ASSESSMENT OF A NOVEL CYANOBACTERIUM ON NATIVE MACROPHYTES AND TOXICITY POTENTIAL FOR TROPHIC TRANSFER TO AQUATIC PREDATORS

by

MELISSA KATHERINE MARTIN

(Under the Direction of Susan B. Wilde and John C. Maerz)

ABSTRACT

Aetokthonos hydrillicola (*Ah*) is a novel cyanobacterium that grows on submerged, freshwater macrophytes. The primary substrate for *Ah* is invasive *Hydrilla verticillata,* and the co-occurrence of *Ah* on *Hydrilla* has been linked to neurological impairment and death of waterbirds, turtles, amphibians, and fish. In laboratory feeding trials, juvenile watersnakes became progressively anorectic, and paedomorphic mole salamanders were lethargic and unresponsive after consuming fish, snails, or tadpoles that fed on *Ah*-positive *Hydrilla*. Some watersnakes and salamanders developed intramyelinic vacuoles in the cervical spinal cord. Field surveys confirmed *Ah*-positive *Hydrilla* generally had higher toxin concentrations than the native macrophyte *Najas guadalupensis*; however, some *Ah*-positive *N. guadalupensis* samples did exhibit comparable levels of toxin production earlier in the season. These findings raise concerns for expanded wildlife mortality in natural environments through evidence for potential for the *Ah* toxin in the fall on other aquatic plant species, and higher trophic levels.

INDEX WORDS: cyanobacterium, freshwater macrophytes, *Hydrilla verticillata*, *Aetokthonos hydrillicola*, watersnakes, *Nerodia*, paedomorphic mole salamander, *Ambystoma talpoideum*

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DEDICATION

I want to dedicate this research to my mom and dad who were always supportive in every endeavor and decision I made in life. They have always believed in me and encouraged me in all things.

“Few places in this world are more dangerous than home. Fear not, therefore, to try the mountain passes. They will kill care, save you from deadly apathy, set you free, and call forth every faculty into vigorous, enthusiastic action.” **John Muir**

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cyanobacteria are photosynthetic prokaryotic organisms widely distributed and adapted to broad range of environments. Cyanobacteria occur as microscopic cells, often in colonies or filaments, taxonomically separated by special morphological, physiological, and genetic characteristics. They are able to fix nitrogen from the atmosphere, and survive in a wide range of temperature, light, and anoxic conditions. Their versatility and hardiness has allowed them to colonize virtually all freshwater, marine, and terrestrial (moist soil) ecosystems on Earth (Ferrão-Filho et al. 2011).

Many cyanobacteria produce secondary metabolites that may be toxic to fish, wildlife, and people. The function of the secondary metabolites that constitute cyanotoxins are complex and potentially includes improving iron chelation (Orr and Jones 1998), defense (Rohrlack et al. 1999), photosynthesis/light-related processes (Young et al. 2005), and intraspecies communications (Schatz et al. 2007). Cyanobacteria typically bloom in eutrophic waters with excess phosphorus and nitrogen loading. Recent research on toxin-producing benthic and epiphytic cyanobacteria indicate they may also be enhanced by excess nutrients within lake sediments (Lyngbya refs, Wilde et al 2014).

Cyanotoxins are diverse chemical substances characterized by distinct toxic mechanisms in vertebrates. Some cyanotoxins are strong neurotoxins (anatoxin-a, anatoxin-a(s), and saxitoxins), some are primarily toxic to the liver (microcystins, nodularin, and cylindrospermopsin), and additional natural products (i.e.

lipopolysaccharides) result in various health impairments (i.e. skin rashes and gastroenteritis). They cause damage to wildlife and humans (Wiegand et al. 2005) mainly via ingestion of contaminated food or water sources (Ibelings et al. 2014). Neurotoxic alkaloids are a special group associated with many different types of cyanobacteria including *Aphanizomenon* sp., *Anabaena* sp., *Lyngbya* sp., *Planktothrix* sp., and *Cylindrospermopsis* sp. These toxins can cause disruption of sodium channels in nerve cells leading to paralysis and death by respiratory arrest in mammals after ingesting a neurotoxin. Cyanotoxin exposure may also occur in aquatic food webs through initial consumption/filtration of toxic cells by zooplankton or bivalves that are then eaten by fish and other aquatic consumers. Additionally, some submerged aquatic plants have been shown to have the ability to uptake hepatotoxins from planktonic blooms of *Microcystis* and *Cylindrospermopsis* that could present a risk to herbivores (Wiegand et al. 2005).

Aetokthonos hydrillicola (*Ah*) is a recently described, epiphytic cyanobacterium that grows on submerged aquatic vegetation (SAV); especially invasive *Hydrilla*, *Hydrilla verticillata*, Brazilian elodea, *Egeria densa*, and Eurasian water milfoil, *Myriophyllum spicatum* (Wilde et al. 2005, 2014). *Ah* appears to seasonally produce a neurotoxin that has been linked to neurological impairment and death among a range of vertebrates (fish, amphibians, turtles, and birds) and invertebrates (*Ceriodaphnia sp.)* that directly or indirectly consume plant material with *Ah* (Fischer et al. 2003; Lewis-Weiss et al. 2004; Rocke et al. 2005; Haynie et al. 2013; Mercurio et al. 2014; Dodd et al. 2016; Maerz et al. In press). Ingestion of toxic *Ah*-positive *Hydrilla* has been linked to the deaths of migratory waterfowl and bald eagle (*Haliaeetus leucocephalus*) in the field, and

is now considered among the largest threats to bald eagle conservation (Thomas et al. 1998, Fischer et al. 2003; Lewis-Weiss et al. 2004). The capacity for trophic transfer of *Ah* toxin to predatory species expands the range of wildlife vulnerable to *Ah* invasion.

To date, most research has focused on threats from *Ah* growing preferentially on invasive SAV. Invasive *Hydrilla*, *Hydrilla verticillata*, Brazilian elodea, *Egeria densa*, and Eurasian water milfoil, *Myriophyllum spicatum* have been linked to avian mortality events (Wilde et al. 2005, Wilde et al. 2014). However, laboratory feeding studies using chickens and mallards showed *Ah* toxicity only when birds were fed *Hydrilla* with dense *Ah* colonies (Birrenkott et al. 2004; Wiley et al. 2007, 2008; Haynie 2008). Recent bioassay-guided fractionation has successfully isolated the neurotoxic compound from *Ah*-positive *Hydrilla*, and led to the development of analytical techniques allowing for detection and relative assessment of potential *Ah* toxin concentration on other plant species (Wiley et al. 2008; Haram 2016).

Recent feeding trials documented neurological impairment and the development of intramyelinated vacuoles consistent with *Ah* toxicity among reptiles and amphibians. Painted turtles (*Chrysemys picta*) fed *Ah*-positive *Hydrilla* for 90 days developed irregular gaits, became anorectic, and developed lesions throughout the brain (Mercurio et al. 2014). Tadpoles of American bullfrogs, *Rana catesbeiana*, green frogs, *R. clamitans*, Southern leopard frogs, *R. sphenocephala,* fed *Ah*-positive *Hydrilla* for 30 days exhibited clinical neuropathy, significant mortality, and characteristic vacuolar lesions in the optic region (Maerz et al. In press). These herpetofauna graze directly on SAV, and therefore, are directly exposed to ingestion of the *Ah*-associated toxin in *Hydrilla* + *Ah* environments.

The broad sensitivity of aquatic fauna to ingestion of *Ah*, the wide range of prey taxa that graze on SAVs colonized by *Ah* and capacity for trophic transfer of toxic materials, and the potential for *Ah* to grow on a range of native and nonnative SAVs suggests the potential risks to wildlife from this novel cyanobacteria are broad. There is an urgent need to evaluate potential exposure and sensitivity of other taxa, particularly predatory species that would be indirectly exposed to *Ah* toxin via their prey, and there is a need to evaluate the potential toxicity of *Ah* growing on a wider range of aquatic macrophytes. We need to determine whether other macrophytes – particularly those that may grow in a wider range of freshwater environments - support *Ah* growth and toxin production, and whether toxin production is comparable in concentration and shows similar seasonal patterns of production.

In this thesis, I examine trophic transfer of *Ah* toxin to two common aquatic predators: Mole salamanders (*Ambystoma talpoideum*) and watersnakes, (*Nerodia* spp.). Mole salamanders are aquatic amphibians. Their geographic range and habitats overlap with known ranges of *Ah*, and they are facultatively paedomorphic, which means that they can remain in aquatic habitats feeding on aquatic prey throughout the year. Mole salamanders occupy vegetated littoral zones and lentic overlapping habitats where they feed on a variety of taxa known to graze SAV including tadpoles, snails, and scuds (Amphipoda, Gammaridae) (Jensen et al. 2008; McAllister et al. 1996). Because mole salamanders consume their prey whole, they would ingest toxins if they were present in any part of the prey (gut contents, liver, muscle, and brain). Therefore, it is probable that mole salamanders would be exposed to *Ah* toxin growing on SAV through the food web.

Northern watersnakes, *N. sipedon*, and banded watersnakes, *N. fasciata*, are common and widespread occupants of freshwater habitats. They feed mainly on aquatic amphibians, fish, and crayfish. Their ranges include the entire eastern half of the United States (U.S.) (Jensen et al. 2008), which includes all known *Ah*-positive water bodies (Wilde et al 2014). Although *Nerodia* are generally inactive during the winter months (Jensen et al. 2008), which overlaps with documented peak *Hydrilla*/*Ah* occurrence in the Southeast (Rocke et al. 2002), they may be exposed to *Ah* toxin in the fall, particularly in the more southern portion of the known distribution of *Ah* in the U.S. Moreover, if *Ah* produces toxins on other SAVs and the phenology of toxin production is different from *Ah* growing on *Hydrilla*, there may be broader exposure periods for *Nerodia* and other wildlife than is currently assumed.

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CHAPTER 2

TROPHIC TRANSFER OF A TOXIN-PRODUCING CYANOBACTERIUM TO PREDATORY PAEDOMORPHIC MOLE SALAMANDERS (*AMBYSTOMA TALPOIDEUM*) [1]

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[1] Martin, M.K., A.C. Camus, B.N. Haram, J. Hunt, J.C. Maerz, R.H. Smith, and S.B. Wilde. To be submitted to Herpetological Conservation Biology.

ABSTRACT

Aetokthonos hydrillicola (*Ah*) is a recently described cyanobacterium that grows on submerged, freshwater macrophytes. Ingestion of *Ah* growing on invasive *Hydrilla verticillata* has been linked to neurological impairment and death in a range of aquatic wildlife. Laboratory feeding trials were used to determine whether consumption of prey that had ingested *Ah*-positive *Hydrilla* would lead to the development of clinical disease among paedomorphic mole salamanders (*Ambystoma talpoideum*), a common aquatic predator. Among salamanders fed prey that ingested *Ah*-negative *Hydrilla*, we did not observe any signs of neurological impairment consistent with cyanotoxicity, and 12 of 13 salamanders had normal brain and spinal cord histopathology. In contrast, several salamanders that consumed prey fed *Ah*-positive *Hydrilla* exhibited diminished responsiveness and paralysis, and 9 of 19 salamanders in the *Ah*-positive treatment had slightly (n = 3) to substantially (n = 6) more vacuolated spaces in the ventral white matter tracts of the spinal cord compared to *Ah*-negative salamanders. These results confirm the sensitivity of mole salamanders to *Ah* toxin and are another demonstration of the potential for exposure to *Ah* toxin among aquatic predators via trophic transfer.

Key Words.—Aetokthonos hydrillicola; cyanobacterium; Hydrilla verticillata; Ambystoma talpoideum; trophic transfer; paedomorphic mole salamanders

INTRODUCTION

Wildlife can be at increased risk in aquatic environments receiving contaminants, excess nutrients, and invasions by non-native species. A newly described cyanobacterium, *Aetokthonos hydrillicola* (*Ah*), which grows on submerged aquatic vegetation (SAV) including invasive *Hydrilla verticillata* has been linked to wildlife

mortality (Wilde et al. 2005). Ingestion of the cyanotoxin results in microscopic intramyelinic vacuoles in the central nervous system (CNS) (Fischer et al. 2002; Augspurger et al. 2003). Clinical signs of neurotoxin exposure may also be observed including uncoordinated movement, lethargic behavior, non-responsiveness or anorexia. (Thomas et al. 1998; Fischer et al. 2003; Mercurio et al. 2014).

Aquatic taxa are exposed to the cyanotoxin through direct consumption of SAV by herbivores or trophic transfer of SAV from herbivore to predators (Thomas et al. 1998; Dodd et al. 2016). Experimental feeding trials demonstrated direct consumption of *Ah*-positive *Hydrilla* induced vacuolar lesions in mallard ducks, *Anas platyrhynchos* (Birrenkott et al. 2004; Rocke et al. 2005; Wiley et al. 2007, 2008; Haynie 2008); grass carp, *Ctenopharyngodon idella* (Haynie et al. 2013); painted turtles, *Chrysemys picta* (Mercurio et al. 2014) and domestic chickens, *Gallus gallus domesticus* (Lewis-Weiss et al. 2004). Trophic transfer was confirmed as a potential route of exposure from coots to red-tailed hawks, *Buteo jamaicensis* (Fischer et al. 2003; Lewis-Weiss et al. 2004) and from island apple snails, *Pomacea maculata* to their predators (Dodd et al. 2016).

In recent trials with amphibians, American bullfrogs, *Rana catesbeiana*, green frogs, *R. clamitans*, Southern leopard frogs, *R. sphenocephala,* tadpoles developed neuropathy and vacuolar lesions after feeding on toxin-producing *Ah* with *H. verticillata* (*Ah*-positive *Hydrilla*) (Maerz et al. In press). These tadpoles directly grazed on SAV, and were directly exposed to toxins via ingestion of the *Ah* growing on the plant surfaces. Whether predatory amphibians that consume prey that have fed on *Ah*-positive *Hydrilla* are sensitive and vulnerable to *Ah* invasion has not been addressed.

Mole salamanders, *Ambystoma talpoideum*, are good focal taxa to test the risks that *Ah* poses to predatory, aquatic amphibians. Their geographic range and habitats overlap with known ranges of *Ah*, and they are facultatively paedomorphic, which means they are reproductive adults that retain larval characteristics. This allows them to remain in aquatic habitats feeding on aquatic prey throughout the year. Mole salamanders occupy vegetated littoral zones of lentic habitats where they feed on a variety of taxa known to graze SAV including tadpoles, snails, and scuds (Amphipoda, Gammaridae) (Jensen et al. 2008; McAllister et al. 1996). Because mole salamanders consume their prey whole, they would ingest toxins if they were present in any part of the prey (gut contents, liver, muscle, and brain). Therefore, it is probable that mole salamanders would be exposed to *Ah* toxin growing on SAV through the food web.

The objectives of this study were to determine (1) if mole salamanders are sensitive to *Ah* toxin and (2) whether the salamander could be sufficiently exposed to the *Ah* toxin via consumption of typical prey that had grazed on *Ah*-positive plant materials. Mole salamanders are expected to become neurologically impaired and develop intramyelinated lesions in the CNS through trophic transfer of the *Ah*-positive *Hydrilla* material.

MATERIALS AND METHODS

Experimental Design.—Twenty aquaria were set up and a randomly assigned to one of two treatments: salamanders fed *Ah*-positive prey (n = 10 aquaria) or salamanders fed *Ah*-negative prey (n = 10 aquaria). Forty mole salamanders were collected and two were placed in each aquaria. The two salamanders in each aquaria were separated by a mesh screen so they could not interact and prey could not move between the two halves.

Salamanders were housed in pairs because prior studies indicate that paedomorphic mole salamanders may undergo metamorphosis if housed in isolation (Anderson et al. 2015). Our design allowed salamanders to sense chemical cues from conspecifics but prevented aggressive interactions between males and allowed for the control and monitor of feeding each individual in each aquarium. Therefore, we used individual salamanders (n = 20 per treatment) rather than individual aquaria as our unit of replication in all analyses. The experiment lasted for 42 days.

Vegetation Collection and Screening.—*Hydrilla* used to feed the *Ah*-positive prey was collected from a site where *Ah* and vacuolar myelinopathy had been documented in previous studies and hydrilla collected to feed to the *Ah*-negative prey was from a lake with no vacuolar myelinopathy or *Ah*. Collection of the *Ah*-positive *Hydrilla* occurred on 10 November 2016 at J. Strom Thurmond Lake, a reservoir where >100 bald eagles (*Haliaeethus leucocephalus*) and large numbers of waterbirds have died from consumption of *Ah*-positive *Hydrilla* (Fischer et al. 2003, 2006; Larsen et al. 2003; Haynie 2008; Haram 2016). *Ah*-negative *Hydrilla* was collected from Lake Wylie, South Carolina, on 21 November 2016, a reservoir where *Ah* has not previously been detected and no *Ah*-associated wildlife deaths have been reported in repeated sampling over multiple years (Haram 2016). A throw rake was used to collect *Hydrilla* from the upper water column (~1 m), which was then placed in 1-gallon zip top plastic bags and transported in separate secure coolers to the lab.

To confirm the presence of *Ah*, replicate glass slides were prepared with 10 randomly selected *Hydrilla* leaves mounted with underside facing up in water with a cover slip. *Hydrilla* leaves were viewed under 200X on Zeiss compound scope using

epifluorescence microscopy on a Rhodamine filter. Relative density of the *Ah* is estimated by viewing each of the ten leaves and calculating the average percent of the *Hydrilla* leaf covered with *Ah* colonies (Wilde et al. 2005; Haynie 2008). *Ah* presence or absence was confirmed by genetic analysis (polymerase chain reaction; PCR) (Wilde et al. 2014). The remaining plant material was placed in a walk-in freezer and stored at -20° C until use in the feeding trials.

Animal Care and Collection.—All methods were approved by the University of Georgia's Institutional Animal Care and Use Committee (A2014 03-018-Y2-A1). Experiments did not involve endangered or threatened species and wildlife collections were permitted by the Georgia Department of Natural Resources (29-WJH-14-170). All animals were housed, monitored and proper procedures followed according to the University of Georgia's Policy on Humane Care and Use of Animals.

Forty paedomorphic mole salamanders were captured with unbaited minnow traps from ponds 4 and 7 in the Whitehall Experimental Forest, where *Ah* has not been previously documented. All salamanders were transported to the University of Georgia Aquatic Biotechnology and Environmental Lab (ABEL) in Whitehall Experimental Forest and placed in 20, 40 - L aquaria filled with dechlorinated tap water. Each aquarium was divided in half by a mesh barrier, and one salamander was placed on each side of the barrier. Two white oak (*Quercus alba*) leaves were bleached with 10% buffered bleach to eliminate any outside bacteria and regulate the pH of the leaves. Leaves were then rinsed, air-dried, and placed in each half of aquaria providing cover for salamanders (Fig. 1.1). Brown contact paper was wrapped around the aquarium reducing light and disturbance to lessen stress on salamanders (Fig. 2.1).

We fed toxic (*Ah*-positive) *Hydrilla* to treatment tadpoles and snails and non-toxic (*Ah*-negative) *Hydrilla* to control tadpoles and snails and used these gut-loaded prey in feeding trials for salamanders. Southern leopard frog egg masses were collected from Fall Lines Sandhill Wildlife Management Area. Egg masses were hatched and raised to a size suitable for salamander consumption at the Herpetology Field Lab. Small aquatic snails (*Physa acuta*) were collected from aquarium tanks maintained in the Whitehall Fisheries Laboratory. To prepare *Hydrilla* for feeding to the salamander tadpole and snail prey items, samples were thawed at 9° C. Tadpoles and snails were placed in aquaria with 15-g of freshly thawed *Ah*-positive or *Ah*-negative *Hydrilla* and allowed to graze for 24 hours prior to being transferred to mole salamander tanks. We euthanized a subsample of tadpoles and snails, necropsied them, dissected the gastrointestinal tract (GIT), and visualized the contents under epifluorescence microscopy using a Rhodamine filter to confirm the presence of *Ah* phycocyanin pigments in gut contents.

Salamanders were randomly assigned to be fed prey that had fed on either *Ah*-positive or *Ah*-negative *Hydrilla*. Salamanders that shared halves of the same tank were assigned to the same treatment. Each salamander was offered the same number of tadpoles and snails each day throughout the feeding trial. Each day remaining prey was extracted and number recorded in order to calculate an individual consumption rate. Weekly cumulative prey consumption was recorded to assess whether salamanders became anorexic over the course of the trial.

Gill length was measured as a proportion of head width each week to determine whether gills were resorbing. A significant reduction in gill length would indicate that the salamander was undergoing metamorphosis. Snout-vent length (SVL), total length

(TL), and wet mass were measured after animals were euthanized and fixed in 10 % buffered formalin (Table 2.1).

Behavior Assessment.—Salamanders were monitored daily to detect clinical signs of mild (twitching, listing, unresponsive) or severe (seizures, paralysis) neurologic impairment. Evaluation of relative responsiveness was determined by first recording whether salamanders were hiding, active or listing. If the salamander was located under oak leaf litter, it was recorded as "hiding", and if it was found in the water column below the boundary of the contact paper, it was considered "active." These were considered normal behaviors. If the salamander was observed above the contact paper it was designated as exhibiting abnormal behavior, or "listing". When a salamander exhibited this "listing" behavior, a plastic drinking straw was used to gently prod the salamander to elicit a response. All responses were recorded, including delayed/no response, swimming away, or aggressive response.

Righting response tests were conducted during weeks four, five, and six of the feeding trial to detect any changes indicative of mild impairment. Each salamander was placed in dorsal recumbency in one inch of water and the time (seconds) required for the animal to return to a normal orientation was measured. This challenge was repeated three times and the average time (seconds) required for righting was used in analysis.

Euthanasia and Histology.—Salamanders exhibiting severe neurologic signs of impairment (paralysis and seizures) were euthanized immediately and all remaining salamanders were euthanized at the end of the experiment. Euthanasia was administered by emersion in a bath of neutral pH-buffered MS-222 (2 g/l). Salamanders were immediately fixed in 10% neutral-buffered formalin for a minimum of 24 hours and

transferred to ethanol for storage. Heads were decalcified with Kristensen's solution for at least 24 hrs. Once decalcified, the head and cervical region of each specimen was transversely sectioned throughout their lengths, targeting the forebrain (telencephalon), midbrain (mesencephalon), hindbrain (metencephalon, myelencephalon), and proximal spinal cord. The transverse sections were placed in tissue cassettes, sectioned at five μm, and stained with hematoxylin and eosin (H&E) for microscopic examination. Histology slides were "blindly" reviewed by a board-certified anatomic veterinary pathologist (HF) from the University of Georgia's Department of Veterinary Medicine for evidence of intramyelinic edema consistent with *Ah* toxicity. Salamander histology results were recorded as "2" (significant vacuolar lesions present), "1" (slightly more lesions than controls), "0" (baseline, no difference from controls).

Statistical Analysis.—Salamander prey consumption, behavioral responses, and histological data were evaluated to determine whether there was a significant effect of *Ah*-positive *Hydrilla* prey feeding treatment. We used linear mixed effects models to conduct a repeated-measures analysis of whether the mean cumulative number of tadpoles and snails consumed, mean gill length, and mean righting response time differed between treatments. We used generalized logistic regression models to determine whether the probability of listing, delayed/no response, swimming away, or aggression, when prodded with a straw differed between treatments over the duration of the trial, and we used a linear model to determine whether the relationship between body mass and length (snout-to-vent length, SVL) differed between treatments at the end of the experiment. We used a Mann-Whitney U test to compare the histopathology scores of salamanders between the two treatments. All analyses were conducted in R version 3.3.1

(R Core Team 2016) with an alpha level of 0.05 used to judge statistical likelihood of treatment effects.

RESULTS

Trial Mortality and Salamander Behavior —During the 6-week feeding trial, 6 salamanders in the *Ah*-positive treatment displayed severe neurologic impairment and were euthanized before the end of the study. Only 1 salamander in the *Ah*-negative treatment had to be euthanized early due to a bacterial infection at a wound site. Salamanders exhibiting mild to moderate neurologic signs of impairment (head twitching, mouth gaping, and muscle atrophy) in both treatment groups were allowed to continue throughout the entire feeding trial.

Behavior.—Salamanders in the *Ah*-positive and *Ah*-negative treatments consumed significantly different cumulative numbers of prey over the duration of the study. *Ah*-positive treatment progressively consumed less prey and gained less mass over time than the *Ah*-negative treatment ($P = 0.03$) (Table 2.2) (Fig. 2.2). There was a tendency for the frequency of listing to increase among salamanders in the *Ah*-positive treatment over time while it remained stable among salamanders in the *Ah*-negative treatment (Fig. 2.3); however, this interaction between treatment and time was marginally non-significant ($P = 0.16$) (Table 2.3). By week three through week six, the 95% confidence intervals around the probability of listing did not overlap between salamanders in the two treatment groups. There was a higher probability of a salamander from the *Ah*-negative treatment being observed in the "active" state compared to a salamander from the *Ah*-positive treatment ($P = 0.04$) (Table 2.4); however, this difference was consistent from the beginning of the trial and did not change over time (Fig. 2.4). The probability of

observing a salamander hiding was low, remained unchanged throughout the trial, and did not differ between treatment groups ($P = 0.38$) (Table 2.5; Fig. 2.5).

During the first few weeks, when listing individuals were prodded with a straw, salamanders from both treatments showed a marginal probability of aggression (biting at the straw), but this behavior declined over time ($P = 0.08$). There was no difference in aggressive responses toward prodding between the two treatments over time ($P = 0.52$) (Table 2.6; Fig. 2.6). Similarly, both treatment groups swam away more often over time, but no significant difference was found between the two treatments ($P = 0.69$) (Table 2.7; Fig. 2.7). Both treatment groups showed more of a delayed/no response when prodded over the six week feeding trial. It appeared as though salamanders in the *Ah*-positive treatment became less responsive to prodding over time compared to salamanders in the *Ah*-negative group (Fig. 2.8); however, there was no statistical significance found between the two groups ($P = 0.85$) (Table 2.8).

Salamanders fed *Ah*-positive prey had significantly higher righting response times than *Ah*-negative salamanders ($P = 0.03$). There was no significant difference of the interaction between treatments during weeks four, five, and six ($P = 0.90$). Five salamanders in the *Ah*-positive group did have righting response times over two seconds as compared to the *Ah*-negative group which were all under two seconds (Table 2.9; Fig. 2.9).

Body Condition.—Mean gill branch length declined over the course of the study in both treatment groups, but there was no significant difference in the mean gill branch length of the *Ah*-positive group versus *Ah*-negative group ($P = 0.73$). Sixteen (43 %) of salamanders fed *Ah*-positive prey and 21 (57 %) *Ah*-negative salamanders had partially or

nearly-fully resorbed their gills by the end of the study (Table 2.10). For a given body length, salamanders in the *Ah*-positive treatment weighed significantly less at the end of the experiment compared to salamanders in the *Ah*-positive treatment (Table 2.11; Fig. 2.10).

Histopathology.—A total of 32 salamanders, 13 *Ah*-negative and 19 *Ah*-positive, were evaluated microscopically. The brains and spinal cords of 12 *Ah*-negative animals were normal (Fig 2.11A). One *Ah*-negative animal exhibited rare, isolated, small, clear vacuolated spaces, some containing axons, primarily in ventral white matter tracts of the spinal cord (Fig 2.11B). Within the *Ah*-positive group, the brains and spinal cords of 10 animals were also microscopically normal. The spinal cords of three *Ah*-positive animals contained scattered vacuolated spaces slightly more numerous than those observed among *Ah*-negative salamanders (Fig 2.11C). Results in these animals were interpreted as equivocal. Changes in two salamanders suggested dilated axon sheaths compatible with lesions consistent with *Ah* toxicity. There was extensive, symmetrical, diffuse vacuolation of white matter areas of the spinal cord. Vacuoles varied in size, were limited by smooth rounded borders, and many contained axons (Fig 2.11D). Some larger vacuoles contained flocculent pale eosinophilic material. Rarely membrane-like eosinophilic strands extended between axons and the vacuole margin (Fig 2.11E). Rare vacuoles were also present in the mesencephalons and brainstems of these two animals. Similar changes were observed in another four salamanders; however, in addition to vacuolation, multiple small foci of hemorrhage were scattered throughout the white matter and immediately beneath the meninges of the brainstem and spinal cord (Fig

2.11F). The *Ah*-positive group had significantly more vacuolation of the white matter of the spinal cord and mesencephalon than the *Ah*-negative group ($P = 0.05$).

Behavioral responses and histopathological results were observationally compared between treatment groups because many individually recorded behavioral responses categorically matched the histopathology results for salamanders. Color coded responses and results were not consistent among salamanders, but there was a pattern between treatment groups. *Ah*-positive treatment showed moderate to severe responses while *Ah*-negative treatment showed none (Table 2.12).

DISCUSSION

The results of this study provide compelling evidence that aquatic salamanders are sensitive to *Ah* toxin and can be exposed via trophic transfer from their prey. Most notably, several salamanders fed *Ah*-positive prey developed impaired righting responses and overall salamanders fed *Ah*-positive prey weighed less per body length at the end of the six week trial. *Ah*-positive individuals were also more likely to be inactive throughout the 6 week feeding trial than *Ah*-negative individuals; however, this pattern was consistent by the first week of the trial and did not change over time. Therefore, we cannot determine whether activity was a random difference between treatment groups present before the experiment or a treatment effect that manifest rapidly among salamanders exposed to *Ah*-toxin. In birds, neurological impairment from exposure to *Ah* toxin can manifest in under five days (Rocke et al. 2002), and among amphibian tadpoles, measurable effects of *Ah* toxin on mortality have appeared in as short as three days (Maerz et al. in press).

It is important that we address the potential for confounding effects of our treatments because our source plant materials each came from one, separate lake. In addition to the presence or absence of *Ah*, there are potentially many other differences between *Hydrilla* collected at the two sites including differences in nutritional quality, metal concentrations, presence of microbes, and the presence of anthropogenic pollutants. We cannot rule out other confounding differences between plant sources in our treatments; however, our results in the context of our other research make it most likely that the effects we observed are related to *Ah* toxin presence and not other factors. First, there have been numerous other feeding trials of wildlife using *Ah*-positive or *Ah*-negative *Hydrilla* from other lakes besides our two source lakes. More than 40 lakes have been surveyed and source *Hydrilla* tested on wildlife to date. Water quality and microbiota differ considerably among those 40 reservoirs. Among the 21 lakes where *Ah*-positive *Hydrilla* was collected, all have been shown to induce impairment in some species of wildlife. Among the 15 *Ah*-negative lakes where *Hydrilla* was collected, that *Hydrilla* has never induced impairment, disease, or death in any wildlife. Therefore, among all prior studies from using *Hydrilla* from many source lakes that vary in many aspects of water quality, only *Ah*-positive *Hydrilla* has ever induced neurological impairment, disease within the CNS, and death among wildlife (Fisher, et al. 2003, Wiley et al. 2007; Wilde, et al 2014, Birrenkott, et al 2004; Lewis-Weiss et al 2004; Wiley et al. 2007; Haynie 2008; Mercurio et al. 2014; Maerz et al. In press). Second, *Ah*-positive *Hydrilla* from our source lake has been collected during the summer – when Ah appear not to produce toxin – and fed to tadpoles, and did not cause any impairment or mortality. Therefore, at other times of the year, *Ah*-positive *Hydrilla* is suitable forage for

amphibians and other wildlife. If something other than *Ah* was responsible for toxicity, it would have to be a factor that also varies seasonally but is currently latent.

Overall, our results are consistent with other studies that report higher rates of abnormal behavior and neurological impairment among animals that had ingested *Ah*-positive plant material, directly or indirectly (Fischer et al. 2003; Wilde et al. 2005; Mercurio et al. 2014; Dodd et al. 2016; Maerz et al. In press; Martin Chapter 3), as was the failure to detect behavioral differences among all individuals within the *Ah*-positive treatment is also consistent with other studies. Not all individuals with intramyelinated vacuoles exhibit clinical signs of neurologic impairment after feeding on *Ah*-positive *Hydrilla* (Rocke et al. 2002; Lewis-Weiss et al. 2004; Wiley et al. 2008). For example, a study conducted on mallards and chickens resulted in few behavioral signs of neurological impairment even though > 50% of the *Ah*-positive birds had characteristic intramyelinated vacuoles consistent with *Ah* toxicity under histological assessment (Birrenkott et al. 2004; Haney et al. 2013; Dodd et al. 2016). One reason why individual animals may vary in the presentation of clinical symptoms or histopathology is that doses or concentrations of the etiologic agent (*Ah* toxin) for all feeding trials, is variable. Animal appetites may vary, and because we are feeding prey plant material from the field, there is likely variation in toxin concentration among the small samples of *Ah*-positive *Hydrilla* provided to each individual.

Vacuoles suggestive of *Ah* toxicity were found primarily in the cervical spinal cord of salamanders. This contrasts with a prevalence of vacuoles within the optic tectum of birds and fish. In addition to vacuolation, small foci of hemorrhage, an ante mortem change, were present in the brainstems and spinal cords of several salamanders. This may

reflect differences of neural functions in amphibians relative to birds and fish. A smaller optic tectum in a salamander may indicate less myelin formation in that region (Butler et al. 2006). Myelin is a coating made of lipid-rich proteins and phospholipids insulating the axons of the CNS and keeping neurons transmitting and responding to nerve impulses. In CNS regions where there is little myelin, we would expect few vacuole development. This means that interspecies comparisons will need to focus on a range of CNS tissues to account for differences in CNS anatomy when evaluating the relative sensitivity of different species to *Ah* toxin.

Although changes in some animals may reflect dilated axon sheaths and intramyelinic edema, similar histologic changes can be a relatively common post mortem artifact. Some indistinguishable pathologies were observed in both *Ah*-negative and *Ah*-positive animals that could be a result of processing artifacts. Such processing or observation error could be exacerbated by small sample sizes, such as was the case in this study, and the inability to control dosing of the toxic compound to individual animals. Nonetheless, the observed differences between salamanders in the treatments were sufficient to suggest sensitivity to trophic transfer of *Ah*-positive materials, and at a minimum warrant additional study.

We do not know whether – in the field – the greater risk from trophic transfer is facilitated simply by prey transferring toxic plant material to the predator, or whether the toxin can bioaccumulate within prey. Recent evidence suggests that *Ah* cyanotoxin can bioaccumulate during digestion by amphipods, snails, and fish (Susan Wilde, pers. comm.). In this study, the short amount of time for prey to feed on *Ah*-positive plant material means that predatory salamanders were vulnerable to toxin exposure simply by

transfer of toxic plant material through prey. There was simply not sufficient time for prey to accumulate toxin in prey tissues. Moreover, lab studies suggest high, rapid mortality among fish, tadpoles and invertebrates when feeding on highly toxic *Ah*-positive *Hydrilla* (Maerz et al. In press; Haram 2016; Martin et al. unpublished obs.). In the field, bioaccumulation of toxin – particularly in more resilient prey or when toxin concentration are sublethal among prey – may be a more important exposure route for predatory species. Moreover, larger, more resilient predatory species may themselves accumulate the toxin, creating increased exposure risks to predatory taxa farther up the food chain. We emphasize that-at this time – there are no documented deaths of amphibians, reptiles, or fish in the field. Therefore we can only consider the risk of *Ah* to these taxa as hypothetical. However, we also note that detecting deaths of these species would be extremely low and there is a reasonable probability that neurologically impaired animals would be preyed upon; therefore, the absence of observed deaths is also not evidence that *Ah* invasion poses no risk to amphibians or other more secretive wildlife.

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Table 2.1. Summary of treatment type (Ah+ = *Ah*-positive prey and Ah- = *Ah*-negative prey), size, sex, mass, snout-vent length (SVL), total length (TL), and assigned aquaria of paedomorphic mole salamanders.

Salamander	Tank	Treatment	Sex	Mass (g)	SVL (mm)	TL (mm)
1	1	Ah+	M	3.124	45.19	88.36
2	1	Ah+	F	3.944	48.11	72.71
3	2	Ah+	M	3.184	47.03	91.62
4	2	Ah+	F	3.623	46.45	76.22
5	3	Ah-	M	2.526	43.95	82.71
6	3	Ah-	F	4.773	51.47	97.35
7	4	Ah+	F	2.342	45.46	79.94
8	4	Ah+	F	3.299	51.26	96.09
9	5	Ah-	M	3.826	48.01	89.49
10	5	Ah-	F	3.327	43.46	83.45
11	6	Ah-	M	3.300	47.43	82.46
12	6	Ah-	F	3.800	46.05	85.31
14	7	Ah+	F	2.929	44.61	89.27
41	7	Ah+	M	2.560	45.94	79.22
42	7	Ah+	F	3.604	47.21	87.05
15	8	Ah+	F	3.808	45.92	85.53
16	8	Ah+	M	2.476	44.63	81.07
17	9	Ah-	M	2.452	44.20	80.57
18	9	Ah-	F	3.584	47.02	87.75
19	10	Ah-	F	4.026	46.48	91.70
20	10	Ah-	M	3.073	47.00	80.48
22	11	Ah-	F	2.711	41.07	79.12
23	12	Ah+	M	3.841	51.18	75.84
24	12	Ah+	F	4.101	53.36	98.06
25	13	Ah+	F	2.630	46.22	77.88
26	13	Ah+	M	3.034	45.66	88.15
27	14	Ah+	F	4.355	47.33	92.29
29	15	Ah-	F	3.119	41.88	78.29
31	16	Ah+	F	3.630	47.98	92.09
32	16	Ah+	M	3.066	46.51	83.70
33	17	Ah+	F	4.244	49.78	90.18
34	17	Ah+	M	3.304	47.43	90.83
35	18	Ah-	F	3.924	45.34	86.52
36	18	Ah-	M	2.468	41.15	82.75
37	19	Ah-	F	4.966	50.11	94.23
38	19	Ah-	M	3.134	47.31	92.04
39	20	Ah-	F	5.050	49.06	93.29

Table 2.2. Results of repeated measures linear mixed effects model of the cumulative number of prey (tadpoles and snails) consumed by salamanders as a function of treatment. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treat), week and the interaction of treatment and week (treat:week) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	175	53016.33	<.0001
treat	1	39	4.98	0.0314
week	1	175	5397.79	<.0001
treat:week	1	175	0.04	0.8398

Table 2.3. Results of generalized logistic regression of the probability of observing a salamander listing as a function of treatment and week of study. Estimates of coefficients, standard error, z statistic (z value), and significance levels (Pr(>|z|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatAh+), week, and interaction of *Ah*-positive treatment and week (treatAh+:week) are listed. (***) indicate significance code of 0.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.63108	0.25599	-6.372	1.87e-10 ***
treatAh+	0.31778	0.33966	0.936	0.349
week	-0.05802	0.06644	-0.873	0.383
treatAh+:week	0.12381	0.08775	1.411	0.158

Table 2.4. Results of generalized logistic regression of the probability of observing a salamander active as a function of treatment and week of study. Estimates of coefficients, standard error, z statistic (z value), and significance levels (Pr(>|z|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatAh+), week, and interaction of *Ah*-positive treatment and week (treatAh+:week) are listed. (***) indicates significance code of 0. (*) indicates significance code of 0.01.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.902061	0.199732	4.516	6.29e-06 ***
treatAh+	-0.576849	0.277428	-2.079	0.0376 *
week	0.005759	0.050642	0.114	0.9095
treatAh+:week	-0.014193	0.071422	-0.199	0.8425

Table 2.5. Results of generalized logistic regression of the probability of observing a salamander hidden as a function of treatment and week of study. Estimates of coefficients, standard error, z statistic (z value), and significance levels (Pr(>|z|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatAh+), week, and interaction of *Ah*-positive treatment and week (treatAh+:week) are listed. (***) indicates significance code of 0.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.77890	0.25310	-7.028	2.09e-12 ***
treatAh+	0.43655	0.35308	1.236	0.216
week	0.01651	0.06363	0.259	0.795
treatAh+:week	-0.08053	0.09178	-0.877	0.380

Table 2.6. Results of generalized logistic regression of the probability a listing salamander responded aggressively to prodding as a function of treatment and week of study. Estimates of coefficients, standard error, z statistic (z value), and significance levels (Pr(>|z|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatpositive), week, and interaction of *Ah*-positive treatment and week (treatpositive:week) are listed. (.) indicates significance code of 0.05.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.6522	1.3611	0.479	0.6318
treatpositive	-0.6361	1.6797	-0.379	0.7049
week	-0.6363	0.3595	-1.770	0.0767 .
treatpositive:week	0.2774	0.4332	0.640	0.5220

Table 2.7. Results of generalized logistic regression of the probability a listing salamander swam away in response to prodding as a function of treatment and week of study. Estimates of coefficients, standard error, z statistic (z value), and significance levels (Pr(>|z|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatpositive), week, and interaction of *Ah*-positive treatment and week (treatpositive:week) are listed.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.8611	1.2802	0.673	0.501
treatpositive	-1.3650	1.5484	-0.882	0.378
week	0.1971	0.3011	0.655	0.513
treatpositive:week	0.1470	0.3667	0.401	0.689

Table 2.8. Results of generalized logistic regression of the probability a listing salamander was non-responsive to prodding as a function of treatment and study week. Estimates of coefficients, standard error, z statistic (z value), and significance levels (Pr(>|z|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatpositive), week, and interaction of *Ah*-positive treatment and week (treatpositive:week) are listed.

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-2.70321	1.60194	-1.687	0.0915
treatpositive	1.82103	1.78772	1.019	0.3084
week	0.15740	0.34485	0.456	0.6481
treatpositive:week	0.07321	0.39036	0.188	0.8512

Table 2.9. Results of repeated measures linear mixed effects model of righting response as a function of treatment and study week. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treat), week and the interaction of treatment and week (treat:week) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	57	37.84311	<.0001
treat	1	33	5.39045	0.0266
week	1	57	0.01017	0.9200
treat:week	1	57	0.01571	0.9007

Table 2.10. Results of repeated measures linear mixed effects model of gill length as a function of treatment and study week. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treat), week and the interaction of treatment and week (treat:week) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	139	637.2695	<.0001
treat	1	35	0.7533	0.3914
week	1	139	55.6393	<.0001
treat:week	1	139	0.1159	0.7341

Table 2.11. Results of linear model of salamander mass and snout-vent length (SVL) as a function of treatment. Estimates of coefficients, standard error, t statistic (t value), and significance levels (Pr(>|t|) for the predicted value of the *Ah*-negative treatment (Intercept), *Ah*-positive treatment (treatpositive) and SVL. (***) indicates significance code of 0. (*) indicates significance code of 0.01.

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	-5.3857	1.4432	-3.732	0.000693 ***
treatpositive	-0.4519	0.1696	-2.664	0.011713 *
svl	0.1941	0.0313	6.200	4.74e-07 ***

Table 2.12. Summary of behavior responses and histopathologic results for paedomorphic mole salamanders. Shades of green and yellow indicate no responses – mild responses. Shades of orange indicate mild-moderate responses. Shades of red indicate severe responses.

Treatment	Histology	Severe Clinical	Righting Response (sec)	Active	Listing	Delayed
Ah-	Normal	No	1.3	67	26	50
		No	1.0	59	10	0
		No	1.1	72	13	0
		No	1.1	56	13	0
		No	1.0	67	0	NA
		No	1.0	54	13	0
		No	0.3	85	10	0
		No	1.0	79	21	0
		No	0.8	86	10	NA
		No	1.0	77	18	0
		No	1.0	84	11	17
		No	1.0	74	3	NA
	NA	No	1.1	87	3	NA
		No	1.0	69	23	0
		No	1.0	64	31	43
		No	1.0	62	5	0
	Mild-moderate	No	1.0	51	28	0
Ah+	Normal	Yes	0.3	53	11	75
		Yes	0.7	79	15	0
		No	1.0	88	12	0
		No	1.2	82	13	0
		Yes	NA	92	3	NA
		No	1.0	11	11	50
		No	NA	77	21	0
		Yes	2.7	54	22	67
	NA	No	3.6	64	15	50
	Mild-moderate	No	1.2	56	33	20
		No	1.0	69	17	100
		No	1.0	46	33	14
	Severe	No	1.1	19	64	67
		Yes	3.3	16	11	25
		No	4.7	63	32	83
		No	2.7	54	36	0
		No	1.0	22	59	92
		No	1.0	48	48	71

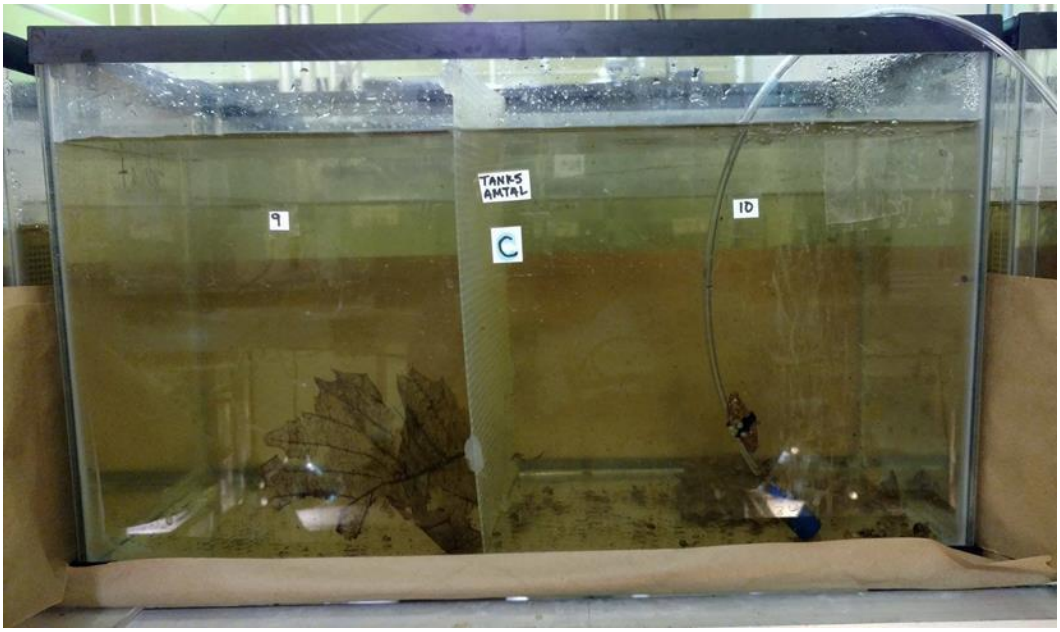

Figure 2.1. Aquaria design for feeding trials with the paper opened on one side to show inside of tank (contact paper fully covered all four sides during the majority of the trial). Tanks were divided in half by a mesh barrier that allowed the movement of chemical cues between halves, but kept the two salamanders and their allocated prey separated. Photograph by Rebecca H. Smith.

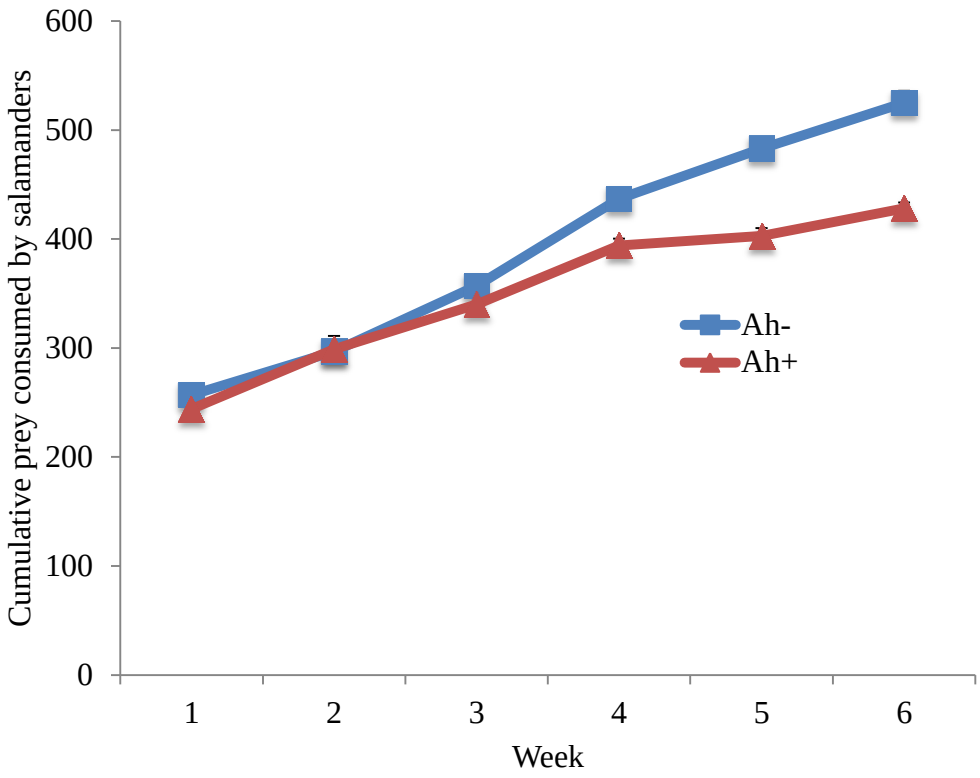

Figure 2.2. Total number of prey consumed by salamanders for each treatment level across six time intervals throughout the 6-week feeding trial. *Ah*-negative prey are represented in blue (*Ah*-) and *Ah*-positive prey consumed are shown in red (*Ah*+).

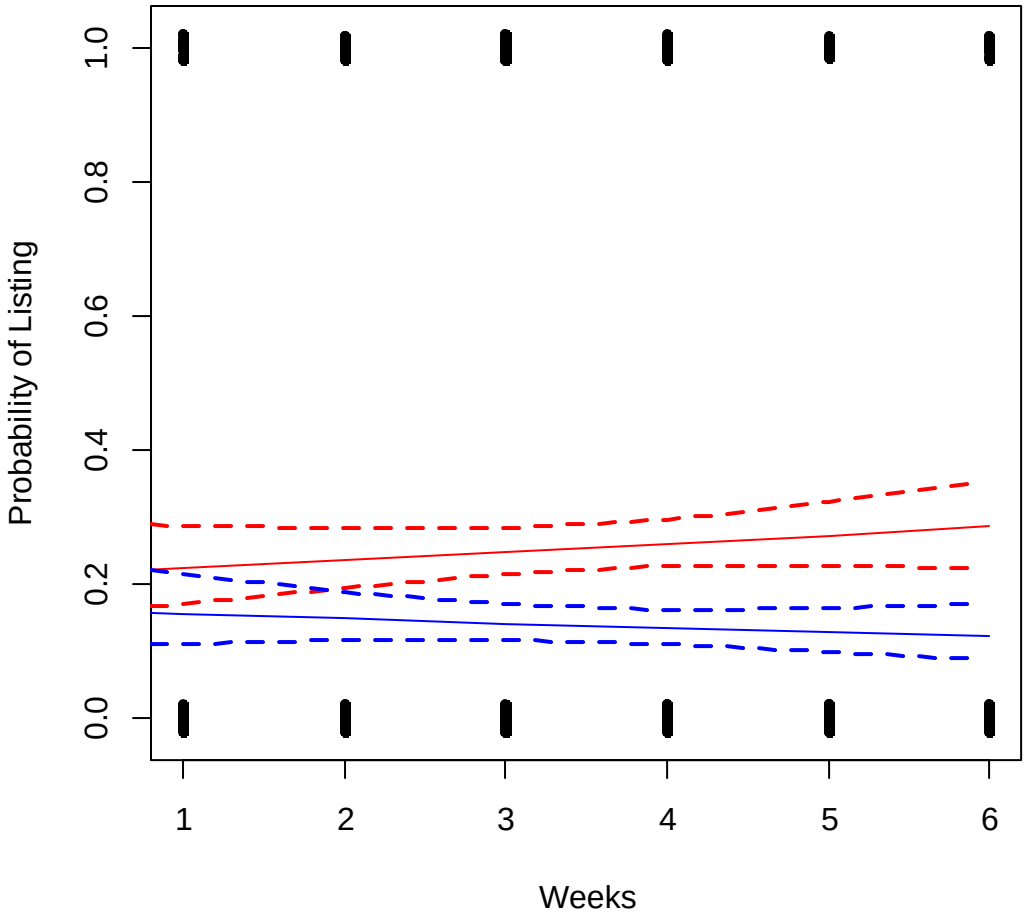

Figure 2.3. Probability of observing a salamander listing as a function of treatment and study week. Blue line represents salamanders in the *Ah*-negative treatment, and the red line represents salamanders in the *Ah*-positive treatment. Dashed lines are 95 % confidence intervals.

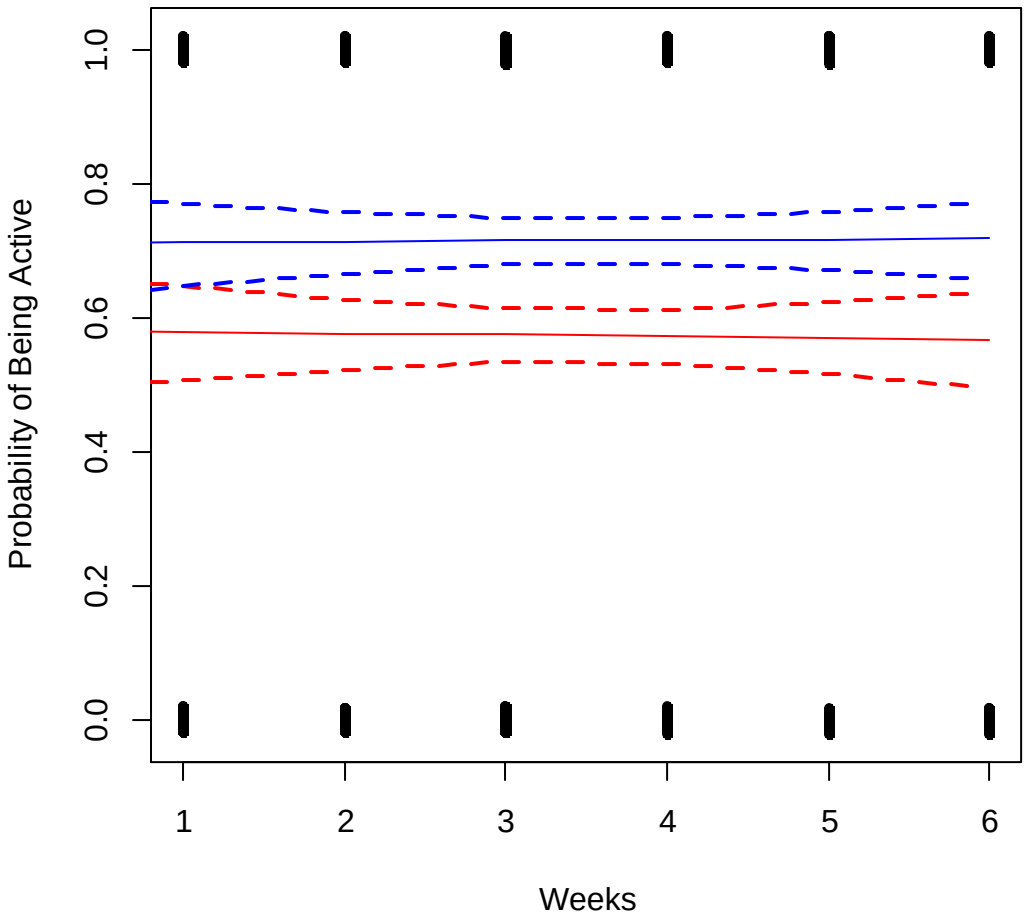

Figure 2.4. Probability of observing a salamander active as a function of treatment and study week. Blue lines represent salamanders in the *Ah*-negative treatment, and the red line represents salamanders in the *Ah*-positive treatment. Dashed lines are 95 % confidence intervals.

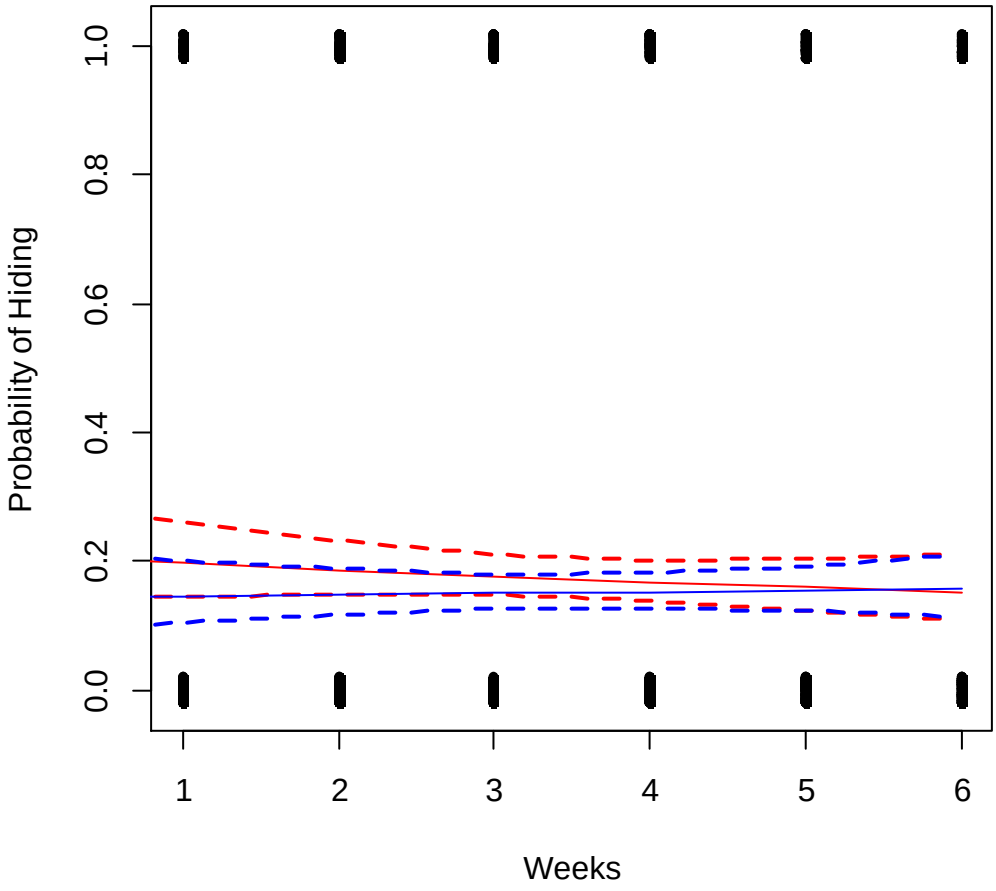

Figure 2.5. Probability of a observing a salamander hiding as a function of treatment and study week. Blue line represents salamanders in the *Ah*-negative treatment, and the red line represents salamanders in the *Ah*-positive treatment. Dashed lines are 95 % confidence intervals.

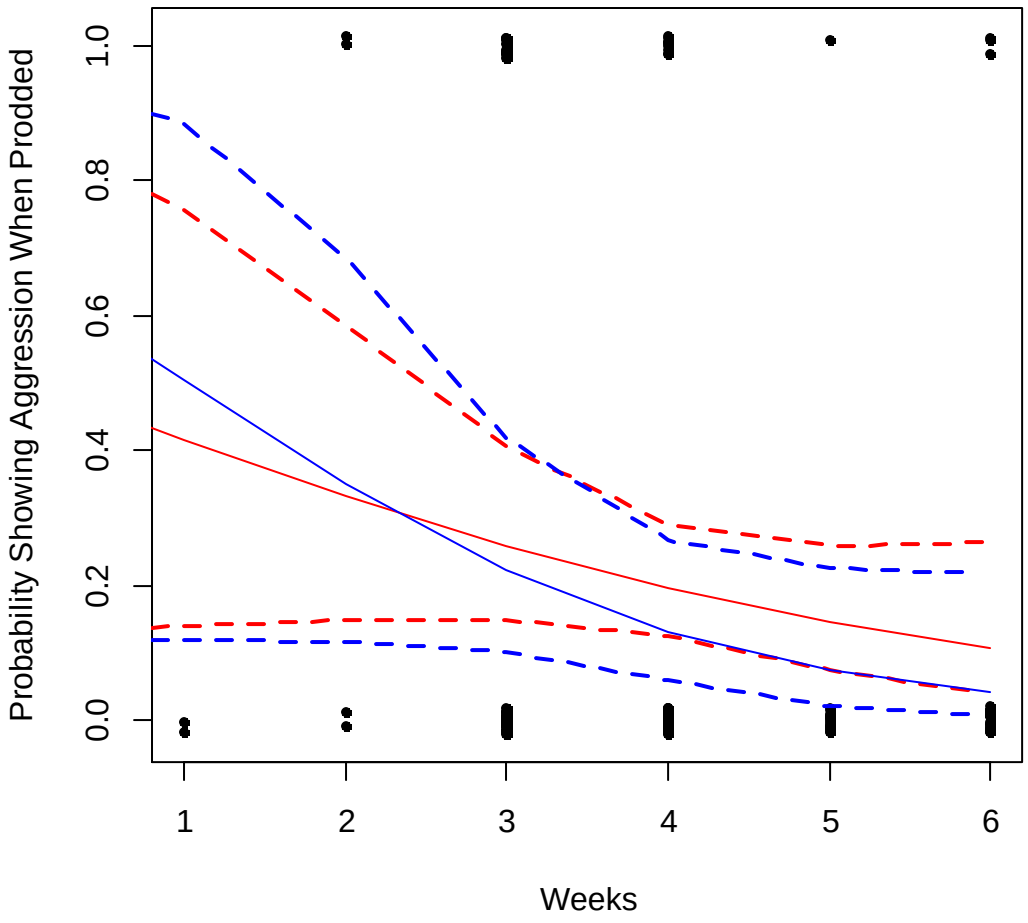

Figure 2.6. Probability a listing salamander responded aggressively to prodding as a function of treatment and study week. Blue line represents salamanders in the *Ah*-negative treatment, and the red line represents salamanders in the *Ah*-positive treatment. Dashed lines are 95 % confidence intervals.

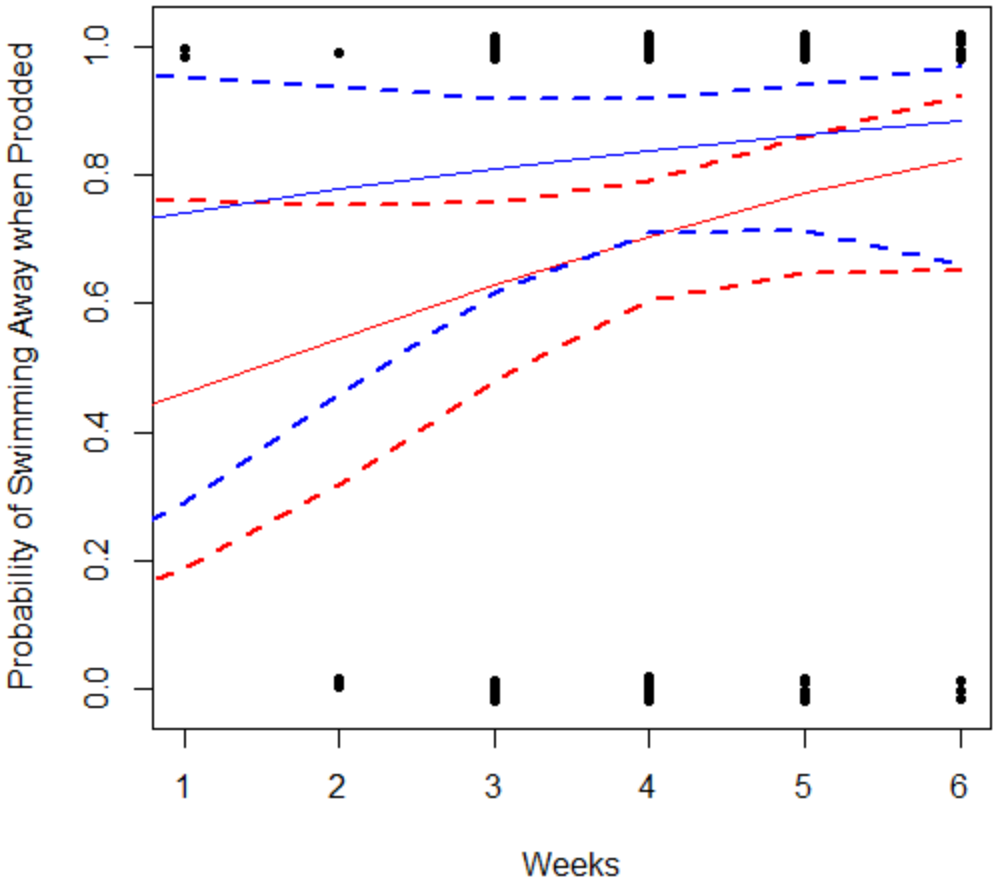

Figure 2.7. Probability a listing salamander responded to prodding by swimming away as a function of treatment and study week. Blue line represents salamanders in the Ah-negative treatment, and the red line represents salamanders in the Ah-positive treatment. Dashed lines are 95 % confidence intervals.

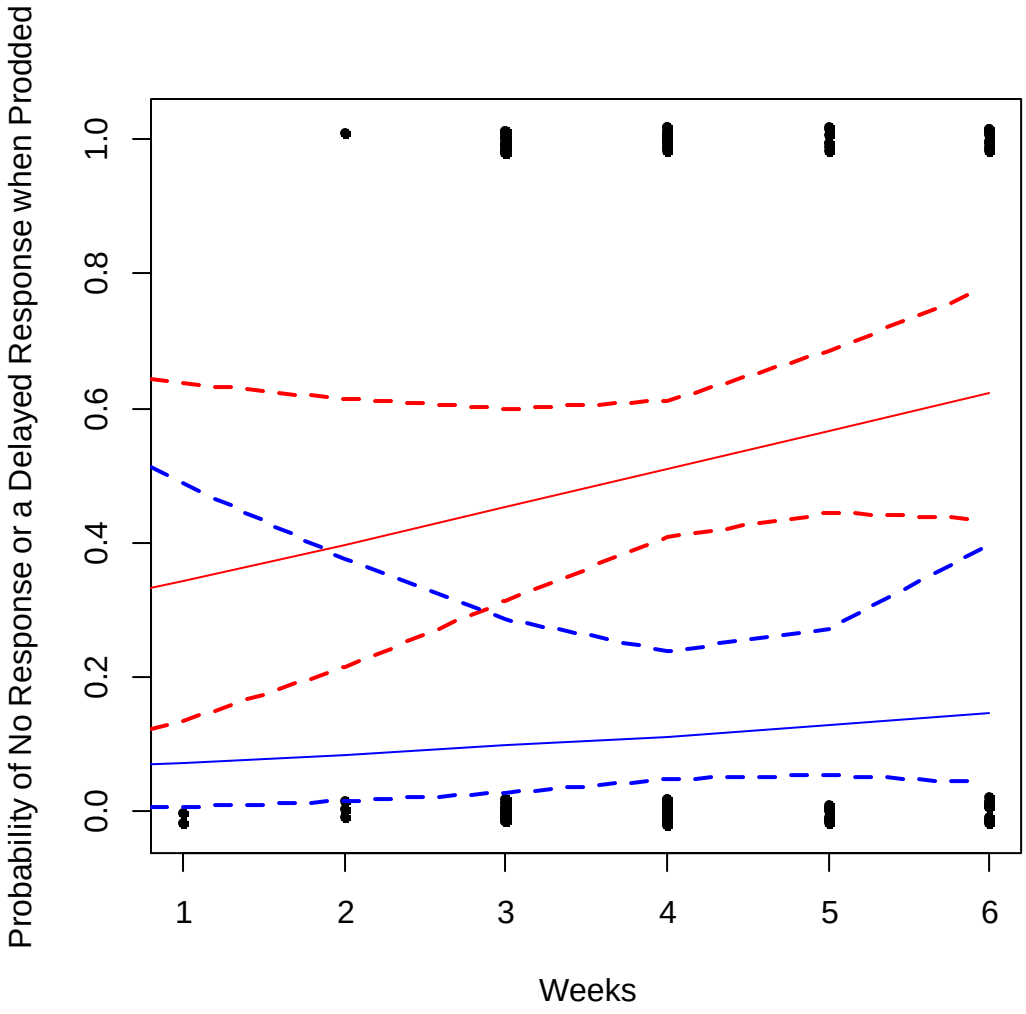

Figure 2.8. Probability a listing salamander was non-responsive to prodding as a function of treatment and study week. Blue line represents salamanders in the *Ah*-negative treatment, and the red line represents salamanders in the *Ah*-positive treatment. Dashed lines are 95 % confidence intervals.

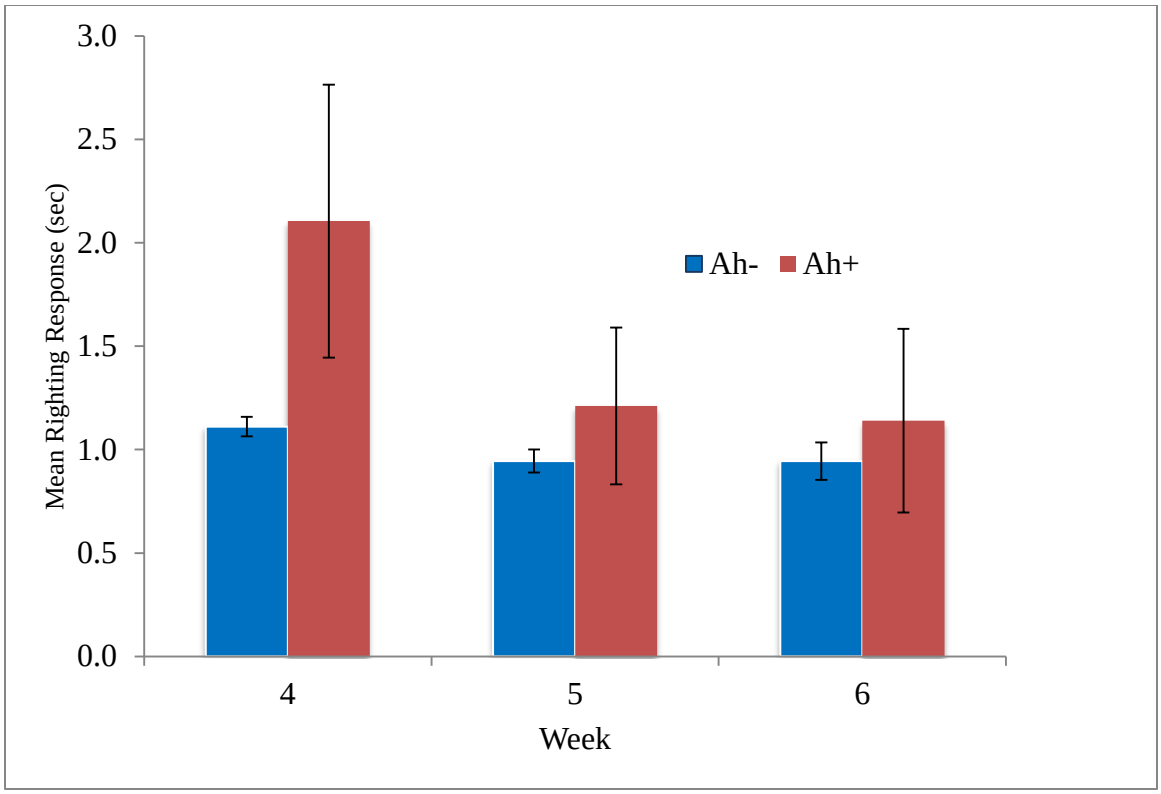

Figure 2.9. Average righting response times (sec) for *Ah*-positive and *Ah*-negative treatment salamanders in weeks 4, 5, and 6 with 95 % confidence intervals represented by error bars for each treatment week.

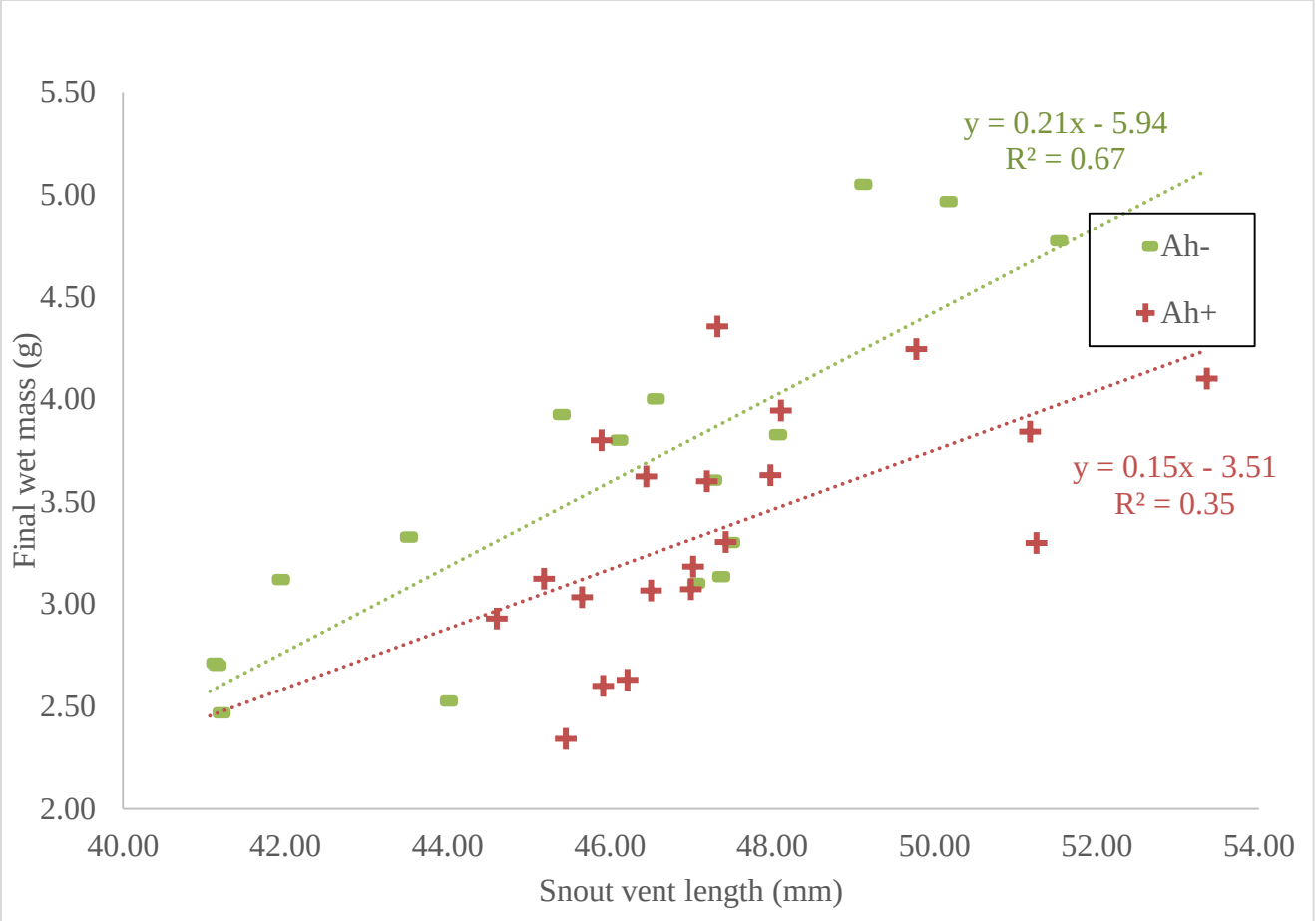

Figure 2.10. Linear regression of *Ah*-positive vs *Ah*-negative snout vent length vs wet mass. Regression equations and that the *Ah*-negative salamanders have a steeper slope (m = 0.21) and less variability ($R^2 = 0.67$) than the *Ah*-positive group (m = 0.15; $R^2 = 0.35$).

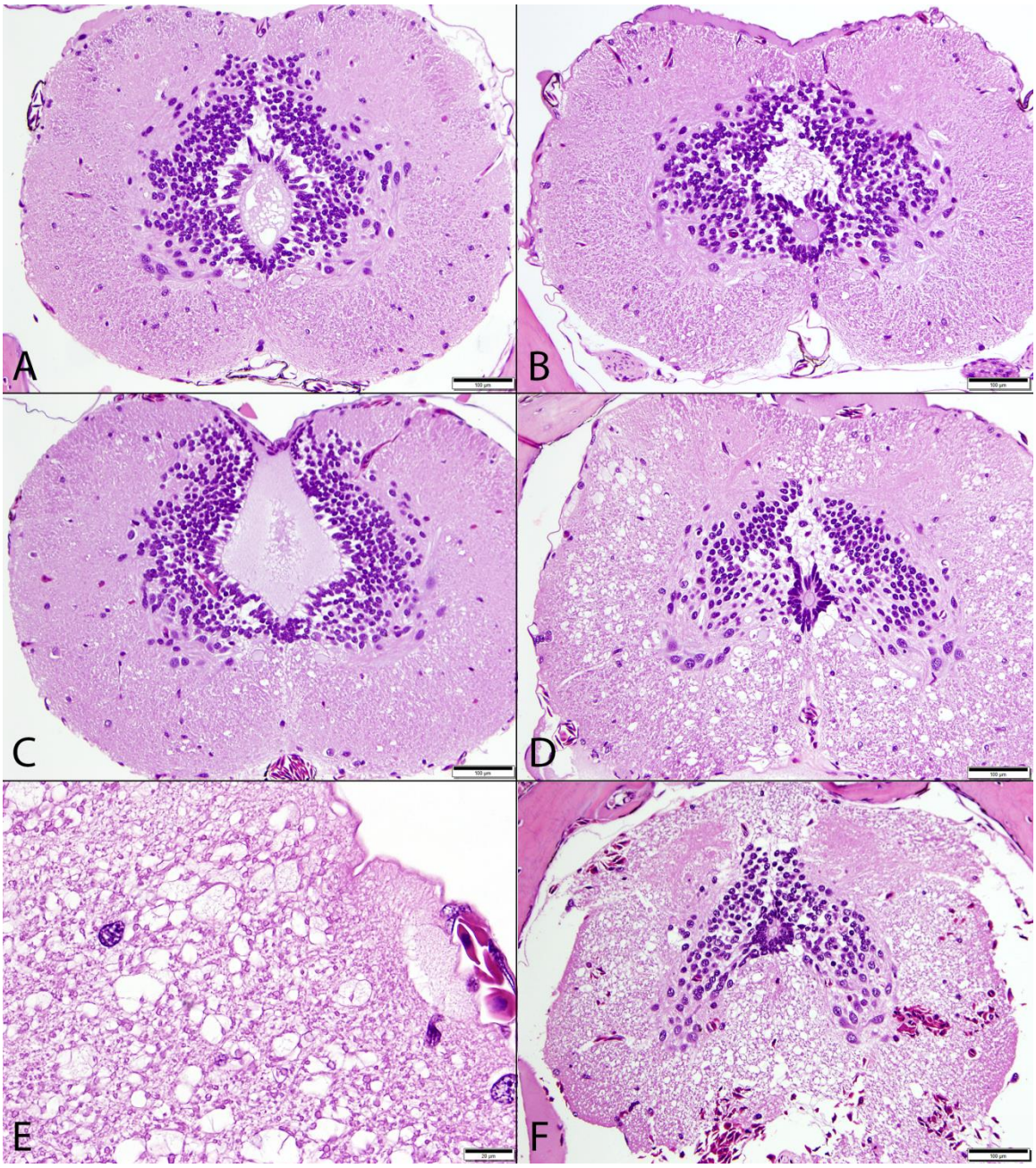

Figure 2.11. Histological images of hematoxylin and eosin (H&E) stained spinal cord sections from control (A-B) salamanders and treatment (C-F) salamanders fed the *Ah* toxic prey. A) Note the uniform pale eosinophilic appearance of the spinal white matter in this normal control (Bar = 100 μm). B) In this control salamander, there are scattered clear vacuolated spaces in ventral spinal white matter interpreted as artifact (Bar = 100

μm). C) The scattered vacuolated spaces in ventral white matter of this treatment animal are indistinguishable from artifactual changes in the previous control (Bar = 100 μm). D) Treatment salamander with extensive symmetrical vacuolation of spinal white matter compatible with *Ah* toxicity (Bar = 100 μm). E) Higher magnification image of the previous treatment animal with severe widespread vacuolation of white matter areas. Vacuoles varied in size, were smoothly bordered, and many contained axons. Some vacuoles contained flocculent material and strands of membrane-like material between axons and the vacuole margin (Bar = 20 μm). F) In addition to vacuolation, the spinal cords of some treatment animals contained small foci of hemorrhage (Bar = 100 μm).

CHAPTER 3

ASSESSING THE TOXICITY OF A NOVEL CYANOBACTERIUM THROUGH A WATERSNAKE TROPHIC TRANSFER STUDY [1]

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[1] Martin, M.K., A.C. Camus, H. Fenton, B.N. Haram, J. Hunt, J.C. Maerz, A. Vaughn, and S.B. Wilde. To be submitted to Herpetological Conservation Biology.

ABSTRACT

Aetokthonos hydrillicola (*Ah*) is a recently described cyanobacterium that grows on submerged, freshwater macrophytes. The primary attachment substrate of *Ah* is invasive *Hydrilla verticillata*, and the co-occurrence of *Ah* on *Hydrilla* has been linked to neurological impairment and death in waterbirds, turtles, amphibians, and fish. The goal of this study was to screen the sensitivity of additional taxa to the cyanotoxin, specifically predatory watersnakes that would be exposed by feeding on prey fed *Ah*-positive *Hydrilla* [trophic transfer]. We used laboratory feeding trials of juvenile northern (*Nerodia sipedon*) and banded watersnakes (*Nerodia fasciata*) fed fish that consumed either *Ah*-negative or *Ah*-positive *Hydrilla*. Behavior tests including tongue flicking, righting response, swimming agility, and appetite were conducted to screen for evidence of neurological impairment, and histopathological slides were screened for vacuolar lesions. Among snakes in *Ah*-negative treatments, histopathology of two of six animals was normal. In contrast, five of six snakes in the *Ah*-positive treatment had slightly (n = 3) to substantially (n = 2) more vacuolated spaces of the cervical spinal cord compared to *Ah*-negative salamanders. The *Ah*-positive snakes became progressively anorectic compared to *Ah*-negative snakes. These results confirm the sensitivity of watersnakes to *Ah* toxin and are another demonstration of the potential for exposure to *Ah* toxin among aquatic predators via trophic transfer.

Key Words.—Aetokthonos hydrillicola; banded watersnake; Nerodia fasciata; Nerodia sipedon; northern watersnake; Hydrilla verticillata; cyanobacterium; trophic transfer

INTRODUCTION

An uncharacterized cyanotoxin produced by a newly described cyanobacterium, *Aetokthonos hydrillicola* (*Ah*), growing on submerged aquatic vegetation (SAV) is hypothesized to be the source of wildlife poisonings (Wilde et al. 2005). This epiphytic cyanobacterium grows densely on *Hydrilla verticillata* (hereafter *Hydrilla),* Brazilian elodea, *Egeria densa*, and Eurasian water milfoil, *Myriophyllum spicatum.* Consumption of the *Ah*-positive *Hydrilla* has been shown to induce microscopic intramyelinic vacuoles in the white matter of the central nervous system (CNS). Focal neurologic signs of exposure may also be present; warning signs and symptoms of toxin contamination include uncoordinated movement (flight and swimming), and inability to right oneself or forage for food. (Thomas et al.1998).

The cyanotoxin exposure is either through direct consumption of SAV by herbivores or trophic transfer of SAV by predators (Thomas et al. 1998; Dodd et al. 2016; Chapter 2). Experimental feeding trials demonstrated direct consumption of *Ah*-positive *Hydrilla* induced vacuolar lesions in mallard ducks, *Anas platyrhynchos* (Birrenkott et al. 2004; Rocke et al. 2005; Wiley et al. 2007, 2008; Haynie 2008); domestic chickens, *Gallus gallus domesticus* (Lewis-Weiss et al. 2004); grass carp, *Ctenopharyngodon idella* (Haynie et al. 2013); painted turtles, *Chrysemys picta* (Mercurio et al. 2014), and amphibian tadpoles (Maerz et al. In press). Trophic transfer via prey was confirmed as a potential route of exposure from coots to red-tailed hawks, *Buteo jamaicensis* (Fischer et al. 2003; Lewis-Weiss et al. 2004), from island apple snails, *Pomacea maculata* to their predators (Dodd et al. 2016), and from tadpoles and snails to paedomorphic mole salamanders, *Ambystoma talpoideum* (Chapter 2).

The objectives of this study were to determine (1) if predatory northern watersnakes, *Nerodia sipedon,* and banded watersnakes, *N. fasciata,* are sensitive to the *Ah* toxin and (2) whether the watersnakes could be sufficiently exposed to the *Ah* toxin via consumption of typical prey that had grazed on *Ah*-positive plant materials. Watersnakes are expected to become neurologically impaired and develop intramyelinated lesions in the CNS through trophic transfer of the *Ah*-positive *Hydrilla* material. Watersnakes are good focal taxa to test the risks that *Ah* poses to predatory, aquatic reptiles. Northern and banded watersnakes feed mainly on aquatic animals including fish, various amphibians, and crayfish. Their range extends across the entire eastern half of the United States (U.S.) (Jensen et al. 2008); overlapping with locations of all known SAV/*Ah* reservoirs (Wilde, et al 2014). *Nerodia* inhabit higher and lower gradient streams that flow into lakes and reservoirs (Cecala et al. 2010) where *Hydrilla* and other SAVs that host *Ah* are common. Although *Nerodia* are less active during the cooler later fall and winter months when peak toxicity is detected on *Ah*-positive *Hydrilla,* they will be active on warmer days when they may continue feeding (Jensen et al. 2008). In the more southern portion of their range, watersnakes can remain active year round.

MATERIALS AND METHODS

Experimental Design.—Twelve aquaria were set up and a randomly assigned to 1 of 2 treatments: snakes fed *Ah*-positive prey (n = 6) or snakes fed *Ah*-negative prey (n = 6). One watersnake was housed in each aquaria. The experiment lasted 93 days.

Vegetation Collection, Screening, and Preparation.—*Hydrilla* was collected from 2 different sites in South Carolina. Collection of the *Ah*-positive *Hydrilla* occurred

on 10 November 2016 at J. Strom Thurmond Lake, a reservoir where fatalities of multiple waterbird species have been linked to consumption of *Ah*-positive *Hydrilla* (Fischer et al. 2003, 2006; Haynie 2008; Haram 2016). *Ah*-negative *Hydrilla* was collected from Lake Wylie, South Carolina, on 21 November 2016, a reservoir where the *Ah* has not previously been detected and no *Ah*-associated wildlife deaths have been reported despite repeated sampling over multiple years (Haram 2016). To collect the *Hydrilla*, a throw rake was dragged across the bottom in 1 m water to loosen and extract the plant material. The *Hydrilla* was then placed in four liter zip top plastic bags and transported in separate secure coolers to the lab. In the lab, replicate subsamples of *Hydrilla* were taken by randomly choosing 20 *Hydrilla* leaves from samples. Leaves were wet mounted on Fisherbrand 25 x 75 x 1.0 mm microscope slides and the presence of *Ah* on *Hydrilla* was initially confirmed using epifluorescence microscopy on a Rhodamine filter (Wilde et al. 2005; Haynie 2008). *Ah* presence or absence was confirmed with genetic analysis (polymerase chain reaction, PCR) (Wilde et al. 2014). The remaining plant material was placed in a walk-in freezer and stored at -20º C until use in the feeding trials.

To prepare the *Hydrilla*, a four liter bag from each sampled lake was thawed at the Whitehall Experimental Forest Herpetology Field Lab in Athens, Clarke County, Georgia at 9º C or room temperature for 24 hours if it was quickly needed. Temperature was measured with a Fisherbrand 76 mm immersion glass thermometer. After thawing, *Hydrilla* was stored at 9º C until needed. As *Hydrilla* was ready and needed, each four liter-sized bag was drained of excess water in ½ mm x 2 mm 2-ply mesh screens, and samples weighed and blended to create a fine green paste, then placed in the appropriate

tank for *ad libitum* feeding of fish. All samples were taken from the four liter zip top bags, hand squeezed to release excess water, and weighed with a Mettler Toledo model PB3002 scale at 30 g each. Samples were then blended in a stainless steel, 240 volt, Waring commercial blender with either 3 ¾ (*Ah*-negative *Hydrilla*) or 4 oz (*Ah*-positive *Hydrilla*) of dechlorinated water. More water was needed to blend the *Ah*-positive *Hydrilla* to prevent clogging the blender. After samples were finely blended, they were each placed in a 7.62 cm Farberware hand strainer. The excess water was removed by shaking the strainer and pressing the *Hydrilla* into the strainer with a 22.23 cm nickel-stainless steel lab spoon until a loose paste formed. Any leftovers from the blender were extracted by rinsing with dechlorinated water into the strainer and pressing it to release the excess water. The samples were then placed in labeled 4-qt zip top plastic freezer bags stored and cooled at 9º C until needed. Each bag was labeled with the date, day of treatment, and treatment type. All *Ah*-positive samples were stored separately from *Ah*-negative samples to reduce the possibility of contamination.

Each day the correctly labeled zip top bag was chosen, transported to the University of Georgia's Aquatic Biotechnology and Environmental Lab (ABEL) at Whitehall Experimental Forest where the samples were dislodged from the bags and placed in their perspective tanks. Thirty eight liter aquaria were used for all *Hydrilla*/fish habitats. *Hydrilla* was placed in habitats 24 – 48 hrs, in advance to allow fish time to feed on *Hydrilla* before the fish were fed to the snakes. All unused treated *Hydrilla* was autoclaved with the Brinkmann Tuttnauer 2340M and then disposed.

Animal Collection and Care.—All methods were approved by the University of Georgia's Institutional Animal Care and Use Committee (A2014 03-018-Y2-A1).

Experiments did not involve endangered or threatened species and were permitted by the Georgia Department of Natural Resources (29-WJH-14-170). All watersnakes along with Nile tilapia, *Oreochromis niloticus*, fathead minnows, *Pimephales promelas*, and guppies, *Poecilia reticulate*, were housed and monitored and proper procedures followed according to the University of Georgia's Policy on Humane Care and Use of Animals.

Nile tilapia was aqua-cultured at ABEL until they were deemed ready to feed on *Hydrilla*. Tilapia was the preferred fish to use for this study since it was currently being raised in-house. Unfortunately, there was a system failure and female breeders didn't survive which substantially depleted the quantity of available feeder fish. Guppies and fathead minnows were available and used as alternatives to accommodate the feeding trial until a viable tilapia population could be established again. All guppies used in the project were purchased from commercial live fish retailers in Athens, Georgia. All fathead minnows were purchased from bait shops in South Georgia. Most fish purchased outside the facility were acclimated to the temperature of the water for at least 20 minutes and then placed in holding tanks where they were fed Spirulina flake food once per day for a balanced diet until it was time to be transferred to treatment aquaria.

Between September and October 2016, 12 watersnakes were collected from various locations in Georgia where *Ah* has not been previously detected nor documented. Three snakes were collected from Harris Shoals Park in Watkinsville, GA on 14 September 2016; 1 was collected at Lake Herrick in Athens, GA on 15 September 2016; 1was collected at Pond 4 in Whitehall Experimental Forest in Athens, GA, October 2016, and the remaining seven were collected from Columbia County, GA on 2 September 2016 and 8 October 2016 (Table 3.1). Snakes were transported in mesh bags and then

housed in individual containers in an ambient temperature of 22º C with water and ad libitum fish and tadpole prey.

For the feeding experiment, each watersnake was transferred to 38 liter aquaria (50.8 cm x 25.4 cm x 30.48 cm) lined with pine shavings and containing 10 cm x 9 cm polyvinyl chloride (PVC) pipe as a retreat. A ceramic heat lamp with 120 volt bulb was provided at one end of each aquarium allowing snakes to bask and a water bowl was placed in each aquarium for feeding and soaking. Tanks were cleaned every 2 -3 weeks. Snakes were randomly assigned to 1 of 2 possible treatments: prey that had been fed *Ah*-positive *Hydrilla* (hereafter, *Ah*-positive treatment, n = 6) or prey that had been fed *Ah*-negative *Hydrilla* (hereafter, *Ah*-negative treatment, n = 6).

To prepare the treatment prey, six aquaria were filled with 30 – 34 liters of 25º C, dechlorinated water 5 cm from the lip and supplied with air stones. Three aquaria were designated *Ah*-positive and three *Ah*-negative approximately 48 h before being used as prey. Fish were placed in a treatment aquarium for 48 h, giving them adequate time to feed on the *Hydrilla*. After 48 h, few fish were haphazardly sampled, euthanized in a 3 cm deep bath of seltzer water, and transported to the Fish Lab where the total length and mass of each fish was measured and gastrointestinal tract (GIT) removed via dissection. The GIT was then wet mounted on Fisherbrand 25 x 75 x 1.0 mm microscope slides and coverslip was placed on top of the slide to keep the GIT in place. The slides were then observed under epifluorescence microscopy on a Rhodamine filter (Haynie 2008; Wilde et al. 2014), which confirmed the presence/absence of the *Ah* by detecting pigmentation of the colonies. Each microscopic GIT sample was photographed and preserved. Once the samples were confirmed to contain *Hydrilla* with or without *Ah*, 2 of the remaining fish

in the aquarium were allocated to each snake by placing them in a freshwater bowl. Each day we recorded the number of fish consumed and replaced any consumed and uneaten fish with two new individuals. By rotating fish into the treatment aquaria with fresh *Ah*-positive or *Ah*-negative *Hydrilla*, we were able to maintain a relative high rate of prey availability for all snakes. No snake consumed all its prey over the entire study.

Behavioral Measures.—We monitored snake appetite as one measure of neurologic impairment. Other studies have documented anorexia, slower mass gain, or mass loss as an effect of *Ah* exposure (Mercurio et al. 2014; Chapter 2). We monitored appetite as the cumulative fresh mass of fish consumed by each snake over time.

Tongue Flick Rates.—Tongue flicking is a common way to measure sensory responses of snakes (Burghardt 1966; Gove et al. 1983; Bennett et al. 2006), so a series of tongue flick response tests were used to determine whether snakes showed a latency to attractive or aversive chemical stimuli. Tongue flick rates were measured once prior to starting experimental treatments (2 January 2017) and 30, 48, and 74 days after treatments started.

Tests consisted of placing snakes in a 76 liter aquarium (76.84 cm x 31.75 cm x 32.39 cm). The number of tongue flicks, for 60 s were counted and then a cotton swab soaked in 91% isopropyl alcohol was presented as an aversive stimulus (Bennett et al. 2006). The swab was extended to within 2 cm of the snake's snout and we measured the tongue flick rate toward the swab and the time it took a snake to turn away. Finally, a cotton swab soaked in canned sardines was presented to snakes as an attractive cue and the number of tongue flicks were recorded for 60 s (Burghardt 1966; Gove et al. 1983; Bennett et al. 2006). All tests were video recorded for measurements.

Righting Response.—The final behavioral assay used to evaluate neurological impairment among snakes was a righting response test. Righting response tests were conducted five days before experimental diet treatments were initiated and 26, 56, and 76 days after diet treatments started. A healthy snake should recover from dorsal recumbency quickly (Bennett et al. 2006). For each test, a snake was placed in dorsal recumbency within a 76 liter aquarium. We measured the time in seconds required for a snake to right itself. We repeated this three times per snake per test and used the average righting response time in our analysis.

Euthanasia and Histopathology.—Snakes were euthanized by injection of pH-neutral buffered tricaine methanesulfonate (MS-222) and was dissolved in 1 ml of deionized water. Three quarters of the MS-222 was first injected intracelomically, and once the snake was sedated, the remaining MS-222 was injected into the heart. After death, the heads were immediately severed and representative samples of various organs were immediately fixed in 10 % neutral buffered formalin for a minimum of 24 hours. The fixed organs were embedded in paraffin wax and heads were processed with Kristensen's decalcification solution. Once decalcified, heads and cervical regions were transversely sectioned throughout their lengths, targeting the forebrain (telencephalon), midbrain (mesencephalon), hindbrain (metencephalon, myelencephalon), and proximal spinal cord. The transverse sections were placed in tissue cassettes, processed routinely, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for microscopic examination. The remaining tissues were stored at 9º C. Histology slides were "blindly" reviewed by a board-certified anatomic veterinary pathologist (HF) from the University of Georgia's Department of Veterinary Medicine for evidence of intramyelinic edema

consistent with *Ah* toxicity. Snake histology results were recorded as "2" (significant vacuolar lesions present), "1" (slightly more lesions than normal), "0" (baseline, microscopically normal).

Statistical Analysis.—We conducted a repeated measures analysis of tongue flick responses to the three stimuli (non-stimulus, alcohol, and sardine stimuli) and righting response time using a linear mixed effects model with treatment and time as fixed factors and the individual snake as a random variable. We used a similar model for the repeated measures analysis of the cumulative mass of fish consumed over time with the addition of snake body mass as a covariate. All analyses have an alpha value = 0.05 unless indicated otherwise and were conducted using the nlme package in R version 3.3.1 (R Core Team 2016).

RESULTS

Two *Ah*-positive and one *Ah*-negative snakes died during the feeding trial from injury and disease unrelated to treatments. One *N. sipedon* (*Ah*-negative) was found with a foreign body lodged in its oral cavity and was immediately euthanized. One *N. fasciata* (*Ah*-positive) died from trauma due to tissue damage. Another *N. fasciata* (*Ah*-positive) possessed skin lesions that were present before the feeding trial, consistent with snake fungal disease (SFD) which was euthanized as well. This left three *N. sipedon* in the *Ah*-positive treatment and one in the *Ah*-negative treatment. One *N. fasciata* was left in the *Ah*-positive and four *N. fasciata* in the *Ah*-negative treatment. No overt signs of neurologic impairment were observed with any snakes. All snakes ate and gained mass during the experiment.

Behavioral Responses.—The cumulative mass of fish consumed was positively correlated with snake mass. Snakes in the *Ah*-positive treatment consumed significantly less cumulative fish mass over the course of the study and gained less mass compared to snakes in the *Ah*-negative treatment ($P = 0.001$) (Table 3.2; Fig. 3.1). The *Ah*-negative snakes consumed a cumulative mass of 1,554 g of fish while *Ah*-positive snakes consumed a cumulative mass of 1,715 g fish over the 93 day feeding trial. This is an 11 % increase.

The number of non-stimulus *Ah*-positive tongue flicks varied among days 0, 30, 48, and 74 ($P = 0.01$), but the effects of treatment and interaction of non-stimulus tongue flicks on these days did not statistically vary between treatment groups ($P = 0.52$) (Table 3.3; Fig. 3.2). The mean number of tongue flicks in response to alcohol swab also did not differ significantly between treatments or vary significantly with time ($P = 0.44$) (Table 3.4; Fig. 3.3). The mean number of tongue flicks in response to sardine swab did not differ significantly between treatments or vary significantly with time ($P = 0.91$) (Table 3.5; Fig. 3.4). The mean number of seconds snakes took to right themselves did not differ significantly between treatment groups or vary significantly with time ($P = 0.52$) (Table 3.6; Fig. 3.5).

Histopathology.—Upon close examination of internal body structures after necropsies, five snakes that consumed *Ah*-negative prey and four snakes that consumed *Ah*-positive prey tested positive for parasites (cestodes or nematodes) which should not have affected results. The brains and proximal spinal cords of 12 snakes, six *Ah*-negative and six *Ah*-positive, were examined and found to be microscopically normal in two *Ah*-negative and one *Ah*-positive animal. Indistinguishable vacuolation was widespread,

primarily in ventral white matter areas, in the spinal cords of four *Ah*-negative (Fig. 3.6A) and three *Ah*-positive (Fig. 3.6B) animals. Vacuoles were relatively uniform in size, slightly more irregular than those observed in salamanders (Martin, Chapter 2), and most contained a distinct axon. In two *Ah*-positive snakes, vacuolation was diffuse throughout the spinal white matter tracts (Fig. 3.6C). Similar to that seen in salamanders (Martin, Chapter 2), vacuoles varied in size and had smooth rounded borders. Axons were frequently displaced peripherally against the margin of the vacuole (Fig. 3.6D).

DISCUSSION

The results of this study provide some evidence that watersnakes (*Nerodia* spp.) are sensitive to *Ah* toxin and can be exposed via trophic transfer from herbivorous fish that feed on *Hydrilla* or other macrophytes that host *Ah*. Though we did not observe clinical signs of impairment of righting responses, tongue flick rates towards prey, or aversion to repulsive chemical cues, we did see clear evidence of progressive anorexia among snakes fed *Ah*-positive prey and evidence of disease in the central nervous system consistent with exposure to *Ah* toxin. These two observations are consistent with studies of other wildlife, most notably a recent study on aquatic turtles (Mercurio et al. 2014). Some painted turtles fed *Ah*-positive *Hydrilla* developed mild and inconsistent ataxia after 96 days of feeding on *Ah*-positive *Hydrilla*. More notably, turtles fed *Ah*-positive *Hydrilla* showed progressive reductions in feeding rate compared to turtles fed *Ah*-negative *Hydrilla*, and all turtles fed *Ah*-positive *Hydrilla* had disease in brain tissues. It is notable that signs of impairment were mild even after 93 days in snakes (this study) and 96 days in turtles (Mercurio et al. 2014) and there was no mortality in either study, but in birds, neurological impairment from exposure to *Ah* toxin can manifest in under

five days (Rocke, et al 2002, Haram, 2016), and among amphibian tadpoles, measurable effects of *Ah* toxin on mortality have appeared in as short as three days (Maerz et al. In press). This illustrates that either exposure dosage or sensitivity to *Ah* toxin can vary greatly among aquatic fauna.

It is important that we address the potential for confounding effects of our treatments because our source plant materials each came from one, separate lake. In addition to the presence or absence of *Ah*, there are potentially many other differences between *Hydrilla* collected at the two sites including differences in nutritional quality, metal concentrations, presence of microbes, and the presence of anthropogenic pollutants. We cannot rule out other confounding differences between plant sources in our treatments; however, our results in the context of our other research make it most likely that the effects we observed are related to *Ah* toxin presence and not other factors. First, there have been numerous other feeding trials of wildlife using *Ah*-positive or *Ah*-negative *Hydrilla* from other lakes besides our two source lakes. More than 40 lakes have been surveyed and source *Hydrilla* tested on wildlife to date. Water quality and microbiota differ considerably among those 40 reservoirs. Among the 21 lakes where *Ah*-positive *Hydrilla* was collected, all have been shown to induce impairment in some species of wildlife. Among the 15 *Ah*-negative lakes where *Hydrilla* was collected, that *Hydrilla* has never induced impairment, disease, or death in any wildlife. Therefore, among all prior studies from using *Hydrilla* from many source lakes that vary in many aspects of water quality, only *Ah*-positive *Hydrilla* has ever induced neurological impairment, disease within the CNS, and death among wildlife (Fisher, et al. 2003, Wiley et al. 2007; Wilde, et al 2014, Birrenkott, et al 2004; Lewis-Weiss et al 2004; Wiley et al.

2007; Haynie 2008; Mercurio et al. 2014; Maerz et al. In press, Chapter 2). Second, *Ah*-positive *Hydrilla* from our source lake has been collected during the summer – when Ah appear not to produce toxin – and fed to tadpoles, and did not cause any impairment or mortality. Therefore, at other times of the year, *Ah*-positive *Hydrilla* is suitable forage for amphibians and other wildlife. If something other than *Ah* was responsible for toxicity, it would have to be a factor that also varies seasonally but is currently latent.

In the spinal cords of snakes that ingested the *Ah*-positive treatment indirectly via prey sources, results demonstrate microscopic changes that were suggestive of, but not definitive for pathology associated with *Ah* exposure among other vertebrates. Bald eagles that had consumed American coots that fed on *Ah*-positive treatment had lesion development in the cerebellum, cerebrum, brain stem, spinal cord, and concentrating in the optic tectum which includes vacuolation at all levels of the brain and in the spinal cord. Additionally, small hemorrhages have been reported in the thalamus and brainstem of affected eagles (Thomas et al. 1998). Ultrastructurally, histopathology of *Ah*-exposed animals is characterized by damage to lipid-rich myelin sheaths that surround and insulate nerve axons primarily in the central nervous system. Specifically, splitting of the myelin lamellae occurs at the intraperiod line, a change consistent with intramyelinic edema (Fisher et al. 2003).

Unlike in birds and fish, vacuolation in the snakes examined was confined almost entirely to the snakes' spinal cords. Because reptile movement is thought to be controlled by the spinal cord and not the brain (Bennett and Mehler 2006), if lesions form here, we might expect to see this affect neurological impairment in *Ah*-positive treatment. *Ah*-positive snakes were hypothesized to show neurologic deficits in tongue flick and

righting response tests, but differences among snakes were not related to the treatment. A possible reason for a snake's lack of response in these behavioral measures may be related to undeveloped protocol for evaluating responses to stimuli in reptiles (Bennett and Mehler 2006). It is possible the stimuli we used to acquire responses were not adequate.

Although these changes in some animals may reflect dilated axon sheaths and intramyelinic edema, similar histologic changes are a relatively common post mortem artifact and must be interpreted with caution. Indistinguishable changes were present in both *Ah*-negative and *Ah*-positive animals and likely reflect post mortem or processing artifacts. The result of finding one microscopically normal *Ah*-positive snake may be attributed to its short length of time in the feeding trial. This snake died during week seven (1/2 way through the feeding trial, not due to any treatment issues). This snake may not have received an adequate dose of *Ah*-positive prey for it to develop vacuoles. Additional potential complicating factors include the small sample size used in the trial and inability to control dosing of the toxic compound to individual animals. While electron microscopy is needed to confirm splitting of myelin lamellae, the observed changes, while subjective, are considered significant and sufficient to warrant further study in these species.

Because vacuoles suggestive of *Ah* toxicity were mainly found in the cervical spinal cord of watersnakes contrasting with where vacuolation was concentrated in birds, may reflect sizes of brain and optic regions in snakes. Neural functions in reptiles are not as abundant as in higher vertebrates such as birds according to Butler et al. 2006. A smaller optic tectum in a snake might indicate less myelin formation in that region.

Myelin is a coating made of lipid-rich proteins and phospholipids insulating the axons of the CNS and keeping neurons transmitting and responding to nerve impulses. Additionally, if a snake's optic region is proportionally smaller and has fewer neurons than a bird's optic region, a decreased quantity of myelin would lead to fewer intramyelinated vacuoles causing neuron cell degeneration in the optic tectum regions of the watersnakes compared to other wildlife. Therefore, as we recommended in Chapter 2, interspecies comparisons will need to focus on a range of CNS tissues to account for differences in CNS anatomy when evaluating the relative sensitivity of different species to *Ah* toxin.

This research suggests watersnake vulnerability to the *Ah* cyanotoxin in another experimental feeding trial in a lab setting. Although researchers do not know the dosage threshold causing lesions or neurologic signs of impairment (Rocke et al. 2002), if *N. fasciata* and *N. sipedon* are exposed to the *Ah* cyanotoxin in their natural environment, they will be susceptible to the effects of *Ah* toxicity through consumption of prey.

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Table 3.1. Snake species, collection site, treatment, beginning and ending mass, and beginning and ending snout-vent length (SVL).

Species	*Site ID*	*Snake*	*Treatment*	*Begin Mass (g)*	*Begin SVL (mm)*	*End Mass (g)*	*End SVL (mm)*
N. sipedon	Oconee Co.	1	*Ah*-	3.19	151	3.47	152
N. sipedon	Oconee Co.	2	*Ah*+	5.15	175	8.9	202
N. sipedon	Oconee Co.	3	*Ah*+	5.81	178	10.5	206
N. sipedon	Clarke Co.	6	*Ah*-	5.68	181	15.1	243
N. fasciata	Columbia Co.	7	*Ah*-	12.83	237	24.9	265
N. fasciata	Columbia Co.	8	*Ah*+	16.34	279	21.48	374
N. fasciata	Columbia Co.	9	*Ah*+	16.44	286	----	----
N. fasciata	Columbia Co.	10	*Ah*-	8.92	225	14.1	229
N. fasciata	Columbia Co.	11	*Ah*-	20.94	294	31.2	307
N. fasciata	Columbia Co.	13	*Ah*-	30.42	324	42.8	343
N. fasciata	Columbia Co.	14	*Ah*+	17.77	279	21.7	389
N. sipedon	Clarke Co.	15	*Ah*+	38.95	387	44.2	282

Table 3.2. Results of repeated measures analysis using a linear mixed effects model of the cumulative mass of fish consumed by watersnakes. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treatment), week, snake mass (snmass), and the interaction of treatment and week (treatment:week) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	122	538.5971	<0.001
treatment	1	9	136.7959	<0.001
week	1	122	754.5040	<0.001
snmass	1	9	34.3985	<0.001
treatment:week	1	122	19.3389	<0.001

Table 3.3. Results of repeated measures analysis using a linear mixed effects model of the number of tongue flicks by watersnakes toward a non-stimulus swab as a function of treatment and day. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treatment), day, and the interaction of treatment and day (treatment:day) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	27	46.23558	<.0001
treatment	1	10	0.67452	0.4306
day	3	27	4.19163	0.0147
treatment:day	3	27	0.75631	0.5284

Table 3.4. Results of repeated measures analysis using a linear mixed effects model of the number of tongue flicks by watersnakes toward an aversive alcohol swab as a function of treatment and day. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treatment), day, and the interaction of treatment and day (treatment:Day) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	17	9.196425	0.0075
treatment	1	10	1.370225	0.2689
Day	2	17	1.976212	0.1692
treatment:Day	2	17	0.860907	0.4404

Table 3.5. Results of repeated measures analysis using a linear mixed effects model of the number of tongue flicks by watersnakes toward a positive, sardine-scented swab as a function of treatment and day. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treatment), day, and the interaction of treatment and day (treatment:Day) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	20	37.36742	<.0001
treatment	1	10	0.01748	0.8974
Day	2	20	0.20308	0.8179
treatment:Day	2	20	0.09818	0.9069

Table 3.6. Results of repeated measures analysis using a linear mixed effects model of the righting response time by watersnakes as a function of treatment and day. Degrees of freedom (numerator and denominator), *F* statistics (F-value), and significance levels (p-value) for the predicted value of the *Ah*–negative treatment (Intercept), *Ah*-positive treatment (treatment), day, and the interaction of treatment and day (treatment:Day) are listed.

	numDF	denDF	F-value	p-value
(Intercept)	1	20	2.8629490	0.1062
treatment	1	10	0.7390927	0.4101
Day	2	20	1.3761314	0.2755
treatment:Day	2	20	0.6803712	0.5178

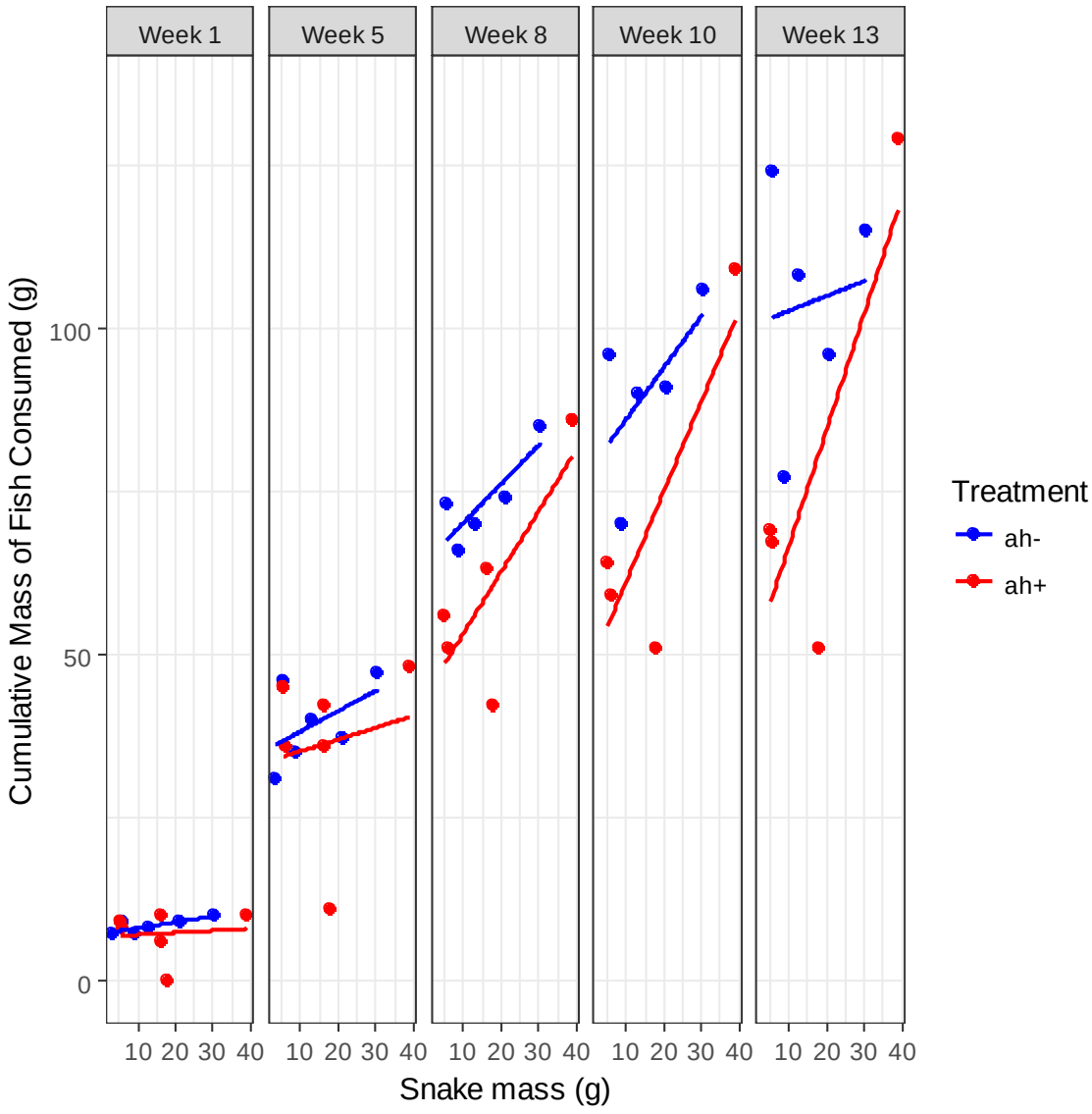

Figure 3.1. Cumulative mass of fish consumed by watersnakes by weeks 1, 5, 8, 10, and 13 as a function of mass of the snake and treatment. Shows progressive anorexia in *Ah*-positive treatment between weeks.

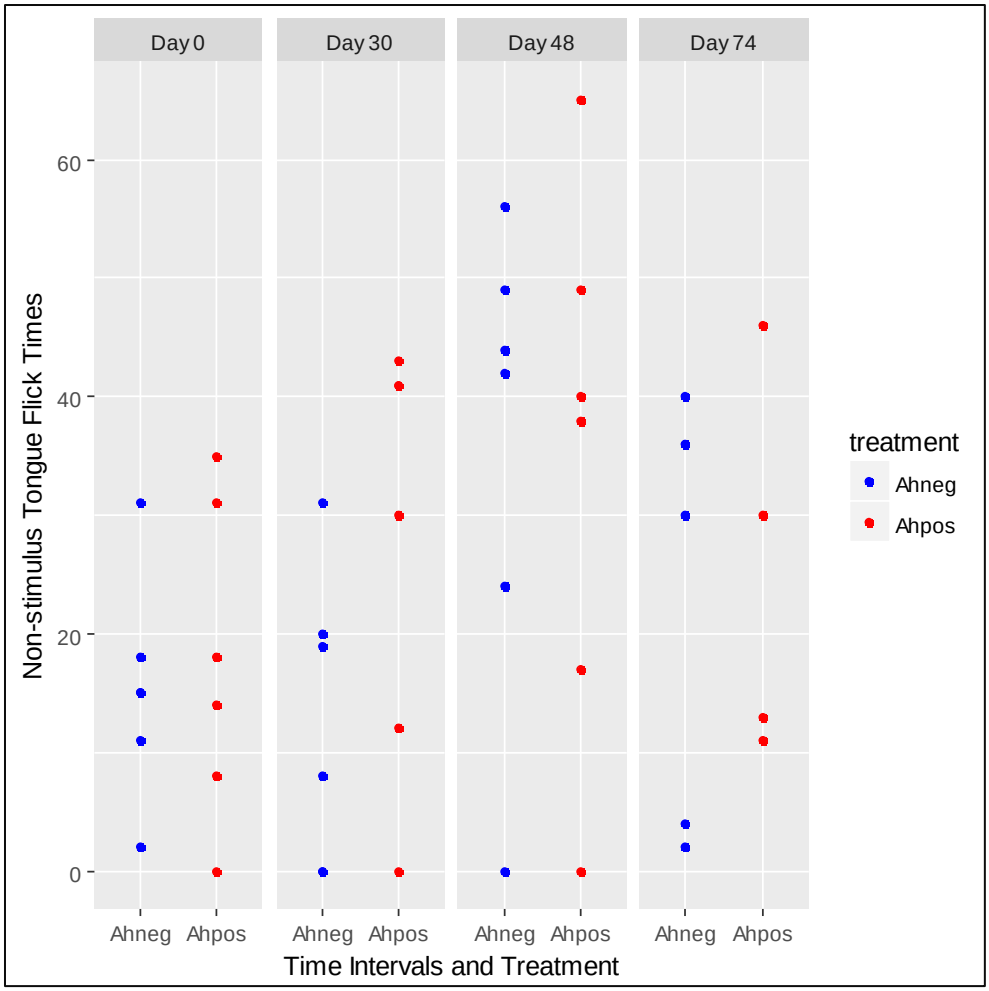

Figure 3.2. Number of tongue flicks in 60 seconds by watersnakes toward a non-stimulus swab as a function of treatment and day.

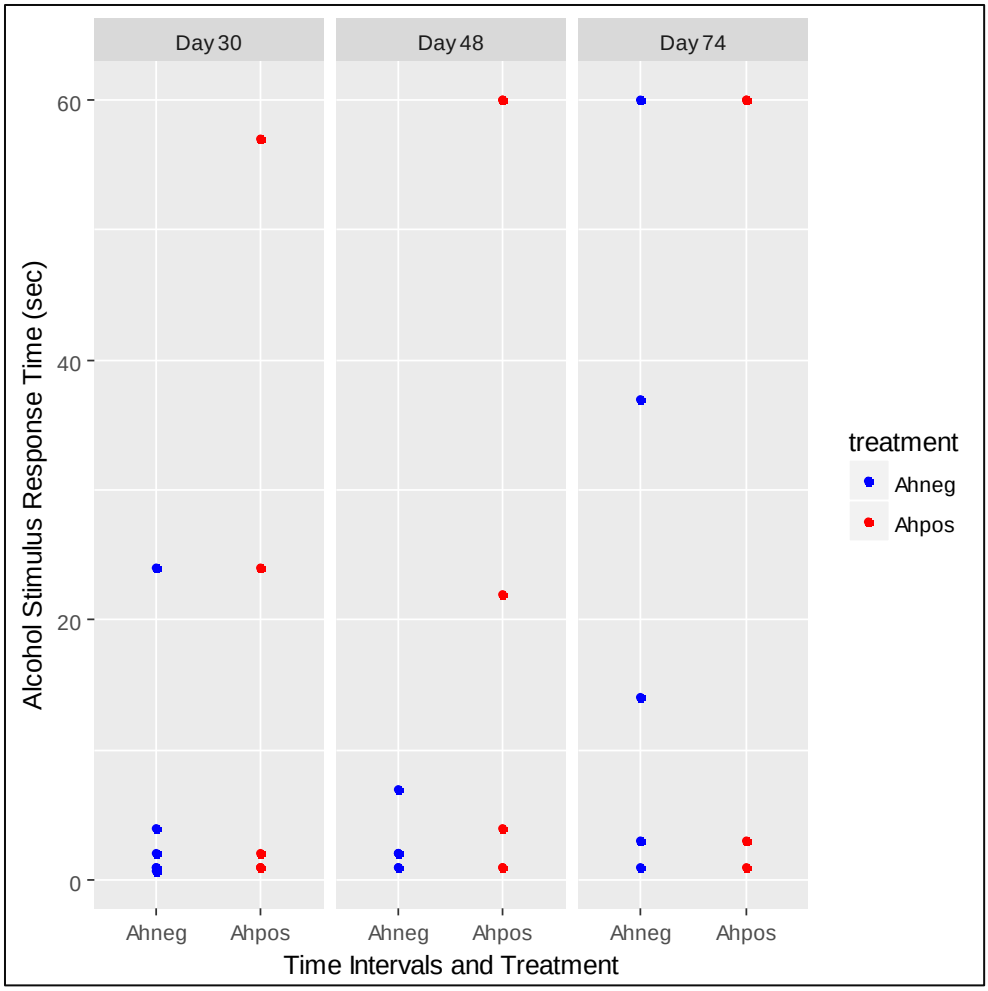

Figure 3.3. Number of tongue flicks in 60 seconds by watersnakes toward an aversive alcohol swab as a function of treatment and day.

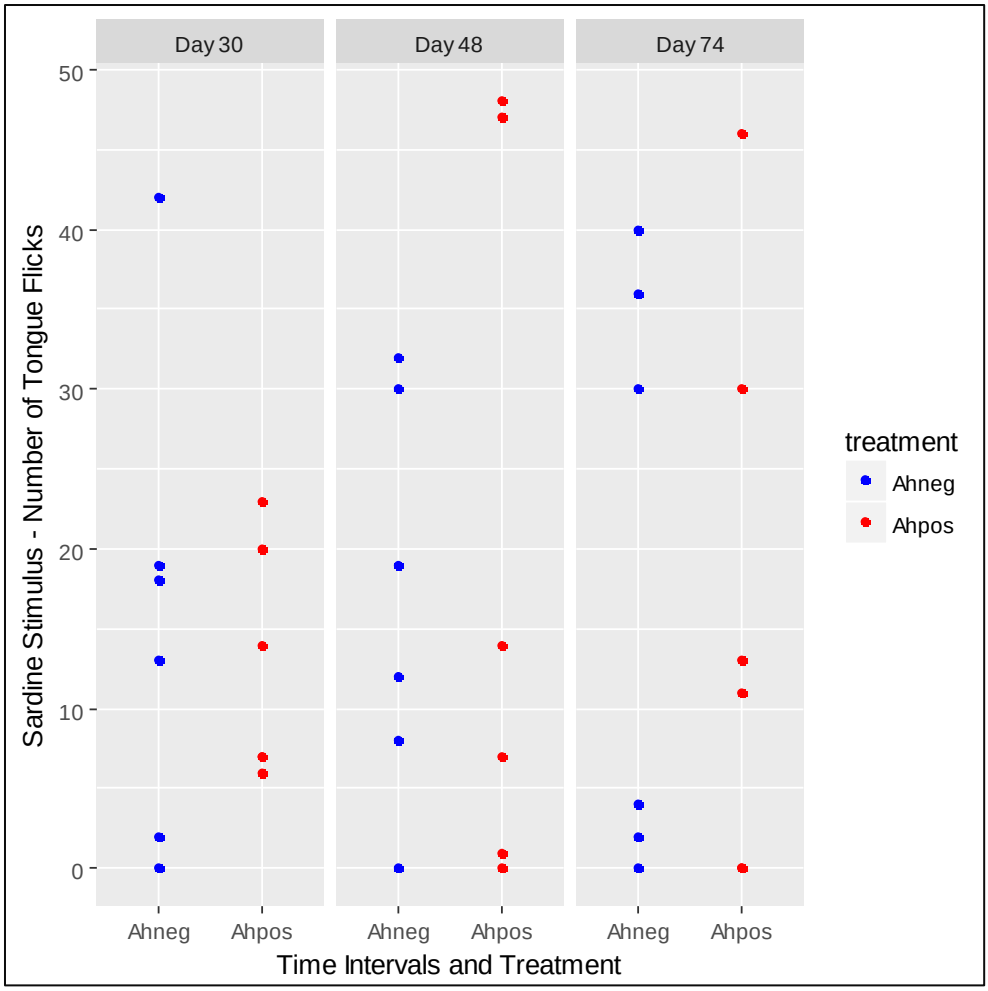

Figure 3.4. Number of tongue flicks in 60 seconds by watersnakes toward a positive sardine-soaked swab as a function of treatment and day.

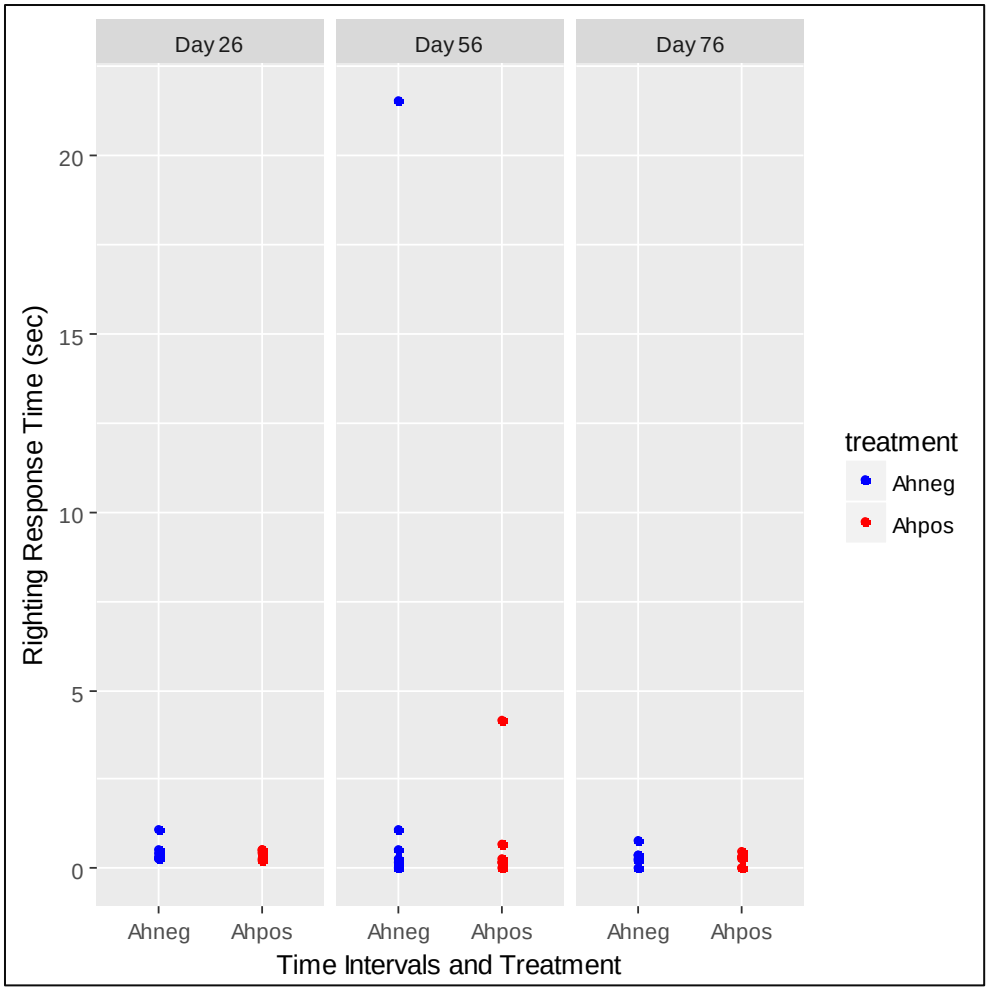

Figure 3.5. Mean righting response times of watersnakes as a function of treatment and day.

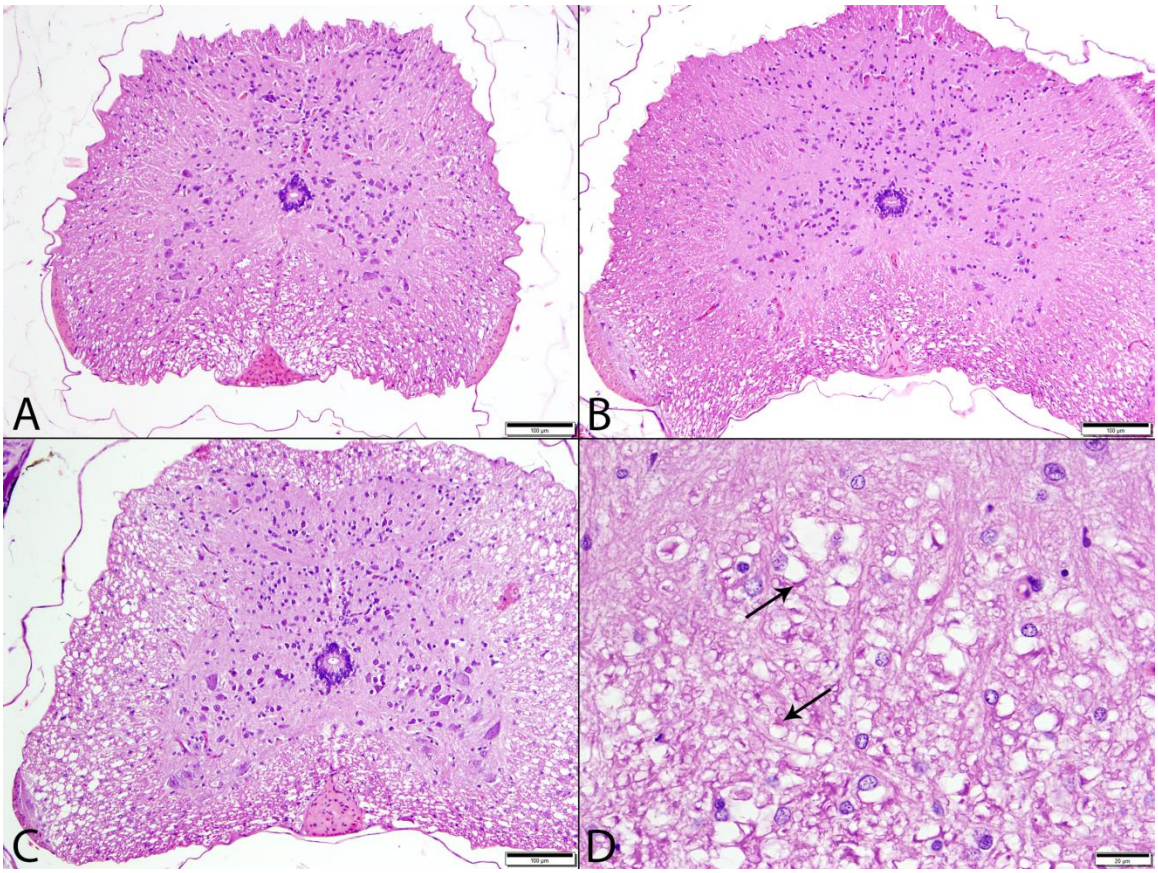

Figure 3.6. Histologic images of H&E stained spinal cord sections from control snakes and treatment snakes fed *Ah* toxic prey. There is widespread indistinguishable vacuolation, primarily in ventral white matter, in the spinal cords of this control (A) and treatment (B) snake. The vacuoles were relatively uniform in size, irregular, and most contained a distinct axon (Bars = 100 μm). C) In contrast, vacuolation of white matter was diffuse in white matter tracts of this treatment animal (Bars = 100 μm). D) At higher magnification, the vacuoles varied in size and had smooth rounded borders. Axons were frequently displaced peripherally against the margin of the vacuole (arrows) (Bars = 20 μm).

CHAPTER 4

COMPARING RELATIVE DENSITY OF *AETOKTHONOS HYDRILLICOLA* AND TOXIN CONCENTRATION ON A NATIVE MACROPHYTE VS NONNATIVE *HYDRILLA VERTICILLATA* [1]

[1] Martin, M.K., B.N. Haram, J.C. Maerz, T.J. Phillips, and S.B. Wilde. To be submitted to Harmful Algae.

ABSTRACT

Aetokthonos hydrillicola (*Ah*) is a recently described cyanobacterium that grows on submerged, freshwater macrophytes, primarily invasive *Hydrilla verticillata*. Neurological impairment and death in waterbirds, turtles, amphibians, reptiles, and fish have been documented in 20 reservoirs with *Hydrilla* and epiphytic colonies of *Ah (Hydrilla/Ah)*. Seasonal consumption of *Ah*-positive *Hydrilla* results in characteristic vacuolar myelinopathy (VM). Although *Hydrilla* is the most common plant harboring the cyanobacterium, *Ah* does grow on other macrophytes and it is not known whether *Ah* produces toxin on alternative host plants. The potential for toxin associated with additional submerged aquatic plants would expand the potential for the disease. The goal of this study was to determine whether there was detectible toxin in native macrophytes colonized by *Ah*. We collected co-occurring samples of *Hydrilla* and southern naiad, *Najas guadalupensis*, from Clarks Hill/J. Strom Thurmond reservoir on five different dates in the fall of 2017. Samples were evaluated microscopically for relative *Ah* surface area coverage and relative toxin concentration was determined using liquid chromatography-mass spectrometry (LC-MS). *Hydrilla* consistently had higher surface area coverage by *Ah* and higher toxin levels compared to *Najas*, except during a mid-November sampling date where *Najas* had a higher toxin concentration than *Hydrilla*.

Key Words.—Aetokthonos hydrillicola; Najas guadalupensis; Southern Naiad; Hydrilla verticillata; macrophyte; Desmethyl bromethalin; liquid chromatography-mass spectrometry

INTRODUCTION

The recently described epiphytic cyanobacterium, *Aetokthonos hydrillicola* (*Ah*), has been linked to vacuolar myelinopathy in freshwater birds, reptiles, amphibians, and fish in the United States (Haynie 2013; Wilde et al. 2014; Mercurio et al. 2014; Maerz et al. In press). *Ah* has been documented on a number of species of invasive submerged aquatic vegetation (SAV) including: *Hydrilla verticillata, Egeria densa*, and *Myriophyllum spicatum*. Laboratory feeding trials have documented neuropathy and death in organisms that consume SAV colonized by *Ah*, or prey that have consumed hydrilla/*Ah* (Fischer et al. 2003; Birrenkott et al. 2004; Wilde et al. 2005; Dodd et al. 2016; Martin, Chapter 2 and 3). An analytical method to detect the neurotoxic compound was developed upon discovery of a unique chromatographic peak characteristic of all samples that produce *Ah* toxicity in vertebrate and invertebrate bioassays (Haram 2016).

The majority of studies on *Ah* toxicity have focused on *Hydrilla-Ah* complex due to the prevalence of *Hydrilla* in locations where *Ah* toxicity has been observed (Wilde, et al 2005, Wilde, et al 2014). However, *Ah* has also been documented on other SAV species including several widespread species native to the US. A number of these species are found throughout the same invasive range as *Hydrilla*, will co-occur with *Hydrilla*, and can also form dense mats when not competing with other invasive SAV species (https://plants.sc.egov.usda.gov/java/). The ability for *Ah* to grow on other SAVs – particularly species with wider distributions than *Hydrilla* or that can occupy a wider range of waterbody types – would dramatically expand the risk that *Ah* poses to wildlife. However, information on whether *Ah* produces the neurotoxin at similar concentrations when growing on other SAVs is lacking.

The objectives of this study were to (1) evaluate percent *Ah* surface area coverage on Southern Naiad (*Najas guadalupensis*) and *Hydrilla* and (2) quantify relative *Ah* toxin levels in extracted *N. guadalupensis*-*Ah* and Hydrilla-*Ah* samples through liquid chromatography-mass spectrometry (LC-MS).

Najas guadalupensis is widely distributed in lakes, reservoirs, ponds, and other slow-moving bodies of water. It can be abundant and form dense mats near the surface on long, branching stems from the substrate or while floating (*Najas guadalupensis*. UF/IFAS. Available from https://plants.ifas.ufl.edu/plant-directory/najas-guadalupensis/. [Accessed 25 June 2018]). These characteristics make *Najas* an adaptable species that might harbor excessive colonies of *Ah* which produce the *Ah*-toxin potentially harming wildlife.

MATERIALS AND METHODS

Study Site.—*Hydrilla* and *Najas* were collected from two different locations on Clarks Hill/J. Strom Thurmond reservoir (Table 4.1), a site where numerous herbivorous and predatory birds have been affected by *Ah* toxicity (Fischer et al. 2003, 2006; Haynie 2008; Haram 2016). This constructed reservoir is located on the Savannah River northwest of Augusta, Georgia. Created in the 1950s, it is managed by the U.S. Army Corp of Engineers (USACE) primarily for flood control, hydropower, and recreation. Existing land management is controlled by Georgia and South Carolina Department of Natural Resources along with USACE. At full capacity, it encompasses over 71,000 acres of water and 1,200 miles of shoreline. Plant samples were collected from 2 locations, Cherokee Creek Ramp (33°43’7.62”N, 82°20’47.89”W) and Powerline Cove (33°43’1.58”N, 82°20’54.14”W). (J. Strom Thurmond Dam and Lake. U.S. Army Corps

of Engineers. Available from http://www.sas.usace.army.mil/About/Divisions-and-Offices/Operations-Division/J-Strom-Thurmond-Dam-and-Lake/)/. [Accessed 25 June 2018]).

Vegetation Collection and Screening.—*Ah*-positive *Hydrilla* and *Najas* from two different locations on Clarks Hill/J. Strom Thurmond reservoir were collected on 12, 26 October and 8, 16, and 30 November 2017. A throw rake was used to collect *Hydrilla* within 1 m of the surface. The samples were then placed in two-liter zip top plastic bags and transported in separate secure coolers to freezers in the lab.

In the lab, subsamples of *Hydrilla* were collected by randomly choosing 20 individual leaves. Subsamples of *Najas* were collected by randomly selecting two stem sections (1 – 2") with leaves. All subsamples were wet mounted on glass microscope slides and visually estimated for percent *Ah* surface area coverage using epifluorescence microscopy with a Rhodamine filter (Wilde et al. 2005; Haynie 2008). Remaining plant material was stored at -20° C for use in extractions.

Extraction, Filtration, and Evaporation.—*Hydrilla* and *Najas* samples, separated by collection date, species, and location separately, were dried in a Quincy Lab Model 40 GC lab oven for 48 hours at 40° C. When dry, each sample was ground with a Coors Tek porcelain ceramic chemical and heat resistant pestle and mortar to a coarse powder. A 3.0 g biomass of each sample was weighed with a (OHAUS Adventure Pro) balance. Each sample was then transferred to individual flasks and added 150 ml of 100 % methanol. We covered each flask with paraffin, wrapped them in aluminum foil, and stored them under a fume hood for 24 hours. All samples were swirled for 2 min every 15 min, totaling 60 min.

Each sample was filtered through qualitative grade filter paper each with a pore size of 4 – 7 µm (Qualitative Diameter 90 mm GE Healthcare Life Sciences Whatman) to remove particulates. Each filter was rinsed with 100% methanol until the liquid ran clear. Each sample extract was evaporated on a BUCHI Rotavapor R-200 at 40^{o} C. Dry extracts were transferred to 2.0 ml pre-weighed microcentrifuge tubes by re-suspending them in 100% methanol, then finished drying on a LABCONCO CentriVap at 34^{o} C. All tubes with dried extract were weighed and mass recorded (Haram 2016).

Liquid Chromatography-Mass Spectrometry (LC-MS).—To estimate *Ah* toxin concentration in each sample, a recently developed liquid chromatography method (Haram 2016) was modified to LC-MS on an Agilent 1200 series (Phillips 2018). Each dried extract sample was transported to the U.S. Environmental Protection Agency Office of Research and Development, Athens, Clarke Co., Georgia. The LC-MS was calibrated with an internal standard, desmethyl bromethalin (DMB), to get a linear curve between 0.015625 and 1.0 ppm in a 90:10 deionized water:propylene glycol solution (PGDI). Desmethyl bromethalin was used to calculate *Ah* toxin concentration because it shares similar pathophysiology with the unknown *Ah* toxin.

Crude extracts were resuspended in 500 µl of 90:10 PGDI by vortexing for 10 s, sonicated for 10 min, and vortexed again for 20 s. Each sample was then centrifuged for up to an hour at 4,500 rpm, and 120 µl of each supernatant was transferred into 200 µl gas chromatography (GC) vial inserts. Each sample was spiked with 15 µl of one ppm stock solution of DMB for a final internal standard concentration of 0.11538 ppm.

Each sample was analyzed, in random order, on LC-MS, including standard curves before and after extract samples. Blank samples were included between each

extract sample to confirm no carryover occurred. A blank spike (0.11538 ppm DMB) was also run before and after the extract samples. Agilent ChemStation software was used to integrate sample peaks and calculate concentrations using the linear equation for the line of best fit for both DMB stock dilution curves. Concentrations were adjusted for matrix effects using detected concentration of DMB internal standards within each sample, as well as adjusted for the sample dilution when the 15 µl spike was added to 120 µl of the sample. Final concentrations were calculated using a molar conversion to account for the difference in molecular weight between DMB and the *Ah* toxin. It was reported as ng of *Ah* toxin per g of dried plant material.

***Statistical Analysis*.—**A Shapiro-Wilk test was used to determine if the observations met normality assumptions. Because normality was not found, a Wilcoxan rank sum nonparametric test for medians was conducted. A linear model was used to determine whether the relationship between *Ah* colony density and *Ah* toxicity differed between the plant treatments at the end of the experiment.

RESULTS

The visual estimates of average percent coverage by *Ah* colonies growing on *Hydrilla* ranged from 56% on the first sampling date in early October to 28% on the final sampling date in November. Average percent coverage on *Najas* was 58% on samples collected in the initial October sampling date and 17% by the final date in late November. (Figure 4.1). *Hydrilla*/*Ah* confirmed toxin levels were low during the first sampling date in early October, higher (60.8 ng/g of plant) by the end of October and highest on final sampling date at the end of November (70 ng/g). *Najas*/*Ah* toxin concentration was also low in early October but the highest levels measured where in mid-November (60.1

ng/g). *Najas*/*Ah* toxin production was low during the final late November sampling (14.9 ng/g). (Figure 4.2). There was a significant difference of the *Ah* toxin concentration levels between the *Hydrilla* (n = 5) and *Najas* (n = 5) samples over the 5 dates tested (P = 0.002). Also, percent of *Ah* colony density did not predict *Ah* toxicity levels over the 5 dates (P = 0.91) (Figure 4.3)

DISCUSSION

These results indicate the novel neurotoxin was produced by the *Ah* colonies growing on *Najas* at significant levels similar to those produced by *Ah* from *Hydrilla* on dates tested in October and November 2017. *Najas* may be senescing earlier than *Hydrilla* and this might increase toxicity levels in mid-November. Also, other parameters that might influence increased *Ah* toxin production on aquatic macrophytes may include water temperature, light, and nutrient and pH changes. Optimal growth of *Ah* cultures isolated from Clarks Hill/J. Strom Thurmond reservoir were at temperatures of < 10º C reduced nutrient levels (10 % BG11) and low light conditions of < 10 µmol photons $m^{-2}s^{-1}$ (Wilde et al. 2014). Unfortunately, growth specifications such as nutrient, water temperature, light, and length of day are unknown for *Najas* (Hasandras et al. 2015). It is possible that when these optimal conditions for *Ah* growth are not met on *Hydrilla*, they might be met on other plant sources such as *Najas* which could be a reason for the different toxin levels documented in these *Hydrilla* and *Najas* samples. Conditions may not be optimal for *Najas* to persist during other times it was tested in this study.

Ah colony density did not predict toxicity levels. During the collection dates, it was difficult to find *Najas*. *Najas* was less abundant and not as easy to locate as compared to *Hydrilla* in Clarks Hill/J. Strom Thurmondr. When *Hydrilla* establishes and

becomes highly abundant in aquatic environments, it can increase the alkalinity of water, eventually preventing native species from growing (California Department of Food and Agriculture. The threat of *Hydrilla*. Available from https://www.cdfa.ca.gov/plant/ipc/hydrilla/pdfs/why_hydrilla_is_bad.pdf [Accessed 20 June 2018]). If alkalinity in *Hydrilla* and surrounding water increases, *Ah* may produce toxin because of cell changes (Aniszewski et al. 2007). These alkalinity changes in the surrounding water column may allow *Najas* to become more alkaline and *Ah* to produce more toxin as documented in the 16 November 2017 sample.

Another reason for lower toxin concentrations in some *Najas* samples may be due to the invasive structural and functional qualities of some non-native aquatic macrophyte species. For instance, *Hydrilla* spreads rapidly throughout an aquatic environment and outcompetes native aquatic macrophytes by its growth habits of thick, floating surface mats (Aullbach-Smith et al. 1996). These dense mats of *Hydrilla* may support increased *Ah* growth which may increase toxin production. Also, the toxin itself may prevent other cyanobacterial species from growing (Wilde et al. 2014).

Dangerous levels of cyanotoxin may not be found on *Najas* at other times of the year due to the structure of the plant. In a previous study, *Najas* collected in September, and tested on tadpoles, resulted in no significant mortality, neurologic signs of impairment, or myelinopathy (Maerz et al. In press). Also, *Hydrilla* may provide more surface area for *Ah* than *Najas* (Wilde et al. 2005).

Study replication was difficult because *Najas* was not abundant or located easily. Without replication, results are not statistically powerful. For future studies, more intensive sampling of native aquatic macrophyte species over a longer period of time

would be informative. To really understand whether native SAV pose as great a threat to wildlife as *Hydrilla*, in terms of *Ah* toxicity, it is important to know what conditions are required for *Ah* toxin production (The United States Environmental Protection Agency. 2014. Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems. https://www.epa.gov/sites/production/files/201408/documents/cyanobacteria_factsheet.pdf [Accessed 24 June 2018]).

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Table 4.1. Submerged aquatic vegetation (SAV) species sampled, SAV locations, SAV collection date, initial dry biomass, methanol solvent volume, and extract dry weight.

Species Sample	Location	Collection Date	Biomass (g)	Methanol (ml)	Evap. (g)
H. verticillata	CCR	10-12-17	3.0	150	0.0474
N. guadalupensis	CCR	10-12-17	3.0	150	0.1389
H. verticillata	CCR-PLC	10-26-17	3.0	150	0.0996
N.guadalupensis	CCR-PLC	10-26-17	3.0	150	0.1768
H.verticillata	CCR-PLC	11-8-17	3.0	150	0.1923
N. guadalupensis	CCR-PLC	11-8-17	3.0	150	0.1899
H. verticillata	CCR-PLC	11-16-17	3.0	150	0.1187
N. guadalupensis	CCR-PLC	11-16-17	3.0	150	0.1228
H. verticillata	CCR-PLC	11-30-17	3.0	150	0.1080
N. guadalupensis	CCR-PLC	11-30-17	3.0	150	0.1175

Table 4.2. Results of linear model of *Ah* toxicity and *Ah* colony density as a function of the treatments (*Hydrilla* and *Najas*). Estimates of coefficients, standard error, t statistic (t value), and significance levels (Pr(>|t|) for the predicted value of the *Hydrilla* treatment (Intercept), *Najas* treatment (naiad) and colony (colony density).

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	48.63975	27.45557	1.772	0.120
naiad	-21.03332	15.71412	-1.338	0.223
colony density	-0.06376	0.56177	-0.113	0.913

Figure 4.1. *Aetokthonos hydrillicola* (*Ah)* microscopic cyanobacterial percent coverage estimation for the Fall 2017 sampling dates.

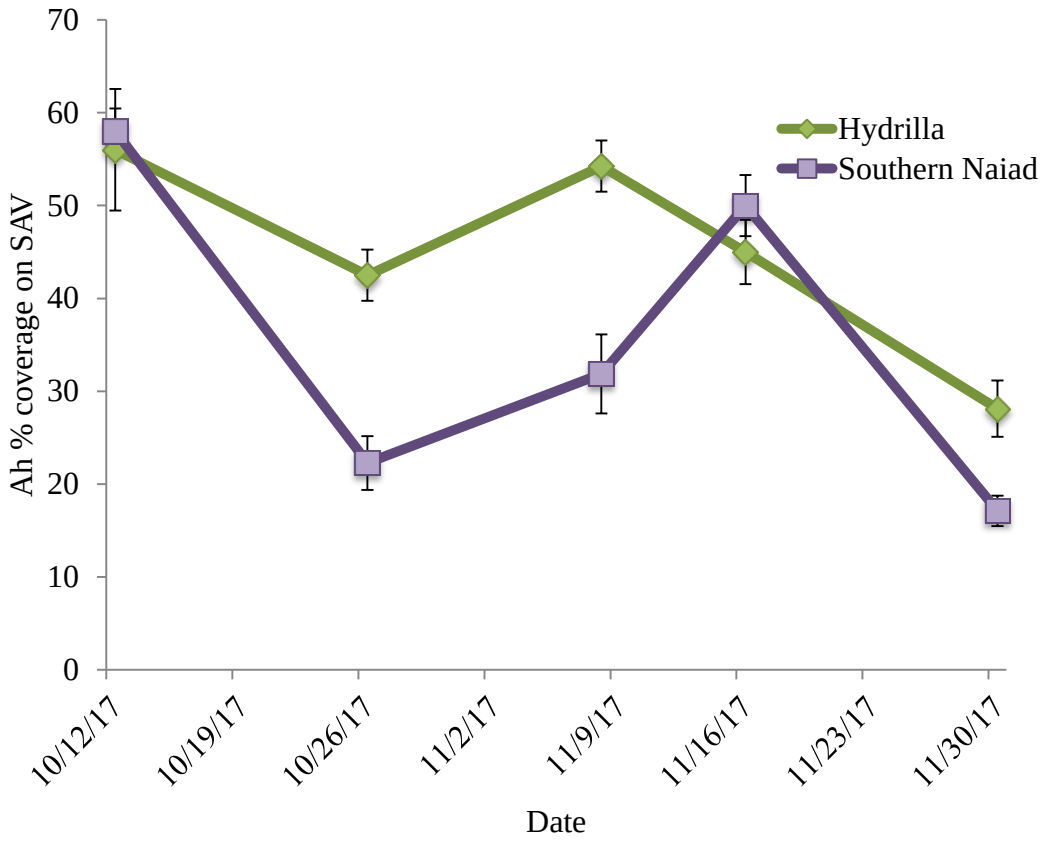


Figure 4.2. *Aetokthonos hydrillicola* (*Ah)* cyanotoxin concentration in nanograms per gram of *Hydrilla* (*H. verticillata*) and southern naiad (*N. guadalupensis*) biomass from various collection dates in October and November 2017. Study site information available from http://www.sas.usace.army.mil/About/Divisions-and-Offices/Operations-Division/J-Strom-Thurmond-Dam-and-Lake/)/ [Accessed 11 June 2018]) (Table 4.1) (Figure 4.1).

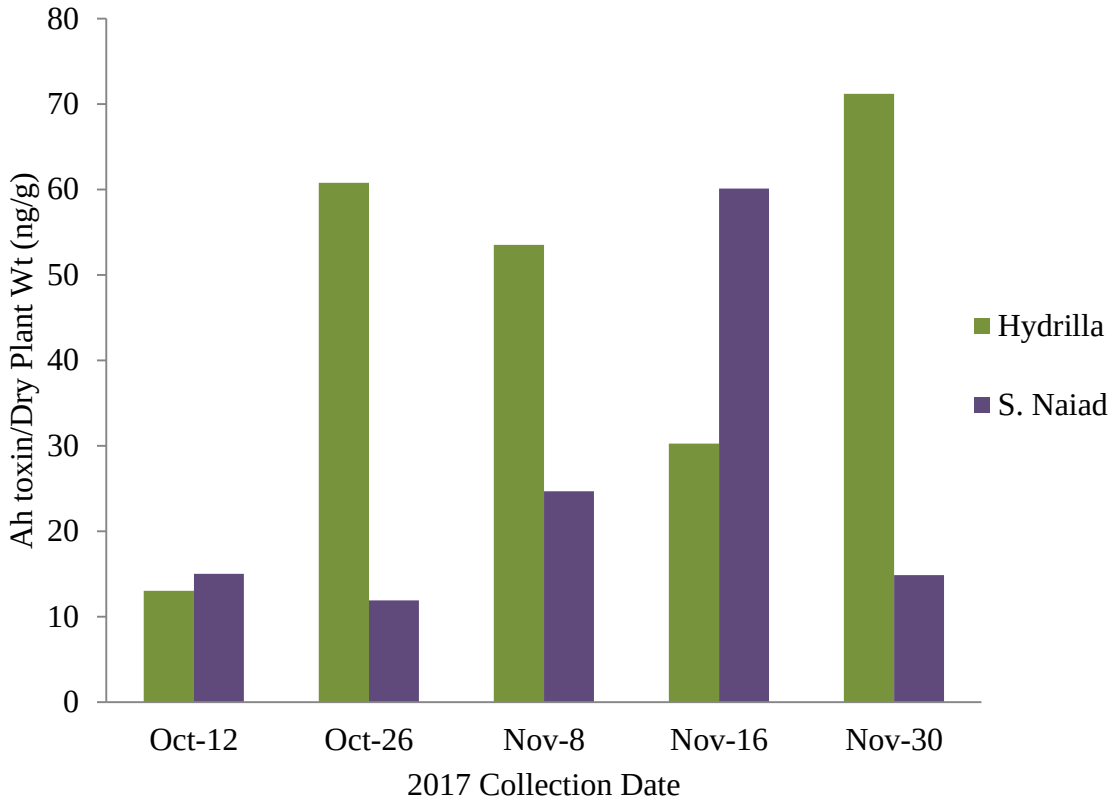
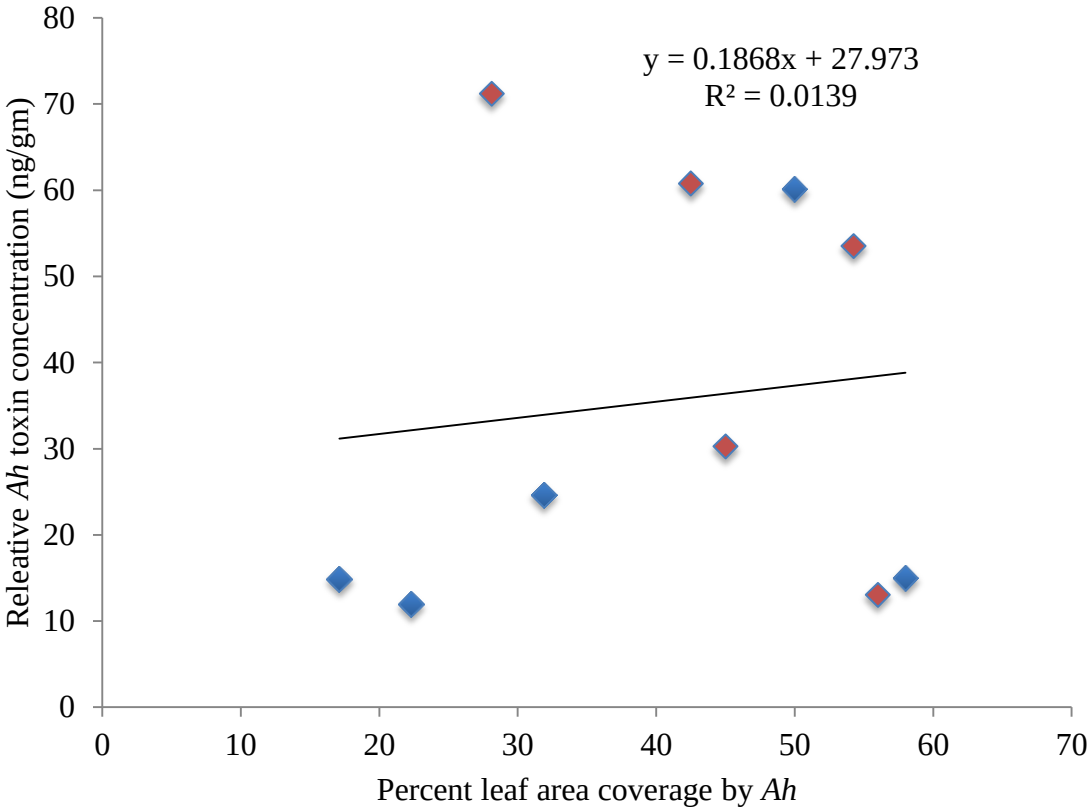


Figure 4.3. *Aetokthonos hydrillicola* (*Ah)* microscopic cyanobacterial percent coverage vs toxin concentration over the Fall 2017 sampling dates.



CHAPTER 5

CONCLUSIONS

This research project confirms that reptiles and amphibians could be at risk if consuming prey that had consumed the novel neurotoxin produced by *Aetokthonos hydrillicola* growing on invasive *Hydrilla verticillata*. Additionally, significant toxin levels were confirmed in an extract from a native submerged aquatic plant (*Najas guadalupensis*), which expands the geographic extent of potential disease locations.

Salamanders.—The results of the salamander feeding trial provides compelling evidence that aquatic salamanders are sensitive to *Ah* toxin and can be exposed via trophic transfer from their prey. Most notably, several salamanders fed *Ah*-positive prey developed impaired righting responses and overall salamanders fed *Ah*-positive prey weighed less per body length at the end of the six week trial. *Ah*-positive individuals were also more likely to be inactive throughout the 6 week feeding trial than *Ah*-negative individuals.

Overall, our results are consistent with other studies that report higher rates of abnormal behavior and neurological impairment among animals that had ingested *Ah*-positive plant material, directly or indirectly (Fischer et al. 2003; Wilde et al. 2005; Mercurio et al. 2014; Dodd et al. 2016; Maerz et al. In press; Martin Chapter 3). The failure to detect behavioral differences among all individuals within the *Ah*-positive treatment is also consistent with other studies. Not all individuals with intramyelinated vacuoles exhibit clinical signs of neurologic impairment after feeding on *Ah*-positive

Hydrilla (Rocke et al. 2002; Lewis-Weiss et al. 2004; Wiley et al. 2008). For example, a study conducted on mallards and chickens resulted in few behavioral signs of neurological impairment even though > 50 % of the *Ah*-positive birds had characteristic intramyelinated vacuoles consistent with *Ah* toxicity under histological assessment (Birrenkott et al. 2004; Haney et al. 2013; Dodd et al. 2016). One reason why individual animals may vary in the presentation of clinical symptoms or histopathology is that doses or concentrations of the etiologic agent (*Ah* toxin) for all feeding trials, is variable. Animal appetites may vary, and because we are feeding prey plant material from the field, there is likely variation in toxin concentration among the small samples of *Ah*-positive *H. verticillata* provided to each individual.

Vacuoles suggestive of *Ah* toxicity were found primarily in the cervical spinal cord of salamanders. This contrasts with a prevalence of vacuoles within the optic tectum of birds and fish. In addition to vacuolation, small foci of hemorrhage, an ante mortem change, were present in the brainstems and spinal cords of several salamanders. This may reflect differences of neural functions in amphibians relative to birds and fish. A smaller optic tectum in a salamander may indicate less myelin formation in that region (Butler et al. 2006). Myelin is a coating made of lipid-rich proteins and phospholipids insulating the axons of the central nervous system (CNS) and keeping neurons transmitting and responding to nerve impulses. In CNS regions where there is little myelin, we would expect few vacuole development. This means that interspecies comparisons will need to focus on a range of CNS tissues to account for differences in CNS anatomy when evaluating the relative sensitivity of different species to *Ah* toxin.

Although changes in some animals may reflect dilated axon sheaths and intramyelinic edema, similar histologic changes can be a relatively common post mortem artifact. Some indistinguishable pathologies were observed in both *Ah*-negative and *Ah*-positive animals that could be a result of processing artifacts. Such processing or observation error could be exacerbated by small sample sizes, such as was the case in this study, and the inability to control dosing of the toxic compound to individual animals. Nonetheless, the observed differences between salamanders in the treatments were sufficient to suggest sensitivity to trophic transfer of *Ah*-positive materials, and at a minimum warrant additional study.

We do not know whether – in the field – the greater risk from trophic transfer is facilitated simply by prey transferring toxic plant material to the predator, or whether the toxin can bioaccumulate within prey. In this study, the short amount of time for prey to feed on *Ah*-positive plant material means that predatory salamanders were vulnerable to toxin exposure simply by transfer of toxic plant material through prey. There was simply not sufficient time for prey to accumulate toxin in prey tissues. Moreover, lab studies suggest high, rapid mortality among fish, tadpoles and invertebrates when feeding on highly toxic *Ah*-positive *H. verticillata* (Maerz et al. In press; Haram 2016; Martin et al. unpublished obs.). In the field, bioaccumulation of toxin – particularly in more resilient prey or when toxin concentrations are sublethal among prey – may be a more important exposure route for predatory species. Moreover, larger, more resilient predatory species may themselves accumulate the toxin, creating increased exposure risks to predatory taxa farther up the food chain. We emphasize that-at this time – there are no documented deaths of amphibians, reptiles, or fish in the field. Therefore we can only consider the risk

of *Ah* to these taxa as hypothetical. However, we also note that detecting deaths of these species would be extremely low and there is a reasonable probability that neurologically impaired animals would be preyed upon; therefore, the absence of observed deaths is also not evidence that *Ah* invasion poses no risk to amphibians or other more secretive wildlife.

Watersnakes.—The results of this study provide some evidence that watersnakes (*Nerodia* spp.) are sensitive to *Ah* toxin and can be exposed via trophic transfer from herbivorous fish that feed on *H verticillata* or other macrophytes that host *Ah*. Though we did not observe clinical signs of impairment of righting responses, tongue flick rates towards prey, or aversion to repulsive chemical cues, we did see clear evidence of progressive anorexia among snakes fed *Ah*-positive prey and evidence of disease in the CNS consistent with exposure to *Ah* toxin. These two observations are consistent with studies of other wildlife, most notably a recent study on aquatic turtles (Mercurio et al. 2014). Some painted turtles fed *Ah*-positive *H. verticillata* developed mild and inconsistent ataxia after 96 days of feeding on *Ah*-positive *H. verticillata*. More notably, turtles fed *Ah*-positive *H. verticillata* showed progressive reductions in feeding rate compared to turtles fed *Ah*-negative *H. verticillata*, and all turtles fed *Ah*-positive *H.verticillata* had disease in brain tissues. It is notable that signs of impairment were mild even after 93 days in snakes (this study) and 96 days in turtles (Mercurio et al. 2014) and there was no mortality in either study, but in birds, neurological impairment from exposure to *Ah* toxin can manifest in under 5 days (Rocke, et al 2002, Haram, 2016), and among amphibian tadpoles, measurable effects of *Ah* toxin on mortality have appeared in

as short as 3 days (Maerz et al. In press). This illustrates that either exposure dosage or sensitivity to *Ah* toxin can vary greatly among aquatic fauna.

It is important that we address the potential for confounding effects of our treatments because our source plant materials each came from one, separate lake. In addition to the presence or absence of *Ah*, there are potentially many other differences between *H. verticillata* collected at the two sites including differences in nutritional quality, metal concentrations, presence of microbes, and the presence of anthropogenic pollutants. We cannot rule out other confounding differences between plant sources in our treatments; however, our results in the context of our other research make it most likely that the effects we observed are related to *Ah* toxin presence and not to other factors. First, there have been numerous other feeding trials of wildlife using *Ah*-positive or *Ah*-negative *H. verticillata* from other lakes besides our two source lakes. More than 40 lakes have been surveyed and source *H. verticillata* tested on wildlife to date. Water quality and microbiota differ considerably among those 40 reservoirs. Among the 21 lakes where *Ah*-positive *H. verticillata* was collected, all have been shown to induce impairment in some species of wildlife. Among the 15 *Ah*-negative lakes where *H. verticillata* was collected, *H. verticillata* has never induced impairment, disease, or death in any wildlife. Therefore, among all prior studies from using *H. verticillata* from many source lakes that vary in many aspects of water quality, only *Ah*-positive *H. verticillata* has ever induced neurological impairment, disease within the CNS, and death among wildlife (Fisher, et al. 2003; Birrenkott, et al. 2004; Lewis-Weiss et al. 2004; Wiley et al. 2007; Haynie 2008; Mercurio et al. 2014; Wilde, et al. 2014; Maerz et al. In press; Chapter 2). Second, *Ah*-positive *H. verticillata* from our source lake has been collected

during the summer – when *Ah* appear not to produce toxin – and fed to tadpoles, and did not cause any impairment or mortality. Therefore, at other times of the year, *Ah*-positive *H. verticillata* is suitable forage for amphibians and other wildlife. If something other than *Ah* was responsible for toxicity, it would have to be a factor that also varies seasonally but is currently latent.

In the spinal cords of snakes that ingested the *Ah*-positive treatment indirectly via prey sources, results demonstrate microscopic changes that were suggestive of, but not definitive for pathology associated with *Ah* exposure among other vertebrates. Bald eagles that had consumed American coots that fed on *Ah*-positive treatment had lesion development in the cerebellum, cerebrum, brain stem, spinal cord, and concentrating in the optic tectum which includes vacuolation at all levels of the brain and in the spinal cord. Additionally, small hemorrhages have been reported in the thalamus and brainstem of affected eagles (Thomas et al. 1998). Ultrastructurally, histopathology of *Ah*-exposed animals is characterized by damage to lipid-rich myelin sheaths that surround and insulate nerve axons primarily in the CNS. Specifically, splitting of the myelin lamellae occurs at the intraperiod line, a change consistent with intramyelinic edema (Fisher et al. 2003).

This research suggests watersnake vulnerability to the *Ah* cyanotoxin in another experimental feeding trial in a lab setting. Although researchers do not know the dosage threshold causing lesions or neurologic signs of impairment (Rocke et al. 2002), if *N. fasciata* and *N. sipedon* are exposed to the *Ah* cyanotoxin in their natural environment, they will be susceptible to the effects of *Ah* toxicity through consumption of prey.

Native and invasive SAV/Ah toxin levels.—These results indicate *Ah* did produce the *Ah* cyanotoxin at significant levels when growing on *Najas guadalupensis* harvested in mid-November comparable to toxin concentration detected in *Hydrilla/Ah* extracts in late fall 2017. *N. guadalupensis* generally senesces earlier than *H. verticillata* in these southeastern ponds and reservoirs which could promote toxin production in the cyanobacterium. Additional potential environmental conditions predicted to promote toxin production in *Ah* include water temperature, light, and nutrient and pH changes. Optimal growth for *Ah* cultures isolated from Clarks Hill/J. Strom Thurmond reservoir were at temperatures of < 10º C with reduced nutrient levels (10 % BG11 media) and light condition of < 10 µmol photons $m^{-2}s^{-1}$ (Wilde et al. 2014).

Ah colony density did not predict toxicity levels. During the collection dates, it was difficult to find *N. guadalupensis*. *N. guadalupensis* was less abundant and not as easy to locate as compared to *H. verticillata* in Clarks Hill/J. Strom Thurmond reservoir. When *H. verticillata* establishes and becomes highly abundant in aquatic environments, it can increase the alkalinity of water, eventually preventing native species from growing (California Department of Food and Agriculture. The threat of *Hydrilla*. Available from https://www.cdfa.ca.gov/plant/ipc/hydrilla/pdfs/why_hydrilla_is_bad.pdf [Accessed 20 June 2018]). Increases in *Ah* toxicity could be linked to increases in alkalinity levels within *H. verticillata* beds and surrounding water (Aniszewski et al. 2007). These alkalinity changes in the *H. verticillata* beds may allow *N. guadalupensis* to become more alkaline and *Ah* to produce more toxin as documented in the 16 November 2017 sample.

Another reason for lower toxin concentrations in some *N. guadalupensis* samples may be due to the invasive structural and functional qualities of some non-native aquatic macrophyte species. For instance, *H. verticillata* spreads rapidly throughout an aquatic environment and outcompetes native aquatic macrophytes by its growth habits of thick, floating surface mats (Aullbach-Smith et al. 1996). These dense mats of *H. verticillata* may support increased *Ah* growth allowing for increased toxin production and *H. verticillata* may provide more surface area for *Ah* than *N. guadalupensis* (Wilde et al. 2005). Also, the toxin itself may have allelopathic effects that prevent other cyanobacterial species from growing (Wilde et al. 2014).

Dangerous levels of cyanotoxin may not be found on *N. guadalupensis* during summer and early fall. In a previous study, *N. guadalupensis* collected in September, and tested on tadpoles, resulted in no significant mortality, neurologic signs of impairment, or myelinopathy (Maerz et al. In press).

Study replication was difficult because *N. guadalupensis* was not abundant or located easily. Without replication, results are not statistically powerful. For future studies, more intensive sampling of native aquatic macrophyte species over a longer period of time would be informative. To really understand whether native SAV pose as great a threat to wildlife as *H. verticillata,* in terms of *Ah* toxicity, it is important to know what conditions are required for *Ah* toxin production (The United States Environmental Protection Agency. 2014. Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems. https://www.epa.gov/sites/production/files/201408/documents/cyanobacteria_factsheet.pdf [Accessed 24 June 2018]).

Limitations.—Nile tilapia was the preferred fish to use for the watersnake feeding trial because there was a viable source at ABEL. Unfortunately, the female breeders didn't survive, and it took time to establish a new population. Guppies and fathead minnows were available and used as alternatives when tilapia were too small and/or numbers too low to accommodate the feeding trial. Because all 3 of these species were varying sizes, we used average mass of fish consumed in our linear model for appetite to account for snakes not consuming the same amount of prey each day.

A swim chamber behavioral assessment was conducted on the watersnakes, but was not included in this thesis. We had several issues with this method of evaluating the snake's swimming performance. The swim chamber is an instrument that was created to assess fish movement. For this reason size of the chamber and water levels were limiting factors. We had to use a subsample of our snakes because 3 would not fit the chamber. Also, water levels in the chamber were difficult to judge, and snakes could easily brace themselves to the sides and back of the chamber without needing to swim.

The two tongue flicking stimuli and righting response assessments were not consistently evaluated before the feeding trial began. We did not evaluate tongue flicks with stimuli on day "0" which would have offered baseline data for comparison purposes to data that was acquired during the feeding trial. Also, it was difficult to time the righting responses. Most of the responses happened within 1 second and videotaping initial responses on day "0" would have been beneficial to have conducted.

Because histopathology is such a specialized field and practice, finding certified experts in processing and analyzing snake and salamander brain tissues is difficult. It is also an expensive and time consuming service. The way snake and salamander brain

tissues were processed was not ideal. Tissues went missing and many had artifactual changes that may have been prevented had we been able to consider the situation based on the optimum outcome.

Larger sample sizes would have been preferred with the snake and salamander studies, but wild animals can be difficult to find if they are not widely distributed and abundant. If we were to collect, for instance, several gravid watersnakes each from a different location and raise the young, each gravid female could be the block to our design which would add more power to the results.

If we wanted to redo the chapter 4 study, we would reconsider several elements to achieve stronger outcomes. Time, funds, access to areas, and availability of macrophytes limited sites and locations sampled. It was challenging to find abundant communities of native macrophytes for this study. They were not as readily available and abundant as *H. verticillata*. We suggest intensive sampling efforts targeting macrophytes at multiple lakes and reservoirs to create a larger sample size to replicate our efforts through time making a more robust statistical design. Future directions in research should focus on quantifying other native macrophyte species and comparing their toxicity to *H. verticillata* during more times and seasons.