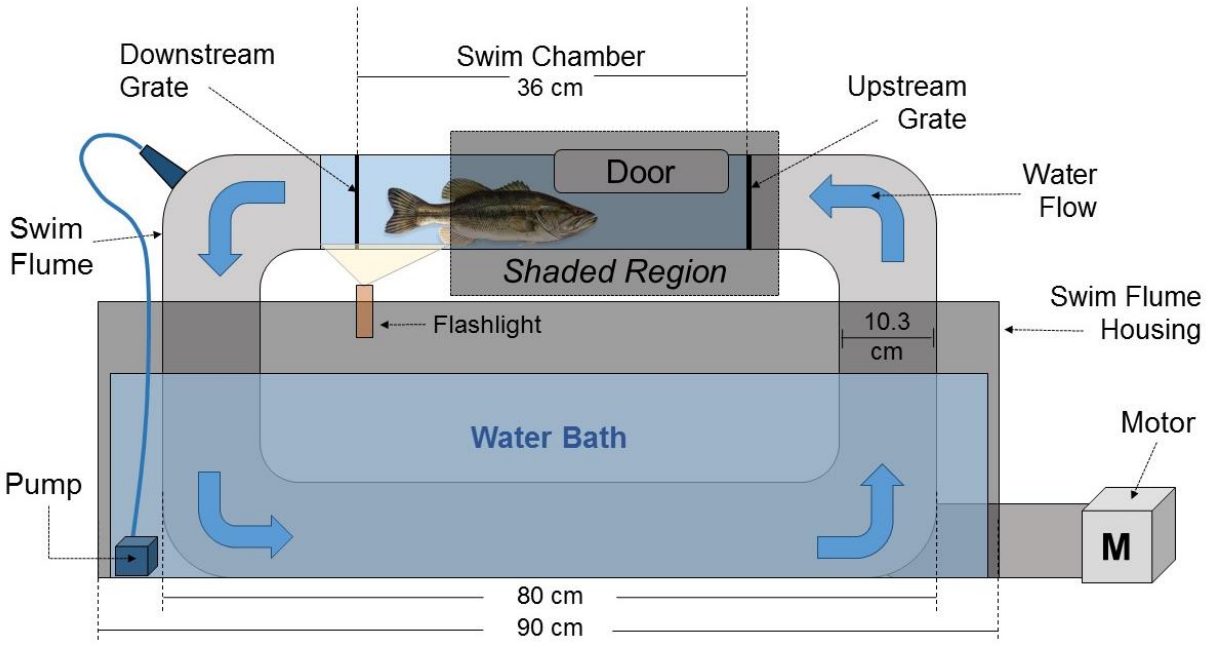

Figure 3.2: Schematic diagram of recirculating swim flume used for burst swimming performance tests.

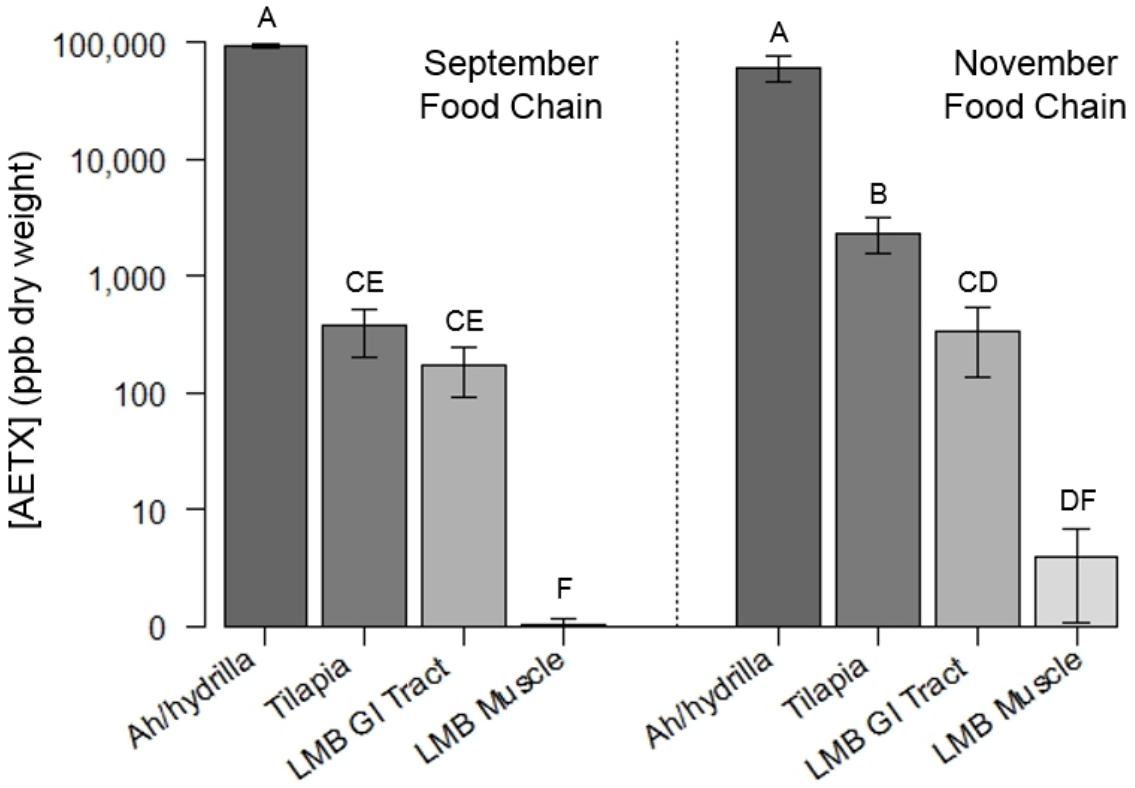

Figure 3.3: Aetokthonotoxin concentrations ([AETX]) in *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla), tilapia (*Oreochromis aureus* x *O. niloticus*), and the gastrointestinal (GI) tracts and axial musculature of largemouth bass (*Micropterus salmoides*, LMB). September *Ah*/hydrilla (n = 3) was collected from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. on 20 September 2017. November *Ah*/hydrilla (n = 3) was collected from a nearby site within the same reservoir on 17 November 2016. September and November tilapia (n = 6 per diet) consumed their respective *Ah*/hydrilla diets *ad libitum* for 5 days. September and November LMB (n = 6 per diet) consumed 1.5% of their body weight in their respective tilapia diet for 14-15 days. Letters designate statistically significant differences in $\log_{10}$ AETX concentrations at $\alpha = 0.05$. Error bars represent 1 se.

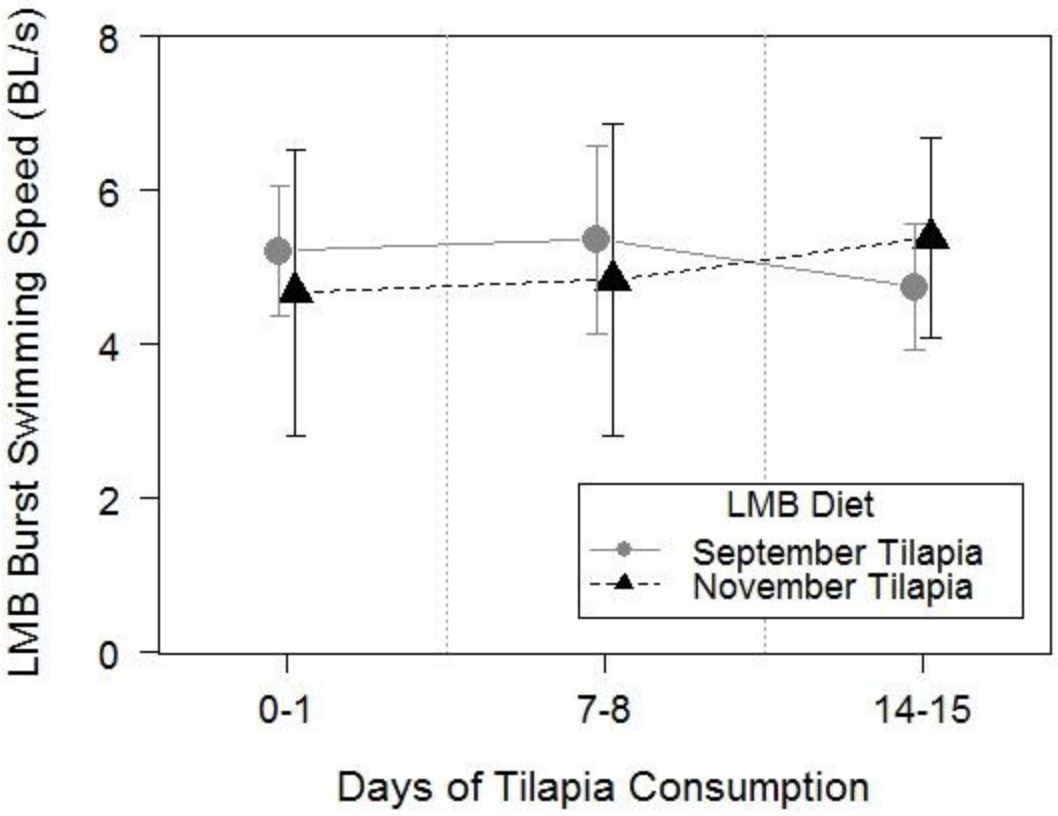

Figure 3.4: Burst swimming speeds (body lengths per second) of largemouth bass (*Micropterus salmoides*, LMB) that consumed tilapia (*Oreochromis aureus* x *O. niloticus*) for 0-1 days, 7-8 days, or 14-15 days. September LMB (circles) consumed tilapia which consumed *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla) collected from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. on 20 September 2017 (September tilapia). November LMB (triangles) consumed tilapia which consumed *Ah*/hydrilla collected from the same reservoir on 17 November 2016 (November tilapia). All tilapia consumed *Ah*/hydrilla for five days before being fed to LMB. Each point represents mean burst swimming speeds of either five (0-1 days feeding) or six (7-8 and 14-15 days feeding) LMB, and error bars represent 1 sd. Two LMB that were swam after 0 days of experimental feeding (n = 1 per diet treatment) were removed from analyses due to observed stress-related behaviors including hiding, erratic swimming, and fasting.

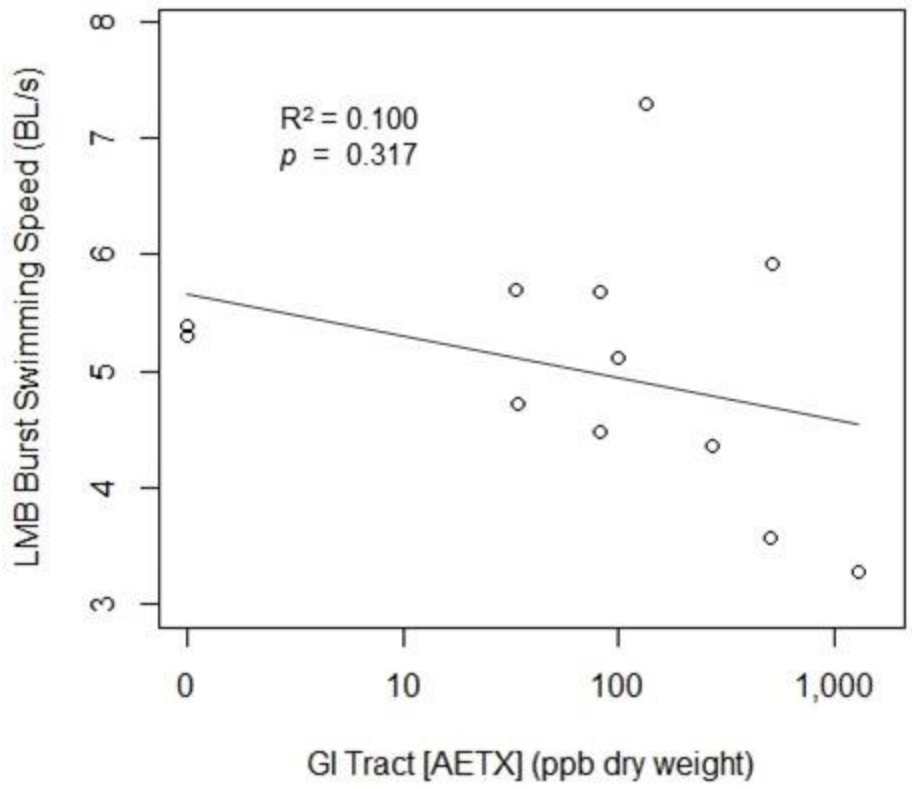

Figure 3.5: Largemouth bass (*Micropterus salmoides*, LMB) burst swimming speed (body lengths per second) was weakly and negatively related to the concentration of aetokthonotoxin ([AETX]) in their gastrointestinal (GI) tracts (n = 12). LMB consumed tilapia (*Oreochromis aureus* x *O. niloticus*) that had eaten *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* for 5 days. LMB consumed 1.5% of their body weight daily in tilapia for 14-15 days. Two of 12 LMB had GI tract AETX concentrations below detection limits (< 7.8 ng per sample) and are reported as containing 0 ppb AETX.

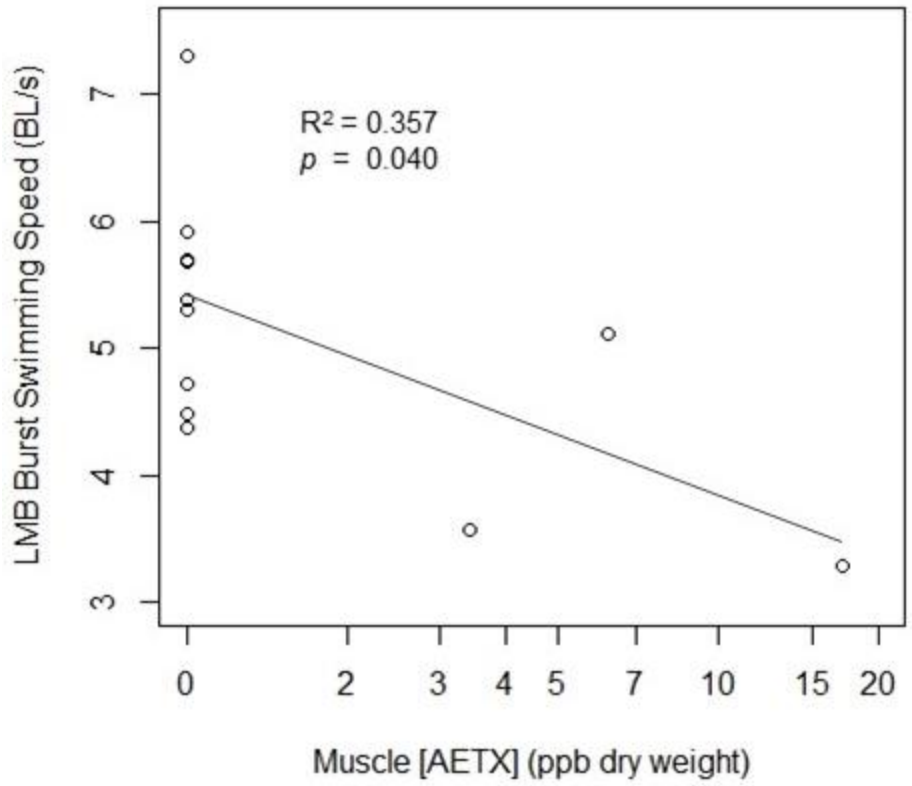

Figure 3.6: Largemouth bass (*Micropterus salmoides*, LMB) burst swimming speed (body lengths per second) was negatively related with aetokthonotoxin concentration ([AETX]) in their axial musculature (n = 12). LMB consumed tilapia (*Oreochromis aureus* x *O. niloticus*) that had eaten *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* for 5 days. LMB consumed 1.5% of their body weight daily in tilapia for 14-15 days. Nine of 12 LMB had muscle AETX concentrations below detection limits (< 7.8 ng per sample) and are reported as containing 0 ppb AETX.

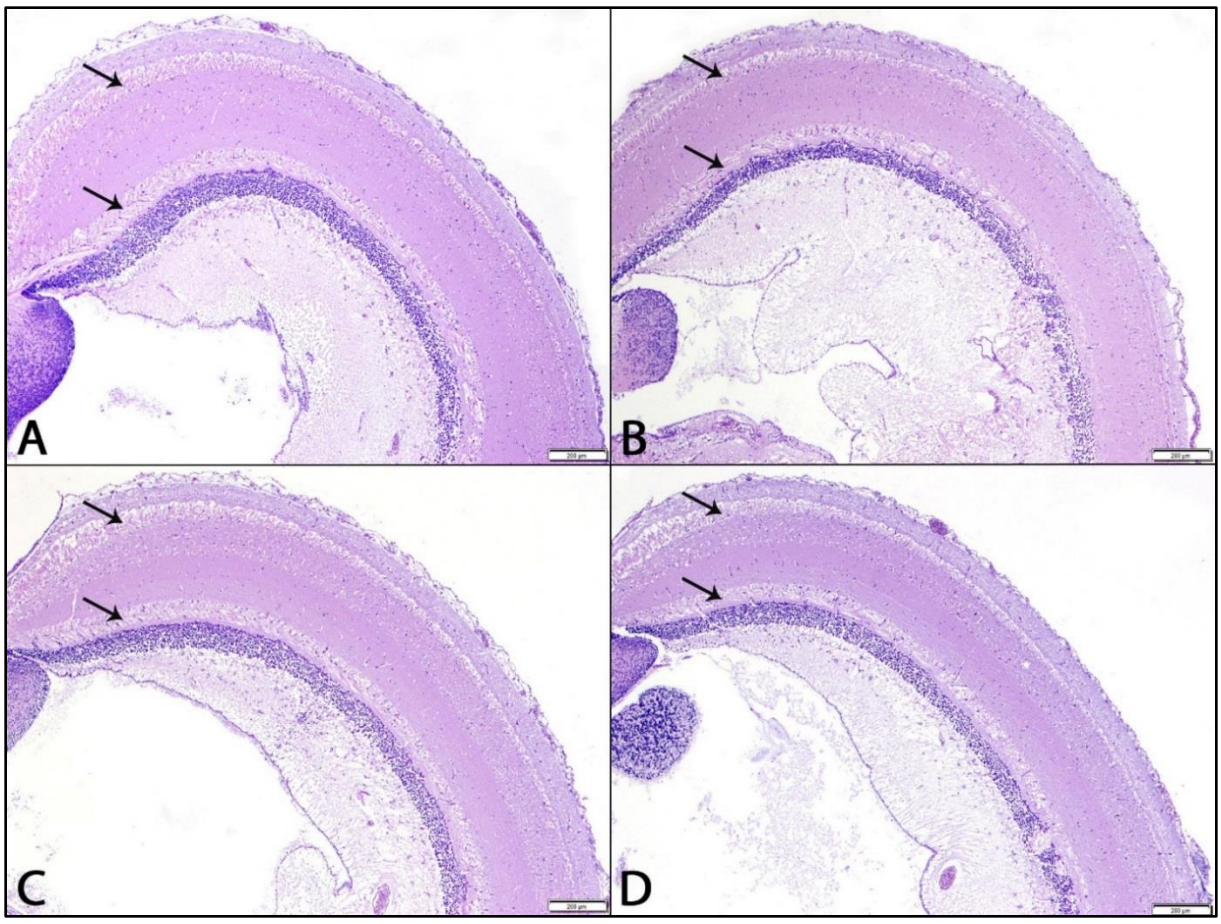

Figure 3.7: Low-magnification histologic images of the optic tecta of largemouth bass (*Micropterus salmoides*, LMB). September LMB consumed tilapia (*Oreochromis aureus* x *O. niloticus*) that consumed *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla) collected from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. on 20 September 2017. November LMB consumed tilapia that consumed *Ah*/hydrilla collected from the same reservoir on 17 November 2016. LMB consumed tilapia for 0-15 days. Widespread vacuolated spaces were interpreted as post mortem and processing artifacts and were present and indistinguishable in the brains of all September and all November LMB. At low magnification, vacuolated spaces are visible as pale staining bands (arrows). **A)** September LMB, 0 days of experimental feeding. **B)** November LMB, 0 days of experimental feeding. **C)** September LMB, 15 days of experimental feeding. **D)** November LMB, 15 days of experimental feeding. Hematoxylin and eosin stain, bars = 200 µm.

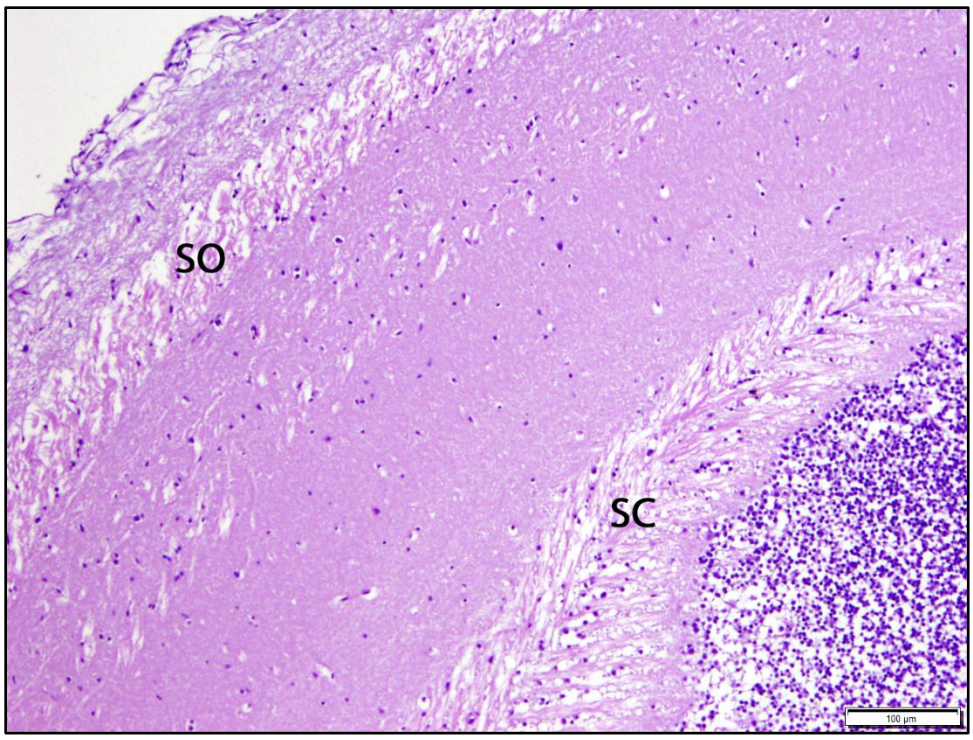

Figure 3.8: Higher-magnification histologic image of the optic tectum of a largemouth bass (*Micropterus salmoides*, LMB). LMB consumed tilapia (*Oreochromis aureus* x *O. niloticus*) that consumed *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla) for five days. September LMB (n = 18) consumed tilapia that consumed *Ah*/hydrilla collected from J. Strom Thurmond Reservoir, Georgia/South Carolina, U.S.A. on 20 September 2017. November LMB (n = 18) consumed tilapia that consumed *Ah*/hydrilla collected from the same reservoir on 17 November 2016. All LMB consumed their respective tilapia diets for 0-15 days. Pictured is the brain of a September LMB that consumed experimental tilapia for 0 days. Indistinguishable, bilaterally symmetrical, vacuolated spaces were present throughout the stratum opticum (SO) and stratum centrale (SC) in all 36 LMB. Vacuoles commonly develop in the SO and SC in birds afflicted with VM. However, vacuoles in LMB brains lacked the round-to-oval shape and smooth, rounded contours typical of VM-related vacuoles. Hematoxylin and eosin stain, bar = 100 µm.

CHAPTER 4

CONCLUSIONS

The discovery of aetokthonotoxin (AETX) in most tissue samples from all five wild fish species suggests that AETX may accumulate in more species and at a greater frequency than described previously. These findings provide ecological context to previous laboratory feeding trials that have shown that fishes (Haynie *et al*. 2013), reptiles (Mercurio *et al*. 2014), and amphibians (Maerz *et al*. 2018) are susceptible to vacuolar myelinopathy (VM) or other adverse effects from consumption of *Aetokthonos hydrillicola*-colonized *Hydrilla verticillata* (*Ah*/hydrilla). AETX concentrations should be surveyed in wild populations from these non-avian species to determine the ecological risks of AETX at the food-web level rather than at the food-chain level as has been investigated via laboratory feeding trials. As data from both wild and laboratory fishes suggest here, attention should be focused on quantifying AETX concentrations in primary consumers of *Ah*/hydrilla because these consumers appear to be exposed to the greatest levels of AETX and are likely responsible for trophic transfer of AETX to secondary and tertiary consumers. Diet studies of predators in *Ah*-inhabited ecosystems should accompany these investigations of AETX concentrations in primary consumers to determine which prey items serve as vectors for transferring AETX through aquatic food webs. Such diet studies would also be helpful for determining which species and trophic guilds of predators may be most at risk for AETX accumulation and therefore VM development.

Although the greatest concentrations of AETX were detected in lower-order consumers compared with higher-order consumers, the results from the wild fish surveys suggests that true bioaccumulation of AETX (i.e., assimilation of AETX into tissue, in this case muscle tissue) occurred at a similar rate in all species regardless of trophic status. The similarity of AETX

concentrations in muscle among all species, despite AETX concentrations in GI tracts (wild fishes) or whole body samples (laboratory tilapia, *Oreochromis aureus* x *O. niloticus*) from lower-order consumers being much greater than those in higher-order consumers, suggests that AETX may not biomagnify up fish food chains. The much greater frequency of VM documentation in primary consumers (mainly American coots, *Fulica americana*, e.g., Thomas *et al*. 1998, Fischer *et al*. 2002, Rocke *et al*. 2002, Dodder *et al*. 2003) compared with secondary consumers (bald eagles, *Haliaeetus leucocephalus*, Thomas *et al*. 1998, Fischer *et al*. 2002, Fischer *et al*. 2006) further supports the claim that AETX may not biomagnify in food chains. Muscle AETX concentrations may have been similar among all four centrarchid fishes due to bioconcentration (i.e., the absorption of a chemical directly from the water column). Bioconcentration of AETX in fish muscle appears plausible given that AETX did not differ with trophic status in wild fishes. However, trophic transfer of AETX (i.e., as opposed to bioconcentration) must explain at least some of the bioaccumulation of AETX in fish muscle because AETX was detected in muscle of largemouth bass (*Micropterus salmoides*) that were never exposed to water containing *Ah*/hydrilla. Trophic transfer and bioaccumulation of AETX from food consumption is therefore suggested, perhaps in combination with bioconcentration, to explain AETX detection in muscle from secondary and tertiary consumers.

The detection of AETX in 18/20 wild fish muscle samples compared with only 3/12 largemouth bass muscle samples from the laboratory feeding trials may suggest that wild fishes have been exposed to AETX either in greater doses or for longer durations compared with the largemouth bass from the laboratory feeding trials. Because only 25% of largemouth bass from the laboratory trials accumulated AETX after 15 days of dietary exposure, compared with detection of AETX in the muscle of 90% of wild largemouth bass from an *Ah*-inhabited reservoir, wild fishes are likely exposed to AETX at least seasonally and possibly year-round. The difference in muscle AETX concentrations between laboratory-raised and wild-caught fishes may also support the claim that AETX may bioconcentrate directly into fish tissues from the

water column. Unlike wild fishes, laboratory-raised largemouth bass were only exposed to AETX through their diet rather than directly through the water column. To determine the duration and extent of AETX exposure in wild populations, additional surveys in a greater diversity of animals are needed throughout the year. Furthermore, quantification of AETX in water samples from *Ah*-inhabited reservoirs would help determine the extent to which bioconcentration may contribute to overall AETX bioaccumulation rates in fishes.

The lack of difference in burst swimming performance between September and November largemouth bass from the laboratory swimming trials was not surprising because AETX concentrations were similar between September and November *Ah*/hydrilla samples. The sublethal effects of AETX on largemouth bass were therefore indeterminable from these trials. Largemouth bass burst swimming performance was negatively related with AETX concentrations in their axial musculature ($n = 12$, $R^2 = 0.357$, $p = 0.040$, Figure 3.5), and the two lowest swimming speeds out of all 12 fish were swam by two of the three largemouth bass in which AETX accumulated. However, the lack of AETX detection in most of these largemouth bass leaves the effect of AETX accumulation on fish motor function in question. In contrast, the 15-day exposure to AETX-contaminated tilapia may not have affected largemouth bass burst swimming performance in these experiments because performances in the study were similar to those from healthy largemouth bass of a similar size that were swam under similar conditions (Kolok 1991). The development of VM and lack of clinical symptoms (i.e., behavioral effects) in grass carp (*Ctenopharyngodon idella*) that were fed *Ah*/hydrilla (Haynie *et al*. 2013) further supports the claim that the motor function of the largemouth bass from our laboratory swimming trials presented herein may have been unaffected by secondary trophic transfer of AETX from consumption of prey fishes. However, the development of VM-related vacuolation in grass carp suggests that some threshold concentration of AETX may elicit sublethal effects which may in turn affect survival and reproductive fitness of these fishes. Additional AETX dietary studies are needed with largemouth bass or other top predators that evaluate the effects of greater doses of

AETX for longer durations. Additional AETX studies with grass carp are also warranted because these fish are one of the only truly herbivorous fishes in North America and may face more risk related to AETX exposure. Grass Carp may serve as a vector of AETX exposure to top predators including larger fish and birds of prey. Grass carp are stocked in reservoirs throughout the U.S.A. to biologically control the spread of invasive hydrilla (see Pípalová 2006 for a detailed review), and future research could also help determine if AETX exposure may reduce their ability control hydrilla abundance.

The successful extraction and quantification of AETX concentrations from plant and animal tissue enables researchers to better understand dose-response relationships of AETX in animals. Dose-response relationships are necessary for understanding threshold AETX concentrations that may affect an organism's health, reproductive fitness, and survival. Bioassays involving cell lines (Wiley *et al*. 2007), cladocerans (*Ceriodaphnia dubia*), chickens (Lewis-Weis *et al*. 2004, Haynie *et al*. 2013, Mercurio *et al*. 2014, Dodd *et al*. 2016), mallard ducks (Birrenkott *et al*. 2004, Rocke *et al*. 2005, Wiley *et al*. 2007, Haynie *et al*. 2013), and zebrafish (unpublished data from Tabitha Phillips *et al*., 2018) have already been used in *Ah*/hydrilla research. However, the use of each of these bioassay organisms can involve financial, regulatory, or logistical challenges. A novel bioassay involving brine shrimp (*Artemia* spp.) nauplii is being developed at the University of Georgia and, if successful, would help alleviate many of these challenges. Brine shrimp are an appealing bioassay species because their eggs (the life stage at which brine shrimp are purchased) are inexpensive and can be stored frozen indefinitely until needed for experimentation. Hatching brine shrimp from eggs requires little physical space, inexpensive equipment, and no more than three days. Finally, because brine shrimp are invertebrates, the regulatory environment is much less restrictive, minimizing the need for permits or administrative approval. However, the effects of AETX on brine shrimp and the similarity of these effects to those that AETX has on other organisms remains unknown. A mammalian bioassay, for example with mice or rats, is therefore

recommended to more appropriately assess the potential effects of AETX on human consumers. Now that AETX has been confirmed in fishes from an *Ah*-inhabited reservoir with high fishing pressure (J. Strom Thurmond Reservoir, W. E. Houck and D. M. Rankin, South Carolina Department of Natural Resources, unpublished data), humans harvesting fishes from these reservoirs may be at risk for AETX exposure. Mammalian bioassays involving AETX would not only help clarify the risk of AETX to humans, but in combination with estimated AETX doses expected in fish fillets such as those reported here, would help determine whether consumption advisories are necessary for fishes harvested from *Ah*-inhabited reservoirs.

The production of AETX was expected to be greatest between mid-October and February in concurrence with the timing of VM outbreaks in birds. However, the similarity of AETX concentrations in *Ah*/hydrilla samples collected from September and November challenges the hypothesis that AETX production varies seasonally. The difference in AETX concentrations between September and November tilapia (Figure 3.2) and the great variability in AETX concentrations within samples from all fish species and tissues together suggest that AETX concentrations vary greatly among *Ah*/hydrilla subsamples taken from the same hydrilla beds. Prevalence of *Ah* colonies within hydrilla beds is greatly heterogeneous, as suggested by patchy and highly variable occurrence of *Ah* among hydrilla stems and even among leaves within individual hydrilla stems. Large samples of *Ah*/hydrilla should be thoroughly homogenized before subsamples are collected to increase the accuracy of inferences that are made about AETX concentrations in *Ah*/hydrilla samples. Additionally, the handling, storage, and processing of September *Ah*/hydrilla samples may have influenced AETX production therein. Because VM outbreaks usually begin during autumn as hydrilla begins to senesce (Rocke *et al*. 2002, Wilde *et al*. 2005, Wiley *et al*. 2007), decreasing temperatures are hypothesized to cue AETX production by *Ah*. The freezing and thawing of *Ah*/hydrilla samples may therefore trigger AETX production by *Ah*, altering AETX levels from what they would have been around the time of sample collection from the field. Understanding the effects of environmental variables on AETX

production would help researchers develop storage and processing methods that would not alter AETX concentrations in collected *Ah*/hydrilla samples.

Understanding AETX production in response to environmental variables would also enable the development of statistical models to help predict the timing and severity of AETX production and therefore the risk for future VM outbreaks to wildlife. Developing predictive models of seasonal and inter-annual AETX concentrations may be the most important subject of future research. The ability to forecast the timing and rate of AETX production in a reservoir would help protect all species by allowing reservoir managers to plan remediation strategies before AETX production begins. The effectiveness of future *Ah*/hydrilla remediation strategies, should such strategies be developed, could also be inferred based on the differences between the amount of AETX produced before and after the execution of those remediation strategies. The effects of physical, chemical, and biological variables on AETX concentrations in *Ah*/hydrilla, water, and animals, as well as *Ah* prevalence on hydrilla and other host plants, should therefore be investigated systematically in multiple reservoirs throughout the year to better understand environmental dynamics of *Ah* and AETX. Such efforts would also help identify factors that may affect or initiate AETX production and would help reveal any relationship between AETX production and VM outbreaks and allow the inference of possible effects to human health.

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APPENDIX A

SUMMARY OF VACUOLAR MYELINOPATHY OCCURRENCE AND THE EFFECTS OF *AETOKTHONOS HYDRILLICOLA*-COLONIZED HYDRILLA EXPOSURE ON ANIMALS

Species	Study Setting[1]	*Ah* Status[2]	Specimen Type	Symptoms		References	Comments
				Clinical[3]	Diagnostic[4]		
Birds							
American coots	DeGray Lake, AR	Present	Wild	Yes	62/77	Thomas *et al*. (1998)	
	Lake Juliette, GA	Present	Wild	Yes	Yes	Fischer *et al*. (2002)	
	Lake Surf[5], NC	Present	Wild	Yes	Yes	Fischer *et al*. (2002)	
	Lake Surf, NC	Present	Wild	Yes	26/26	Larsen *et al*. (2002)	
	Lake Mattamuskeet, NC	Absent	Wild	No	0/12	Larsen *et al*. (2002)	
	Lake Surf, NC	Present	Sentinel[6]	Unrep.[7]	9/13	Rocke *et al*. (2002)	
	Lake Surf, NC	Present	Wild	Yes	Yes	Rocke *et al*. (2002)	
	JSTR[8], GA/SC	Present	Wild	Yes	Yes	Fischer *et al*. (2003)	
	Lake Seminole, GA	Absent	Wild	No	No	Fischer *et al*. (2003)	
	Lake Surf, NC	Present	Wild	Unrep.	16/16	Dodder *et al*. (2003)	
	Lake Surf, NC	Present	Laboratory	3/6	0/6	Rocke *et al.* (2005)	
	Harris Lake, NC	Absent	Laboratory	0/2	0/2	Rocke *et al*. (2005)	
	JSTR, GA/SC	Present	Wild	Yes	Yes	Fischer *et al*. (2006)	
Bald eagles	DeGray Lake, AR	Present	Wild	Yes	14/14	Thomas *et al*. (1998)	
	Lake Hamilton, AR	Present	Wild	Yes	1/1	Thomas *et al*. (1998)	
	Lake Ouachita, AR	Present	Wild	Yes	11/11	Thomas *et al*. (1998)	
	Lake Juliette, GA	Present	Wild	Unrep.	Yes	Fischer *et al*. (2002)	
	Lake Surf, NC	Present	Wild	Unrep.	Yes	Fischer *et al*. (2002)	
	JSTR, GA/SC	Present	Wild	Unrep.	Yes	Fischer *et al*. (2002)	
	Savannah River Site, SC	Present	Wild	Unrep.	Yes	Fischer *et al*. (2002)	
	JSTR, GA/SC	Present	Wild	Unrep.	Yes	Fischer *et al*. (2006)	
Bobwhite quail	JSTR, GA/SC	Present	Laboratory	0/4	0/4	Birrenkott *et al*. (2004)	Dermal exposure
Buffleheads	Lake Surf, NC	Present	Wild	6/6	6/6	Augspurger *et al.* (2003)	
Canada geese	JSTR, GA/SC	Present	Wild	Unrep.	Yes	Fischer *et al*. (2006)	

Chickens	JSTR, GA/SC	Present	Laboratory	5/5	4/5	Lewis-Weis *et al*. (2004)	Trial 1 (fed coot tissues)
	Lake Seminole, GA	Absent	Laboratory	0/1	0/1	Lewis-Weis *et al*. (2004)	Trial 1 (fed coot tissues)
	JSTR, GA/SC	Present	Laboratory	1/7	3/7[9]	Lewis-Weis *et al*. (2004)	Trial 2 (fed coot tissues)
	Lake Seminole, GA	Absent	Laboratory	0/3	0/3	Lewis-Weis *et al*. (2004)	Trial 2 (fed coot tissues)
	JSTR, GA/SC	Present	Laboratory	0/4	3/4	Lewis-Weis *et al*. (2004)	Trial 3 (fed hydrilla)
	Lake Seminole, GA	Absent	Laboratory	0/4	0/4	Lewis-Weis *et al*. (2004)	Trial 3 (fed hydrilla)
	JSTR, GA/SC	Present	Laboratory	0/8	0/8	Wiley *et al*. (2007)	Trial 1
	Lake Marion, SC	Absent	Laboratory	0/8	0/8	Wiley *et al*. (2007)	Trial 1
	Davis Pond, SC	Present	Laboratory	0/15	0/15	Haynie *et al*. (2013)	Fed grass carp tissues
	Lake Marion, SC	Absent	Laboratory	0/9	0/9	Haynie *et al.* (2013)	Fed grass carp tissues
	JSTR, GA/SC	Present	Laboratory	5/5	5/5	Mercurio *et al*. (2014)	
	Lake Seminole, GA	Absent	Laboratory	0/5	0/5	Mercurio *et al*. (2014)	
	Lake Hatchineha, FL	Absent	Laboratory	0/5	0/5	Dodd *et al*. (2016)	Fed hydrilla
	Lake Hatchineha, FL	Absent	Laboratory	0/5	0/5	Dodd *et al*. (2016)	Fed apple snails
	Lake Tohopekaliga, FL	Present	Laboratory	0/10	10/10	Dodd *et al*. (2016)	Fed hydrilla
	JSTR, GA/SC	Present	Laboratory	3/5	5/5	Dodd *et al*. (2016)	Fed apple snails
	JSTR, GA/SC	Present	Laboratory	2/5	5/5	Dodd *et al*. (2016)	Fed hydrilla
Great horned owls	JSTR, GA/SC	Present	Wild	Unrep.	2/2	Fischer *et al*. (2006)	
Killdeer	JSTR, GA/SC	Present	Wild	Unrep.	1/1	Fischer *et al*. (2006)	
Mallard ducks	Lake Surf, NC	Present	Sentinel	Unrep.	31/144	Rocke *et al*. (2002)	
	Lake Surf, NC	Present	Wild	Yes	Yes	Rocke *et al*. (2002)	
	Crystal Lake, NC	Absent	Sentinel	Unrep.	0/13	Rocke *et al*. (2002)	
	Harris Lake, NC	Absent	Sentinel	Unrep.	0/14	Rocke *et al*. (2002)	
	Trace Lake, NC	Absent	Sentinel	Unrep.	0/26	Rocke *et al*. (2002)	
	Lake Surf, NC	Present	Wild	2/2	2/2	Augspurger *et al*. (2003)	
	Lake Surf, NC	Present	Sentinel	No	0/8	Larsen *et al*. (2003)	
	JSTR, GA/SC	Present	Sentinel	0/4	Inconcl.	Birrenkott *et al*. (2004)	Hydrilla exposure 2001
	JSTR, GA/SC	Present	Sentinel	0/9	0/9	Birrenkott *et al*. (2004)	Hydrilla gavage 2001
	JSTR, GA/SC	Present	Sentinel	1/9	6/9	Birrenkott *et al*. (2004)	Hydrilla exposure 2002
	JSTR, GA/SC	Present	Laboratory	0/6	2/6	Rocke *et al.* (2005)	
	Harris Lake, NC	Absent	Laboratory	0/2	0/2	Rocke *et al*. (2005)	
	Lake Surf, NC	Present	Laboratory	0/31	0/31	Rocke *et al.* (2005)	Adults
	Lake Surf, NC	Present	Laboratory	0/20	0/20	Rocke *et al*. (2005)	Ducklings
	Davis Pond, SC	Present	Sentinel	5/15	15/15	Wilde *et al*. (2005)	
	JSTR, GA/SC	Present	Laboratory	0/8	0/8	Wiley *et al*. (2007)	Trial 1 (Oct. 2003)
	Lake Marion, SC	Absent	Laboratory	0/8	0/8	Wiley *et al*. (2007)	Trial 1 (Oct. 2003)
	Davis Pond, SC	Present	Laboratory	0/6	4/6	Wiley *et al*. (2007)	Trial 2 (Nov.-Dec. 2003)
	JSTR, GA/SC	Present	Laboratory	2/6	6/6	Wiley *et al*. (2007)	Trial 2 (Nov.-Dec. 2003)
	Lake Marion, SC	Absent	Laboratory	0/6	0/6	Wiley *et al*. (2007)	Trial 2 (Nov.-Dec. 2003)
	Davis Pond, SC	Present	Sentinel	0/10	6/10	Haynie *et al*. (2013)	

Mallard ducks (cont.)	Davis Pond, SC	Present	Laboratory	1/15	10/15	Haynie *et al.* (2013)	
	Lake Marion, SC	Absent	Laboratory	0/5	0/5	Haynie *et al*. (2013)	
Red-tailed hawks	JSTR, GA/SC	Present	Laboratory	0/5	5/5	Fischer *et al*. (2003)	Fed coot tissues
	Lake Seminole, GA	Absent	Laboratory	0/1	0/1	Fischer *et al*. (2003)	Fed coot tissues
	JSTR, GA/SC	Present	Laboratory	0/1	1/1	Lewis-Weis *et al*. (2004)	Fed coot tissues
Ring-necked ducks	Lake Surf, NC	Present	Wild	2/2	2/2	Augspurger *et al.* (2003)	
Mammals							
Domestic swine	JSTR, GA/SC	Present	Laboratory	0/4	0/4	Lewis-Weis *et al*. (2004)	Fed coot tissues
	Lake Seminole, GA	Absent	Laboratory	0/1	0/1	Lewis-Weis *et al*. (2004)	Fed coot tissues
Reptiles							
Painted turtles	JSTR, GA/SC & Upper Towaliga Reservoir, GA	Present	Laboratory	5/5	5/5	Mercurio *et al*. (2014)	
	Lake Seminole, GA	Absent	Laboratory	0/5	0/5	Mercurio *et al*. (2014)	
Amphibians							
Bullfrogs	JSTR, GA/SC	Present	Laboratory	Yes	Untested	Maerz *et al*. (2018)	
	Lake Wylie, NC & Lake Seminole, FL	Absent	Laboratory	No	Untested	Maerz *et al*. (2018)	
Green frogs	JSTR, GA/SC	Present	Laboratory	Yes	8/10	Maerz *et al*. (2018)	Autumn hydrilla
	JSTR, GA/SC	Present	Laboratory	No	0/10	Maerz *et al*. (2018)	Summer hydrilla
	Lake Wylie, NC & Lake Seminole, FL	Absent	Laboratory	No	1/9	Maerz *et al*. (2018)	
Southern leopard frogs	JSTR, GA/SC	Present	Laboratory	Yes	Untested	Maerz *et al*. (2018)	
	Lake Wylie, NC & Lake Seminole, FL	Absent	Laboratory	No	Untested	Maerz *et al*. (2018)	
Green treefrogs	JSTR, GA/SC	Present	Laboratory	No	0/10	Maerz *et al*. (2018)	
	Lake Wylie, NC & Lake Seminole, FL	Absent	Laboratory	No	0/10	Maerz *et al*. (2018)	
Fishes							
Grass carp	Davis Pond, SC	Present	Sentinel	0/23	7/23	Haynie *et al*. (2013)	
	JSTR, GA/SC	Present	Laboratory	0/100	11/20	Haynie *et al*. (2013)	
	Lake Marion, SC	Absent	Laboratory	0/100	0/20	Haynie *et al.* (2013)	

[1] For wild and sentinel study specimens, study setting represents location of observations. For laboratory study specimens, study setting represents location from which food (*Ah*-colonized hydrilla unless stated otherwise in comments) was collected.

[2] The occurrence of *Aetokthonos hydrillicola* in the study setting. This does not necessarily represent the occurrence of aetokthonotoxin in the study setting.

[3] Clinical symptoms include various types of motor impairment including but not limited to ataxia, seizures, and the inability to walk, swim, wade, fly, or balance. Death is included as a clinical symptom only when accompanied by diagnostic symptoms.

[4] Diagnostic symptoms include the occurrence of intramyelinic edema and vacuolization of white matter in the central nervous system as defined by Thomas *et al*. (1998).

[5] Also known as Woodlake.

[6] Describing study specimens which were introduced manually to the study setting from another location for experimental purposes.

[7] Unreported information.

[8] J. Strom Thurmond Reservoir; also known as Clarks Hill Reservoir.

[9] Gastrointestinal tracts from affected coots were only fed to the three chickens which developed VM.