



# Effects of developmental exposure to neurotoxic algal metabolites on predator-prey interactions in larval *Pimephales promelas*

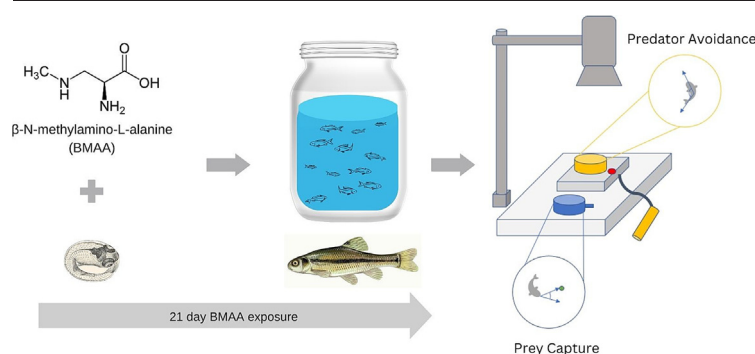
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## HIGHLIGHTS

- $\beta$ -N-methylamino-L-alanine exposure in early development affects Fathead Minnow behavior.
- Predator avoidance escape time is longer in  $\beta$ -N-methylamino-L-alanine exposed fish.
- $\beta$ -N-methylamino-L-alanine exposure slows recognition of vibrational threats in larva.
- $\beta$ -N-methylamino-L-alanine did not strongly impact larval prey-tracking ability.
- Cyanotoxins alter stimulus detection and locomotor performance in Fathead Minnows.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Harmful algal blooms are a growing environmental concern in aquatic systems. Although it is known that some of the secondary metabolites produced by cyanobacteria can alter predator-prey dynamics in aquatic communities by reducing foraging and/or predator evasion success, the mechanisms underpinning such responses are largely unknown. In this study, we examined the effects of a potent algal neurotoxin,  $\beta$ -N-methylamino-L-alanine (BMAA), on the development and behavior of larval Fathead Minnows, *Pimephales promelas*, during predator-prey interactions. We exposed eggs and larvae to environmentally relevant concentrations of BMAA for 21 days, then tested subjects in prey-capture and predator-evasion assays designed to isolate the effects of exposure at sequential points of the stimulus-response pathway. Exposure was associated with changes in the ability of larvae to detect and respond to environmental stimuli (i.e., a live prey item and a simulated vibrational predator), as well as changes in behavior and locomotor performance during the response. Our findings suggest that chronic exposure to neurodegenerative cyanotoxins could alter the outcomes of predator-prey interactions in natural systems by impairing an animal's ability to perceive, process, and respond to relevant biotic stimuli.

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## 1. Introduction

Natural environments are subject to the adverse impacts of human activity, which can have prominent effects on local wildlife (Green and Higginbottom, 2000; Sondergaard and Jeppesen, 2007; Dubois et al., 2017; Hader et al., 2020). In aquatic communities, one pressing ecological problem is the presence of contaminants of emerging concern (CECs; Sauve and Desrosiers, 2014; Nawaz and Sengupta, 2019; Nilsen et al., 2019; Deere et al., 2020; Kroon et al., 2020). Exposure to a variety of CECs has well-documented effects on the physiology, reproduction, and survival of affected organisms (Faucher et al., 2006; Rohr et al., 2006; Weber, 2006; Blaha et al., 2009; Painter et al., 2009; Vasconcelos et al., 2010; Sih et al., 2011; Olson et al., 2018). However, fewer studies have examined how sub-lethal changes in behavior might alter the outcomes of intraspecific and interspecific interactions and shift community dynamics (Relyea and Edwards, 2010; Barbieri et al., 2013; Pan et al., 2017; Saaristo et al., 2018; Sharma, 2019). Consequently, significant gaps exist in our ability to accurately predict the long-term impacts of exposure in natural ecosystems.

Cyanotoxic metabolites associated with harmful algal blooms (HABs) are naturally occurring CECs and are ubiquitous in freshwater systems in the United States and abroad (Blaha et al., 2009; Al-Sammak et al., 2014; Nawaz and Sengupta, 2019). Exposure to some of these metabolites has been linked to alterations in the architecture of the central nervous system (CNS) and disruptions in neurofunction (Cox et al., 2005; Berry et al., 2007; Blaha et al., 2009; Al-Sammak et al., 2014). Because behaviors are regulated through the CNS and proper neuronal function is essential for organismal responses to ecological stimuli, such neurodevelopmental disturbances have the potential to alter the behavior of individuals in important fitness-bearing contexts. In turn, this could reduce larval recruitment into the population or accelerate rates of contaminant transfer through the food chain via increased predation risk (Vasconcelos et al., 2010; Bianco et al., 2011; Bhandari et al., 2015; Bambino and Chu, 2017; Roegner et al., 2019).

Success in two contexts critical to the fitness of fish living in HAB-affected waters – i.e., capturing prey and evading predators – have the potential to be impacted as a consequence of cyanobacteria-induced disruption in neurofunction. During prey-tracking, the fish approaches the prey and periodically adjusts its trajectory to maintain alignment (Lauder, 1980; Bianco et al., 2011; Muto and Kawakami, 2013; Day et al., 2015; Jouary et al., 2016; Thompson et al., 2018). Because fish rely on tightly controlled sensorimotor integration processes during hunting events (Kane and Higham, 2011; Trivedi and Bollmann, 2013; Westphal and O'Malley, 2013; Mearns et al., 2019), foraging success in altered environments could be affected by (i) changes in sensory processes that influence the ability to detect and process cues from prey (Ashur et al., 2017; Draper and Weissburg, 2019); (ii) factors related to locomotor performance, such as the maneuverability, stability, and accuracy of approach (Higham, 2007); or (iii) both.

Contaminants could similarly affect an individual's ability to recognize and respond to potential predators (Faucher et al., 2006; Weber, 2006; Painter et al., 2009; Olson et al., 2018). In larval fish, predator-avoidance behavior is primarily controlled by the Mauthner cells (M-cells), which receive sensory information from the environment and generate a directional, evasive motor response (C-start; Painter et al., 2009; Hitchcock et al., 2015). Exposure to toxicants may impair an individual's ability to detect and recognize a potential threat (Painter et al., 2009; Cripps et al., 2011; Rearick et al., 2018; Saaristo et al., 2018; Sievers et al., 2018), or respond appropriately; for example, via changes in the structure of the M-cells (Shan et al., 2015).

In this study, we used lab-controlled empirical experiments to evaluate the effects of exposure to cyanobacterial metabolites on stimulus detection and locomotor performance in a common freshwater fish, the Fathead Minnow (*Pimephales promelas*) during predator-prey interactions. *Pimephales promelas* are a widespread freshwater fish inhabiting rivers, streams, and lakes throughout North America (CABI, 2012) and are a model species for understanding the implications of environmental change on predator-

prey interactions in freshwater aquatic systems (Ankley and Villeneuve, 2006; Painter et al., 2009; Armstrong et al., 2012; Holbech et al., 2012; Ward et al., 2017). As a species typically found at lower trophic levels, *P. promelas* are an ideal model for this study because they act as both predators and prey; they consume algae, crustaceans, protozoa, and zooplankton, and are predated upon by several game fish species, including *Esox Lucius*, *Salvelinus namaycush*, *Sander vitreus*, and *Perca flavescens* (CABI, 2012).

The central hypothesis was that early developmental exposure to a representative neurodegenerative cyanotoxin alters the dynamics of predator-prey interactions in aquatic communities through impaired behavioral function. To test this hypothesis, we used high-speed video to record hunting sequences and anti-predator responses of freely moving larval minnows reared under control conditions and reared in the presence of a common blue-green algal neurotoxin,  $\beta$ -N-methylamino-L-alanine (BMAA).  $\beta$ -N-methylamino-L-alanine has recently become a CEC of substantial research interest. It is a non-protein amino acid that affects organisms in a concentration-dependent manner and exhibits a neuroexcitatory effect leading to nerve cell death (Spasic et al., 2018). In fish, exposure to BMAA decreases endurance, increases fatigability, produces abnormal spinal axis formation and convulsions, and increases mortality (Purdie et al., 2009; Sher, 2017). Further, BMAA has been detected in the tissues of aquatic organisms at different trophic levels and shows strong evidence of bioaccumulation (Lage et al., 2015; Rogers et al., 2018; Spasic et al., 2018).

Our main objectives were to evaluate the effect of BMAA on prey-capture and predator-escape behaviors. Additionally, our goal was to determine whether the stimulus detection and/or locomotor performance components of the response were similarly affected across both contexts.

## 2. Methods

### 2.1. Experimental design

We conducted two experiments to evaluate and compare the effects of early developmental exposure to BMAA on two innate behaviors: prey strikes and anti-predator C-start escape responses. Fish larvae typically utilize a combination of several locomotor behaviors to accomplish ecologically relevant goals. Compared to free-swimming, where fish exhibit nonspecific bouts of movement, stimulus-response pathways, such as those that characterize hunting and escape behaviors, typically take the form of specialized bouts of movement utilizing context-typical combinations and sequences of behavior (Marques et al., 2017). Both prey strike and predator avoidance responses require the organism to detect a stimulus in the environment [prey/predator], orient the body [to/from], and direct the body [approach/escape] by sequentially updating the relative position of the body to the stimulus. In experiment 1, we examined the behavior and kinematics of 21-day-old larval fish during hunting events. In experiment 2, we examined the response of larval minnows to a simulated predator (Ward et al., 2017; Rearick et al., 2018). All procedures were approved by the Institutional Animal Care and Use Committee at Ball State University (1142896-1).

#### 2.1.1. Subjects, housing, and maintenance

Six-month old *P. promelas* were purchased from a culturing facility (Environmental Consulting and Testing; WI, USA) and sent to Ball State University. Breeding groups consisting of two females and one male were housed in 6-L tanks in a continuous flow-through system (Aquaneering, CA, USA). Each tank contained a PVC spawning tile. All fish used in the experiment were maintained for the duration of the experiment under a 16 h: 8 h light-dark regime at room temperature (mean  $\pm$  SD: 20.6  $^{\circ}$ C  $\pm$  0.86  $^{\circ}$ C). Clutches were removed from the tanks on the day they were laid and randomly assigned to the control or a treatment group. All fish were fed live prey (*Artemia franciscana*; Brine Shrimp Direct, UT, USA) twice daily during the exposure period. However, larvae were not fed for 18 h prior to hunting trials to ensure motivation to forage.

### 2.1.2. Treatments

Powdered BMAA ( $\beta$ -N-methylamino-L-alanine; Sigma Aldrich, Inc., Germany) was dissolved in ultra-pure water (Millipore, MA, USA) and then serially diluted to create concentrated stock solutions. Stock solutions were made on a weekly basis and stored in amber glass bottles at 4 °C. Fresh aqueous solutions were prepared each day via the addition of the appropriate solution of aged, aerated water to create a control (0 ng/L; BMAA<sub>C</sub>) and treatments with nominal concentrations of 5, 25, 125, 625 ng/L BMAA (hereafter referred to as BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub>, respectively). Concentrations of BMAA in waterways have been reported from 110 ng/L up to 25 µg/L (Al-Sammak et al., 2014; Wiltsie et al., 2018; Vo Duy et al., 2019).

Clutches were maintained during the 21-day early developmental treatment period in 750-mL glass vessels fitted with an air stone. The water was exchanged each day with freshly treated medium via a 50 % daily static renewal protocol to account for chemical degradation (United States Environmental Protection Agency, 2002). The fish were monitored daily for mortality, and temperature and total dissolved solids were measured via a hand-held water quality tester (Ubante, Model: YL-TDS2-A). Additional water quality measurements were conducted weekly (pH, conductivity, nitrate, nitrite, free chlorine, total chlorine, hardness, alkalinity; Hach Test Strips, CO, USA; Ubante). Concentrations of stock and aqueous solutions were measured at the start of the experiment via liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, water samples were taken from exposed vessels and frozen in 1 L high-density polyethylene (HDPE) containers for later LC-MS/MS analysis (Indiana State Department of Health) throughout the exposure period.

### 2.1.3. LC-MS/MS analysis

A standard curve from 1 to 1000 µg/L, method blank, laboratory fortified sample, and study water samples were extracted after the addition of 0.3 mL of concentrated HCl on a vacuum manifold with Waters Oasis MCX cartridges. Cartridges were conditioned with 5 mL of methanol followed by 5 mL DI water. Samples were pulled through the cartridges using a vacuum line then eluted into 15-mL glass centrifuge tubes using 10 mL of a mixture of 97 mL methanol and 3 mL ammonium hydroxide. Extracts were blown down to dryness with nitrogen on a TurboVap at 50 °C. Extracts were reconstituted in mobile phase consisting of 63 % acetonitrile, 37 % DI water, and 0.1 % formic acid. All samples were brought to a final volume of 1 mL.

The LC-MS/MS method was modified from Faassen et al. (2012). A Sciex QTRAP 6500+ tandem liquid chromatograph mass spectrometer was used for analysis. The multiple reaction monitoring transitions used for BMAA were 119/88 quantitative and 119/76 qualitative. The LC method consisted of a mobile phase gradient that started at 63:37:0.1 acetonitrile:water:formic acid, then changed to 55:45:0.1 over the first 3 min, and holding for 2 min before returning to the starting conditions for a total run time of 15 min. Retention time of BMAA is about 4.8 min. The column used was a SeQuant ZIC-HILIC 3.5 µm, 100 Å column with dimensions of 2.1 × 150 mm. The autosampler was cooled to 4 °C to prevent degradation of the samples, and the column compartment was held at 30 °C for the duration of the run.

### 2.1.4. Early developmental endpoints of exposure

Individual clutch size was recorded by counting the number of eggs on the tile on day 1 of the exposure period. Hatching success was estimated by counting the eggs that remained on the tile at the completion of hatching divided by the number of eggs laid. Survival to day 21 was estimated by counting the number of larvae in the jar on the day of larval testing divided by the number of embryos that hatched. In addition, clutches were monitored for duration of development (the number of days from being laid to hatching) and the hatching duration (day from first hatch to the day of last hatch). Growth was quantified by measuring the standard length (SL; in mm) of larvae on the day of experimental testing.

### 2.1.5. Statistical analysis

Preliminary analysis indicated that the data for clutch size, number of days until hatching, hatch success, and survival violated parametric assumptions of

normality. Therefore, among-treatment differences in these variables were tested via non-parametric Kruskal-Wallis tests, followed by Dunn post hoc comparisons. Larval standard length was analyzed via a linear mixed model, with total length as the outcome variable and treatment specified as a predictor. Clutch ID was included as a random factor. For all analyses, an  $\alpha = 0.05$  was set as the level of significance for comparison. Statistical analyses were conducted in R (R Core Team).

## 2.2. Behavioral assays

### 2.2.1. Experiment 1: prey capture

Hunting by larval fish includes several unique behavioral maneuvers that are exclusive to the prey-tracking sequence (Bianco et al., 2011; Fig. 1A). Individuals first detect and recognize a potential prey item. After recognition, the fish establishes eye convergence and orients its body in line with prey via a J-turn (Muto and Kawakami, 2013). J-turns are described as multiple caudal bends at an amplitude >90 deg., approximating a “J” shape (McElligott and O'Malley, 2005). Following the J-turn, the fish approaches the prey, periodically updating its trajectory relative to the prey via swim bouts and tailbeat cycles. When the fish is within striking distance, it sucks the prey into its mouth (Lauder, 1980; Bianco et al., 2011; Day et al., 2015; Thompson et al., 2018).

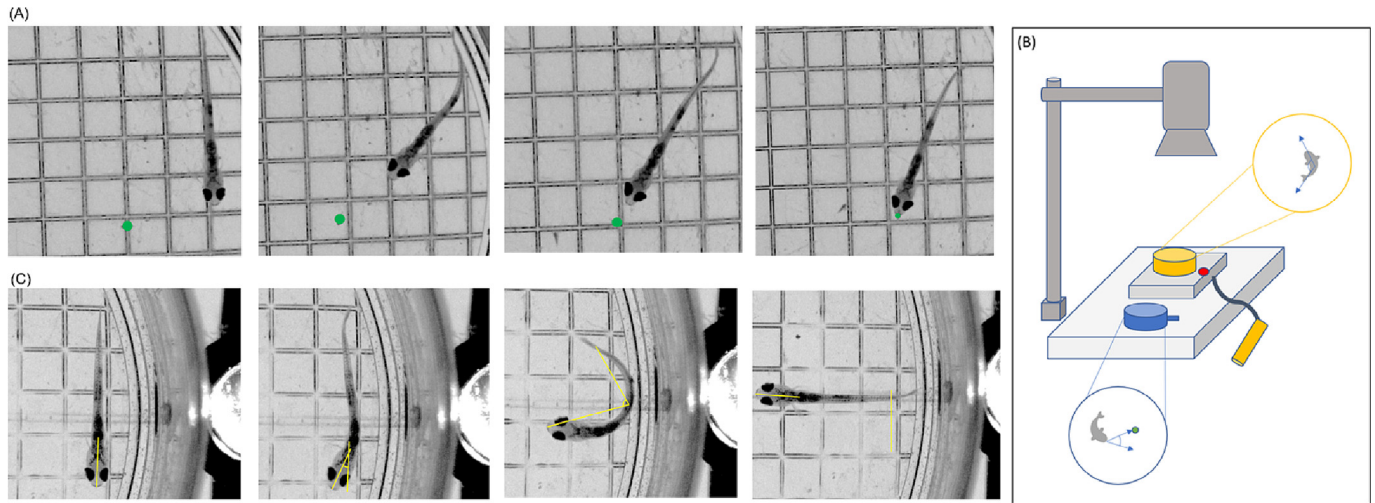
Hunting trials were conducted in a 3.5-cm diameter dish containing 10 mL clean aged water and centered on a no-heat, light-emitting diode (LED) light pad (Tiktek/A4-DWT). Trials were conducted in a darkened room to minimize disturbance. The volume of water in the arena was sufficient to allow unimpeded horizontal movement by the larvae but minimized vertical swimming. A channel built into the wall of the arena allowed prey items to be introduced with minimal disturbance to the fish. At the start of a trial, a focal larva was introduced into the arena via a glass dropper and allowed 60 s to acclimate. Several live *A. franciscana* were then gently injected into the channel using a 2-µL pipette. We recorded full-length prey strikes of individual larvae from the frame immediately prior to the first J-turn made by the fish towards a prey item (i.e., recognition) to successful capture. Prey strikes were recorded at 1000 frames per second (fps) using a high-speed video camera (Phantom VEO-E 340L, Wayne, NJ, USA) fit with a macro lens (ZEISS, Oberkochen, Germany) mounted approximately 30 cm above the arena (Fig. 1B). Each individual was tested once.

Prey strikes were analyzed using Phantom PCC 3.1 software. Each video was calibrated using a 2 × 2 mm grid on the bottom of the arena prior to measurement and the standard length (SL; mm) of the fish was measured from a still frame. To evaluate the effects of exposure on prey detection, we measured the distance of the larva to the prey at the start of the hunting sequence from the anterior midpoint of the head between the eyes. Distances were divided by SL and expressed as a proportion of body length to account for individual variation in size. To evaluate the effects of exposure on prey-tracking performance, we then measured the angle of orientation of the fish to the prey item at 50-frame intervals over the first 1000 frames of the response. We also recorded the number of tracking maneuvers (slow swims and tailbeats) performed by each individual while tracking prey. Last, we quantified the total duration of the hunting event (in ms) from the initial frame of the hunting sequence to the frame in which the prey was successfully consumed.

### 2.2.2. Statistical analysis

Among-treatment differences in the distance of the larva to the prey at the start of the hunting event (scaled by body length) and the duration of the hunting sequence were tested via linear mixed models fit by residual maximum likelihoods and t-tests that used Satterthwaite's method. Clutch ID was included as a random effect. To determine whether focal subjects differed in their prey-tracking precision, we fit a separate linear mixed model with the angle of orientation to the prey specified as the dependent variable, and treatment and time specified as predictors; individual and clutch IDs were included as random effects. Preliminary analysis indicated that the data for the frequencies of tracking maneuvers (slow swims,





**Fig. 1.** Experimental setup and behaviors of *Pinephales promelas*. (A) Initial recognition of the *Artemia franciscana* prey item (green dot), J-turn movement to orient the body, approach trajectory to prey via slow swims, and culmination with successful capture of prey. (B) Experimental camera and arena set up during behavioral tracking assays (orange, predator avoidance; blue, prey capture). (C) Initial detection of the vibrational stimulus, fictive yaw in the first 10 milliseconds, quantification of the deepest body bend, and culmination of the escape trajectory following escape swims. Yellow lines and angles depict angular displacement of the head.

tailbeats) violated parametric assumptions of normality. Therefore, among treatment differences in these variables were tested via non-parametric Kruskal-Wallis tests, followed by Dunn post hoc comparisons.

### 2.2.3. Experiment 2: predator escape

The 'C-start' is an evolutionarily conserved predator-escape behavior exhibited by all teleost fishes (Domenici and Blake, 1997; Hale et al., 2002). The response is primarily controlled by the Mauthner cells (M-cells), which receive sensory information from the environment and generate a directional, evasive motor response (Eaton et al., 1981). When a stimulus is received, fish respond by rapidly contracting the trunk muscles and bending the body into a "C" shape (Hitchcock et al., 2015). Contraction of the muscles generates thrust and causes the head to become displaced relative to its initial orientation in space through the midsagittal plane (i.e., yaw), which alters the trajectory of escape (Hitchcock et al., 2015; Nair et al., 2015). In the final stage of the response, the body straightens and is propelled forward via a rapid escape swim (Domenici and Hale, 2019; Fig. 1C).

Larval predator escape trials were conducted, recorded, and analyzed using the experimental arena set-up and software described in Experiment 1 (Fig. 1B). To begin a trial, a glass dropper was used to place a focal individual into the arena, which was centered on a platform containing a trigger-activated vibrational chip; this vibrational stimulus effectively induces predator avoidance behavior in larval minnows (Painter et al., 2009; Ward et al., 2017; Rearick et al., 2018). Subjects were permitted 60 s to acclimate to the arena before the stimulus was administered. A simultaneously activated small light-emitting diode was used to establish time zero for video analysis. All individuals were administered the stimulus once to prevent potential habituation (Faria et al., 2018).

To evaluate the effects of exposure on stimulus detection, we quantified the latency of the response (ms) from the onset of the stimulus to the frame in which angular displacement of the head was first observed. To evaluate the effects of exposure on behavioral performance over time, we digitized landmarks on the anterior tip of the snout and anterior edge of the dorsal fin at the point of midline insertion and recorded the angle of displacement of the head at 10-frame intervals from the frame of stimulus detection through completion of the escape swim. In addition, we assessed differences in expression of the 'C' maneuver among treatments by measuring the maximum degree of the C-bend. We digitized three points on the body – the tip of the snout, the centerpoint of the body bend on the trunk, and the posterior end of the caudal vertebrae – at the frame of maximum yaw, just before the body unfurled. Last, we measured the total

duration of the response from the frame of stimulus detection through completion of the C response, just before beginning the escape swim.

### 2.2.4. Statistical analysis

Among-treatment differences in the latency to respond, the change in yaw in the first 10 and 20 ms of the response, the extent of the maximal body bend, the total escape angle, and the total duration of the response were tested via linear mixed models, fit by residual maximum likelihoods and *t*-tests that used Satterthwaite's method to compare dependent variables across levels for significant effects. Clutch IDs were included as a random effect in the models. To determine whether focal subjects differed in the expression of the response over time, we fit linear mixed models with the absolute heading angle of the focal subject relative to the initial position (0°) specified as the outcome variable and treatment and time specified as predictors; in this model, individual ID and clutch ID were included as random effects. We assessed whether there was a bias in the direction of C-starts (left-right) via Pearson's chi-squared test ( $\chi^2$ ).

## 3. Results

### 3.1. Early indicators of developmental exposure

#### 3.1.1. Water quality

Table 1 shows measured concentrations of BMAA in the freshly prepared, daily aqueous solutions. Analysis of randomly selected water samples taken 24 h post exposure throughout the 21-day treatment period indicated that BMAA was largely undetectable in the BMAA<sub>5</sub> and BMAA<sub>25</sub> treatments after 24 h and exhibited a wide range of values in BMAA<sub>125</sub> and BMAA<sub>625</sub> concentrations (Table 1), suggesting either

**Table 1**

LC-MS/MS concentrations of  $\beta$ -N-methylamino-L-alanine (ng/L) in freshly prepared aqueous solutions ( $n = 1$  for all concentrations) and in randomly selected water samples taken throughout the 21-day exposure period ( $n = 5$  in BMAA<sub>5</sub> and BMAA<sub>125</sub>,  $n = 6$  in BMAA<sub>25</sub> and BMAA<sub>625</sub>) 24 h post-addition to exposure vessels. Mean and standard deviations are provided in italics for BMAA<sub>125</sub> and BMAA<sub>625</sub> treatments.

Concentration (ng/L)	BMAA <sub>5</sub>	BMAA <sub>25</sub>	BMAA <sub>125</sub>	BMAA <sub>625</sub>
Daily aqueous solutions	4	22	119	609
Post 24 h	ND – 2	ND – 4	8–140 <i>54.6 ± 58.9</i>	110–844 <i>419.5 ± 309</i>

ND = not detected.

substantial degradation of the exposure chemical, uptake via larva, or both. All recovered values were well within reported environmental concentrations (Al-Sammak et al., 2014; Wilitsie et al., 2018; Vo Duy et al., 2019). Water temperature was stable through the exposure period; over all concentrations the mean ( $\pm$  SD) daily temperature recorded was  $20.6 \pm 0.9$  °C. Other measurements of water quality (pH =  $8.4 \pm 0.2$ ; conductivity =  $844 \pm 95$  mS/cm; total dissolved solids =  $397 \pm 45$ ; and chlorine: undetectable) were also consistent throughout the experiment.

### 3.1.2. Survival, hatching success, and larval growth

Fifty-one clutches were collected for analysis ( $n = 14, 11, 10, 7, 9$  for the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively). Individual clutch sizes collected from breeding pairs varied from 8 to 364 (mean  $\pm$  SD;  $102 \pm 84$ ); overall, there was no significant difference across treatments in the average number of eggs per clutch ( $H = 9.38$ ,  $df = 4$ ,  $P = 0.05$ ). The mean ( $\pm$  SD) number of days from the time that the eggs were laid to the first day of hatch was  $5.46 \pm 1.33$ ,  $5.73 \pm 1.10$ ,  $5.86 \pm 1.35$ ,  $6.17 \pm 1.60$ , and  $6.50 \pm 1.05$  in the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively ( $H = 2.74$ ,  $df = 4$ ,  $P = 0.60$ ). We did not observe differences among treatments in either the length of the hatching period ( $2.89 \pm 1.69$ ,  $2.60 \pm 1.43$ ,  $2.80 \pm 1.64$ ,  $3.25 \pm 1.26$ , and  $2.25 \pm 0.50$  in the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively;  $H = 1.38$ ,  $df = 4$ ,  $P = 0.85$ ) or the proportion of eggs that successfully hatched ( $0.72 \pm 0.34$ ,  $0.92 \pm 0.14$ ,  $0.90 \pm 0.08$ ,  $0.91 \pm 0.10$ , and  $0.97 \pm 0.04$  in the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively;  $H = 7.78$ ,  $df = 4$ ,  $P = 0.10$ ).

Survival to day 21 of the experiment was highly variable across clutches, ranging from 0.6 % to 100 %. We did not observe any differences among treatments in the proportion of fish that survived to day 21 after hatching (mean  $\pm$  SD:  $0.51 \pm 0.29$ ,  $0.76 \pm 0.07$ ,  $0.48 \pm 0.32$ ,  $0.60 \pm 0.35$ , and  $0.78 \pm 0.16$  in the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively;  $H = 5.06$ ,  $df = 4$ ,  $P = 0.28$ ). Growth was similar among the larval groups ( $F_{34,185} = 2.02$ ,  $df = 34$ ,  $P = 0.12$ );

at day 21, larvae in the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments had standard length (SL) measurements of  $6.62 \pm 0.70$ ,  $8.02 \pm 1.54$ ,  $7.62 \pm 1.05$ ,  $7.83 \pm 1.50$ , and  $8.68 \pm 1.28$  mm, respectively.

### 3.2. Behavioral assays

#### 3.2.1. Experiment 1: prey capture

In total, we analyzed 185 individual prey strikes ( $n = 34, 29, 43, 38$ , and 41 for the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively). Subjects in all groups initiated the prey-capture sequence at a similar distance to the prey ( $F_{34,185} = 1.24$ ,  $P = 0.29$ ; Fig. 2A).

Consistent with expectations, the absolute change in heading angle decreased gradually over time as subjects approached the prey (Fig. 2B). The model indicated a significant main effect of treatment ( $F_{186,2652} = 4.85$ ,  $P = 0.001$ ) on the change in angular orientation to the prey during approach. Individuals in the BMAA<sub>625</sub> and BMAA<sub>125</sub> groups approached prey from an overall smaller angle of orientation compared to fish in the lower treatments (post hoc comparisons of BMAA<sub>625</sub> vs BMAA<sub>5</sub>, BMAA<sub>25</sub>,  $P_s < 0.05$ ; BMAA<sub>125</sub> vs BMAA<sub>5</sub>, BMAA<sub>25</sub>,  $P_s < 0.05$ ). The model also indicated significant main effects of time since detection ( $F_{186,2652} = 308.49$ ,  $P < 0.001$ ) and a treatment  $\times$  time interaction ( $F_{186,2652} = 2.77$ ,  $P = 0.03$ ) on the angular change in the trajectory of hunting larvae. Across treatments, the angle of orientation to the prey decreased significantly over the first 50 ms of the hunting event (angle at detection vs 50 ms later,  $P < 0.001$ ), before slowing between 50 and 100 ms. Little further change in orientation occurred after 100 ms for the duration of the hunting sequence (comparisons between all subsequent adjacent timepoints,  $P_s > 0.05$ ).

Across treatments, subjects differed in the numbers of behavioral tracking maneuvers performed. Specifically, fish reared under conditions simulating the highest algal density performed fewer swim bouts (slow swims; Fig. 2C;  $H = 11.11$ ,  $df = 4$ ,  $P = 0.03$ ) than those in the lower treatments

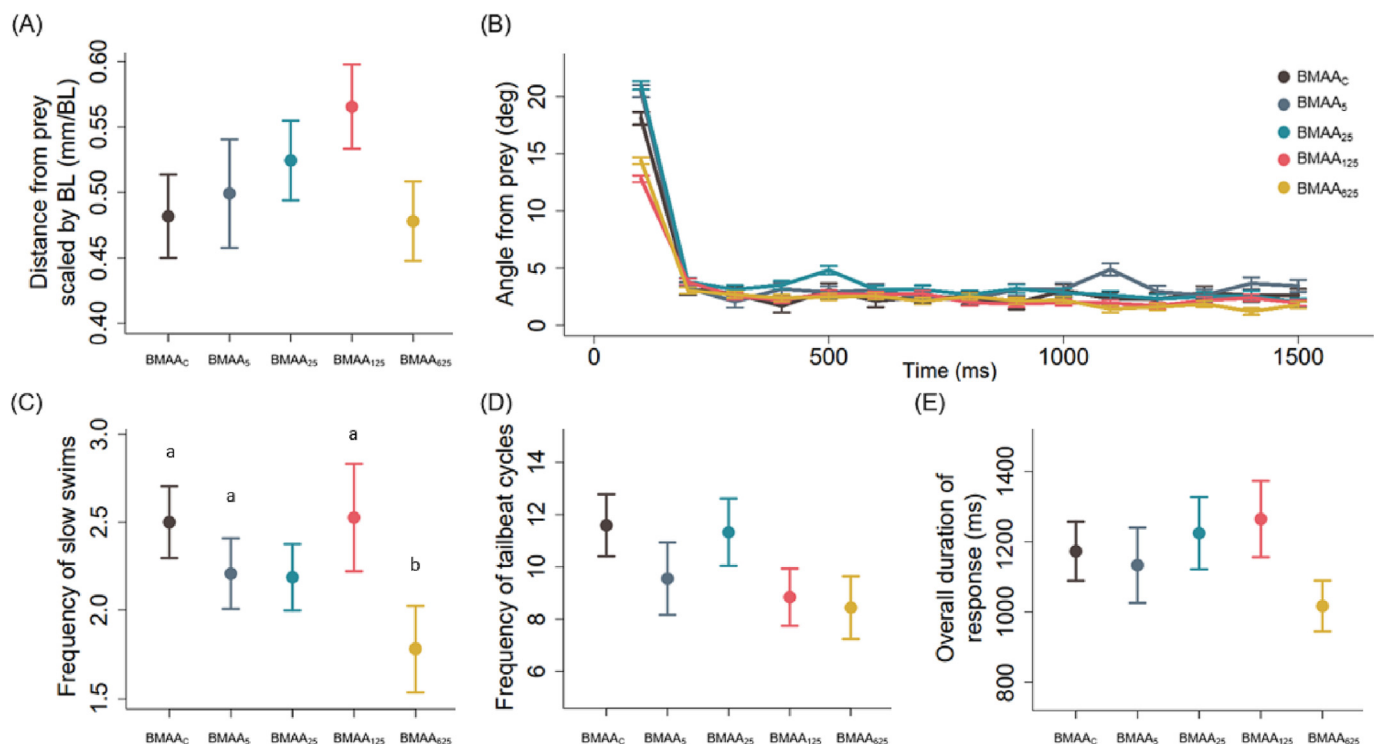


Fig. 2. Effects of developmental exposure to  $\beta$ -N-methylamino-L-alanine on prey-hunting behavior. (A) Distance from *Artemia franciscana* prey at initiation of the prey-capture sequence scaled by body length (BL) in millimeters. (B) Angle of orientation of the head relative to the prey from prey recognition through capture in degrees. (C) Frequency of slow swims. Letters depict significant differences among treatments (BMAA<sub>625</sub> vs. Control, BMAA<sub>5</sub>, BMAA<sub>125</sub>; all  $P_s < 0.05$ ). (D) Frequency of tailbeat cycles. (E) Duration of the response from recognition to prey capture in milliseconds. Marks and whiskers represent means  $\pm$  standard errors. *Pimephales promelas* larvae were tested after 21 days of exposure.

(BMAA<sub>625</sub> vs. Control, BMAA<sub>5</sub>, BMAA<sub>125</sub>; all  $P$ s < 0.05). However, larvae in all treatments performed a similar number of tailbeat cycles ( $H = 7.51$ ,  $df = 4$ ,  $P = 0.11$ ; Fig. 2D). The total duration of the hunting sequence was similar across treatments (i.e., ~1160 ms;  $F_{34,185} = 0.59$ ,  $P = 0.67$ ; Fig. 2E) with the time elapsed between prey recognition and capture ranging from a mean  $\pm$  SD of  $1.02 \pm 0.46$  s in the BMAA<sub>625</sub> group to  $1.22 \pm 0.67$  s in the BMAA<sub>25</sub> group.

### 3.2.2. Experiment 2: predator escape

In total, we analyzed 72 predator escape responses ( $n = 15, 13, 15, 15$ , and 14 for the control, BMAA<sub>5</sub>, BMAA<sub>25</sub>, BMAA<sub>125</sub>, and BMAA<sub>625</sub> treatments, respectively). There was a non-significant but dose-dependent trend associated with exposure treatment, with an increased latency to respond to the stimulus ( $F_{27,72} = 1.39$ ,  $P = 0.28$ ; Fig. 3A). Compared to control individuals, larvae reared in the BMAA<sub>625</sub> treatment demonstrated a 63 % increase in the mean reaction latency, corresponding to an average absolute difference of approximately 108 ms. At recognition, individuals in all treatments initiated the response by bending the body into a C shape with little translational displacement.

Fast starts were equally directed to the left or right across trials and treatments ( $X^2 = 1.43$ ,  $df = 4$ ,  $P = 0.84$ ). A linear mixed model revealed a significant main effect of time on the change in angular heading relative to the starting position ( $F_{27,347} = 129.01$ ,  $P < 0.001$ ; Fig. 3B). As expected, angular displacement of the head (i.e., yaw) increased significantly over the first 40 ms of the response (heading angle at 10 ms vs 20 ms, 20 ms vs 30 ms, 30 ms vs 40 ms,  $P < 0.001$ ) and then showed little further change (comparisons between all subsequent adjacent timepoints,  $P$ s > 0.05). We did not detect a significant main effect of treatment ( $F_{27,347} = 1.36$ ,  $P = 0.26$ ) or a treatment  $\times$  time interaction ( $F_{27,347} = 0.47$ ,  $P = 0.75$ ), suggesting that individuals in all treatments followed an overall similar pattern of response with respect to displacement of the head. However, further examination of the data showed a non-significant but dose-dependent trend in the absolute initial change in yaw over the first 10 ms of the response, with individuals reared in higher concentrations showing smaller angular displacement of the head ( $F_{27,72} = 0.58$ ,  $P = 0.70$ ; Fig. 3B).

Larvae reared under different simulated levels of algal exposure also showed a non-significant, dose-dependent trend in the extent of maximum degree of lateral body bend (i.e., expression of the 'C' shape; Fig. 3C). Compared to control fish, BMAA-exposed individuals showed weaker body curvature, as evidenced by a wider body angle ( $F_{27,72} = 2.05$ ,  $P = 0.13$ ); this

difference was not significant, but fish in the BMAA<sub>625</sub> group expressed a maximum degree of curvature approximately 15 deg. wider than that of controls.

The overall duration of the response from recognition through completion of the C-maneuver also showed a non-significant but dose-dependent trend, with individuals in the higher treatments taking longer to execute the response ( $F_{27,70} = 0.70$ ,  $P = 0.60$ ; Fig. 3D); at the extremes, escape responses of fish in the BMAA<sub>625</sub> treatment were approximately 28 % longer in duration than those of control fish. The overall angle of escape showed a dose-dependent non-significant trend, with individuals reared under conditions simulating higher algal densities escaping at a shallower angle relative to the starting position ( $F_{27,72} = 0.63$ ,  $P = 0.65$ ; Fig. 3E).

## 4. Discussion

In this study, we conducted parallel behavioral performance assays to assess the effects of a common cyanotoxin, BMAA, on larval *P. promelas* in the role of both predator and prey. Our results yielded two main findings; first, we found consistent but largely non-significant trends indicating that exposure to BMAA during early development may be associated with performance-based differences in both larval hunting and antipredator behavior. Second, we found evidence for algal-associated effects on stimulus detection and locomotor performance during predator evasion, but only in locomotor performance during hunting. Although subtle, taken together these data indicate that early developmental exposure to harmful algal blooms can induce deviations from normal behavior, with potentially important fitness consequences.

### 4.1. Experiment 1: prey capture

Data from this study suggest that developmental exposure to algal metabolites does not affect the distance at which prey are detected but may affect how larvae track them. We found no difference among the groups in the distance at which focal subjects initiated the hunting response (i.e., all individuals initiated the hunting sequence at a distance equivalent to approximately half their body length). During a hunting event, individuals in all groups similarly adjusted their heading angle relative to the prey to bring their body in line with the prey over the first 100 ms of the response. Although individuals reared in the BMAA<sub>625</sub> group did approach prey from a significantly shallower overall angle of

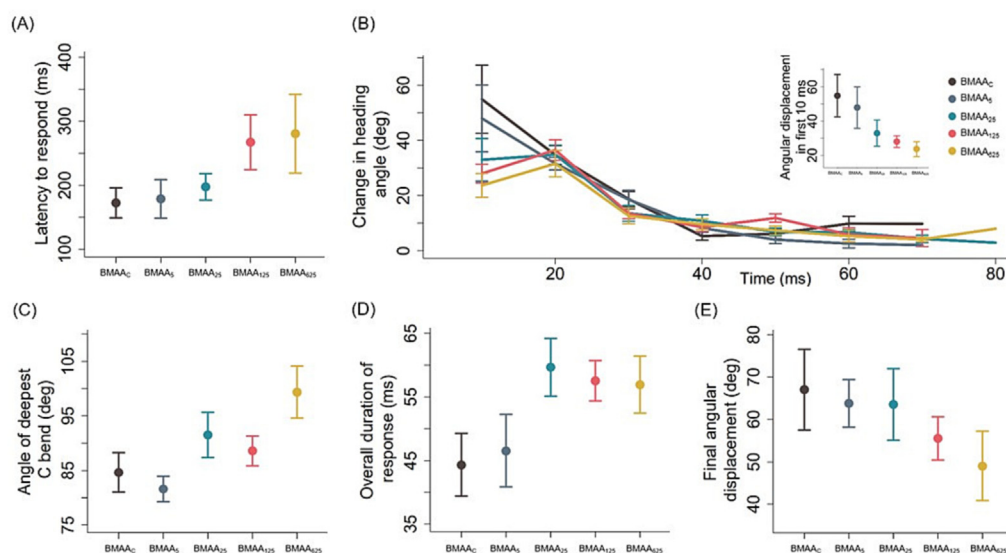


Fig. 3. Effects of developmental exposure to  $\beta$ -N-methylamino-L-alanine on predator-avoidance behavior. (A) Latency to respond to the vibrational stimulus in milliseconds. (B) Change in yaw throughout the predator avoidance sequence in degrees. Inset figure depicts the angular displacement of the head in the first 10 milliseconds. (C) Angular curvature of the body during the deepest C bend in degrees. (D) Overall duration of response in milliseconds. (E) Final angular displacement at the completion of the C bend in degrees. Marks and whiskers represent means  $\pm$  standard errors. *Pimephales promelas* larvae were tested after 21 days of exposure.



orientation compared to individuals in the other treatments, this was likely an artifact of starting position relative to the prey. However, subjects in the BMAA<sub>625</sub> group performed fewer slow swim tracking maneuvers while approaching prey.

Although the treatment-associated differences in hunting behavior that we observed are subtle, they are consistent with previous studies demonstrating that contaminants can modify the behavior of predators (Weis et al., 2001; Relyea and Edwards, 2010; Cripps et al., 2011; McLean and Fetcho, 2011; Bartolini et al., 2015) and reduce foraging success (Scherer et al., 1997; Carvalho et al., 2008; Purser and Radford, 2011; Allan et al., 2013; Figueiredo et al., 2016; Ashur et al., 2017; Sievers et al., 2018). Capturing prey is necessary for survival, and changes in the performance of behaviors that regulate this process early in life could limit larval recruitment into the population (Munday et al., 2010). For example, Allan et al. (2013) exposed both predator and prey fish to waters contaminated with high levels of CO<sub>2</sub>. Predator performance was affected, though the strength of the impact also depended on prey exposure history; predators had greater success if the prey was also exposed to similar levels of contaminant compared to unaffected prey. Additional studies investigating the effects of BMAA on the outcomes of predator-prey interactions following exposure of both the predator and prey are now needed to understand the full impacts of exposure to BMAA on foraging.

#### 4.2. Experiment 2: predator escape

Developmental exposure to BMAA showed dose-dependent trends on the response of larvae to a vibrational, simulated predator threat. Larvae reared in higher concentrations of BMAA (e.g., BMAA<sub>125</sub> and BMAA<sub>625</sub>) had delayed response latencies compared to control fish, with individuals in the BMAA<sub>625</sub> group showing a 63 % longer recognition and 28 % longer total response time. Although not statistically significant, these data suggest that larval fish reared in the presence of BMAA may have a reduced ability to detect a vibrational potential threat.

In addition, the dose-dependent trends from this study show preliminary support for the hypothesis that early developmental exposure to algal neurotoxins alters the performance of the C-start response. First, larvae in the higher treatments exhibited a smaller degree of yaw (and a correspondingly slower angular velocity) over the first 10 ms of the response. Second, fish exposed to BMAA exhibited relatively weaker body curvature, with the degree of curvature being approximately 17 % shallower in fish in the BMAA<sub>625</sub> group than controls. Accordingly, the overall angle of escape in the highest treatment was 18 deg. (36 %) shallower than that of control fish, though the range of escape angles performed by fish in all treatments was consistent with ranges reported in previous literature (Eaton et al., 1988; Tytell and Lauder, 2002).

After the stimulus was detected, C-start activity resulted in a burst contraction of trunk muscles followed by a recoil of the tail with little displacement of the head, all under 1 s (Domenici and Blake, 1997; Eaton et al., 1988). Several studies have shown impaired C-start responses following exposure to contaminants, characterized by increased latency to respond and decreased velocity of the behavior (Weber, 2006; Painter et al., 2009; Cox et al., 2019). Our data are consistent with those of Weber (2006), who documented increases in latency to respond to a vibrational stimulus following sub-lethal developmental exposure to mercury. The same study also documented reduced velocities of head movement in embryonic zebrafish, *Danio rerio*, similar to the decreased displacement of heading angle that we observed following the administration of a vibrational stimulus.

#### 4.3. Action of BMAA on the detection of sensory information and locomotor response

Hunting in larval fish is primarily visually guided (Gahtan et al., 2005; Bianco et al., 2011), but both the lateral line and visual systems function together to detect and locate prey at different parts of the prey-capture sequence (New et al., 2001; Carvalho et al., 2008). Predator avoidance behavior can also be stimulated via the detection of cues in multiple sensory

modes, including acoustic, vestibular, chemosensory, and lateral-line pathways (Eaton and Popper, 1995), but the detection of a vibrational stimulus, such as that used in this study, occurs primarily via the lateral line system. At present, it is unknown whether the changes in the onset of predator avoidance behavior that we observed are due to distorted sensory input or altered sensory processing, both of which have been shown to contribute to variation in performance (Ashur et al., 2017). Various anthropogenic-influenced changes in the environment have been shown to damage the neuromast cells of the lateral line (Lombarte et al., 1993; Harris et al., 2003; Hayashi et al., 2015). However, the effects of BMAA on the function of the lateral line have not yet been explored.

β-N-methylamino-L-alanine exerts toxicity via hyperexcitation of neurons, leading to cell death (Karamyan and Speth, 2008; Cox et al., 2017; Okamoto et al., 2018). Among its mechanisms of toxicity, BMAA acts as a glutamate-receptor agonist, inhibits cystine uptake, and induces oxidative stress, leading to hyperexcitation (Lobner et al., 2006; Lobner, 2009; Okamoto et al., 2018). BMAA is among several environmental toxicants (i.e., cadmium, lead, antidepressants, mercury) that may alter the physiology of neurons, leading to longer or otherwise abnormal responses to ecologically important stimuli (Faucher et al., 2006; Weber, 2006; Painter et al., 2009; Ward et al., 2017; Olson et al., 2018; Rearick et al., 2018). In vivo studies of BMAA in animals report inability to extend legs and stand (Vega et al., 1968), dragging gait (Polsky et al., 1972; Smith and Meldrum, 1990), and whole-body shakes (i.e., convulsions; Polsky et al., 1972; Ross and Spencer, 1987; Smith and Meldrum, 1990; Matsuoka et al., 1993; Karamyan and Speth, 2008). In our study, among-treatment differences in the kinematics and expression of locomotor performance were subtle, and we did not observe tremors or convulsions. However, we anecdotally observed that BMAA-exposed fish frequently showed evidence of jerked swimming movements that were outside the scope of variables quantified in this experiment. Still, fish exposed to BMAA had a decreased frequency of slow swims (Fig. 2C,D), weaker body curvature (Fig. 3C), and smaller angles of escape trajectory (Fig. 3E), suggesting the potential for alterations in locomotor efficiency. It is possible that these changes could have amplified, detrimental effects in natural systems, especially when combined with other neurotoxins (Karamyan and Speth, 2008; Lobner, 2009; Cox et al., 2017).

## 5. Conclusions

Harmful algal blooms are an increasing problem of global proportions (Blaha et al., 2009; Al-Sammak et al., 2014; Nawaz and Sengupta, 2019). The results of this study enhance our current knowledge by suggesting that increases in the frequency and severity of HABs may also alter the function and structure of aquatic communities via changes in individual behavior that have the potential to alter predator-prey interactions, similar to other forms of anthropogenic environmental change. Moreover, because these toxic metabolites are transferred and accumulated up the food chain (Jonasson et al., 2010; Brodin et al., 2014), an increased susceptibility to predation may accelerate rates of transfer and bioaccumulation. Cox et al. (2003) reported that concentrations of toxins can increase by up to two orders of magnitude at each trophic level. Indeed, some fish have been found to contain concentrations of cyanobacterial neurotoxins up to 200-fold higher than the cyanobacteria themselves (Jonasson et al., 2010). Because the consumption of foods contaminated with BMAA has been putatively associated with amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease (Pablo et al., 2009; Bradley et al., 2013), the results of this study also provide insight into the factors affecting routes of human exposure and health risks. These results, taken after exposure to low chemical concentrations, provide an avenue to assess the effects of naturally occurring neurotoxic metabolites in an assay of ecological relevance.

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## CRediT authorship contribution statement

Gina Lamka: conceptualization (equal); funding acquisition (supporting); data curation (lead); investigation (lead); analysis (lead); visualization (lead); writing – original draft (lead); writing – review & editing (equal). Autumn Auxier: funding acquisition (supporting); investigation (supporting). Ally Swank: funding acquisition (supporting); investigation (supporting). Katie Esarey: investigation (supporting). Hannah Mullinax: investigation (supporting). Ryan Seymour: investigation (supporting). Jessica Ward: conceptualization (equal); funding acquisition (lead); supervision (lead); writing – review & editing (equal).

## Data availability

Raw data and code are available on Mendeley Data (DOI:10.17632/rj9zvww5jvw.1).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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