**INTRODUCTION**

-**Background and Rationale**

* **Ecological and Biological Importance of P. promelas (Fathead Minnow)**
  + Discuss the ecological role and relevance of P. promelas as a model organism in behavioral studies.
    - The fathead minnow (*Pimephales promelas*) is an emerging model for studies of anthropogenic effects on behavior (Lavelle and Sorensen 2011; Thunstrom 2017; Vignet and Parrott 2017) which can be bred in captivity and can typically reach maturity at 5 months1.
* **Overview of β-methylamino-L-alanine (BMAA)**
  + Introduce BMAA, its sources (e.g., cyanobacteria), and general effects.
* **Environmental Relevance of BMAA**
  + Explain the presence of BMAA in aquatic environments and potential behavioral impacts on aquatic life.
    - BMAA and its isomers have been detected in waterways (Al-Sammak et al., 2014; Wilitsie et al., 2018; Vo Duy et al., 2019) and bioaccumulated in several taxa (lobsters, Sandhu et al. 2024; humans, Fiore et al. 2020; zooplankton, mussels, oysters, and fishes, Jonasson et al. 2010; plants, Rosen and Hellenas 2008; Regueiro et al. 2017).
    - While boldness, aggressiveness, growth, and reproduction can be unaffected by 14 days of exposure to BMAA, altered gene expression indicates long-lasting effects on the brain in mangrove rivulus fish (*Kryptolebias marmoratus*; Carion et al. 2020).
    - Prey capture, predator avoidance, and maximal swimming speed have all been documented to be influenced by early-life exposure to BMAA (Carion et al. 2018; Carion et al. 2020; Lamka et al. 2023).

- **Behavioral and Personality Traits in Fish**

* **Concept of Animal Personality**
  + Define animal personality and its significance in behavioral ecology.
* **Behavioral Assessments in Fish**
  + Overview of common methods for assessing fish behavior and personality traits, emphasizing the open field test.

-**Previous Research**

* **Behavioral Studies on BMAA**
  + Summarize previous studies on the behavioral effects of BMAA on aquatic organisms.
* **Personality and Repeatability in Fish**
  + Review studies on the repeatability of behavior as an indicator of personality traits in fish.

-**Study Objectives**

* **Research Questions and Hypotheses**
  + State the specific research questions:   
    (1) Are the behaviors recorded during the open field test reflective of underlying personality traits? To test this we will evaluate the repeatability of each behavior across 8 time points.  
    (2) How does BMAA exposure affect behavior in the open field test? To test this we will compare performance among treatment groups.
* **Significance of the Study**
  + Discuss the importance of understanding the impact of environmental toxins on behavioral development and personality traits.

**MATERIALS AND METHODS**

**Subjects and animal care**

The subjects of this study were the progeny of six-month old *P. promelas* purchased from a culturing facility (Environmental Consulting and Testing; WI, USA). Breeding groups, each 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 spawning tile for clutches to be laid upon. Spawning tiles were monitored twice daily, and clutches were removed on the day they were laid and randomly assigned to a control or treatment group. The fish were fed live prey items (*Artemia franciscana*; Brine Shrimp Direct, UT, USA) twice daily and were maintained throughout the experiment under a 16 h: 8 h light-dark regime at room temperature (mean ± SD: 20.6°C ± 0.86°C). Mortality events were monitored twice daily. All procedures were approved by the Institutional Animal Care and Use Committee at Ball State University (1142896-1).

**Treatment regime**

Stock solutions were prepared weekly, consisting of serially diluted solutions of powdered β-methylamino-L-alanine (BMAA; Sigma Aldrich, Inc., Germany) dissolved in ultra-pure water (Millipore, MA, USA), and stored in amber glass bottles at 4°C. Treatments with nominal concentrations of 5 or 25 ng/L BMAA (hereafter referred to as BMAALOW and BMAAHIGH) and a control (0 ng/L) were prepared daily by adding an appropriate concentration of stock solution to aged, aerated water. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure concentrations of stock solutions at the start of the experiment (Indiana State Department of Health; see Lamka et al. 2023). BMAA has been detected in waterways in concentrations as low as 100 ng/L to as high as 25 µg/L2,3 (Wilitsie et al. 2018), therefore we used conservative sub-lethal but environmentally-relevant stock concentrations of the chemical. The water was exchanged daily using a 50% static renewal protocol to account for degradation (USEPA 2002), as a related experiment indicated substantial degradation of BMAA over 24 h (Lamka et al. 2023).

Fish in the BMAALOW and BMAAHIGH groups were exposed to BMAA for the first 21 days post-fertilization (dpf) and subsequently reared in clean water for the remainder of the experiment. Clutches were maintained on the spawning tile in a 750 mL glass vessel fitted with an airstone for the first 5 dpf before being transferred to individual housing containers (6-well plate; Corning, Inc., NY, USA) where they hatched. The fish were housed separately after hatching due to the inability to mark newly-hatched fish4,5. Fish were permitted visual contact to control for potentially confounding effects of social isolation on behavior.5,6 Additionally, chemical cues from the home tanks of fish not used in this experiment were introduced to prevent developmental impediments due to social isolation5,7. Each fish was transferred to a 750 mL glass vessel at 49 dpf, and then to a 1.8 L tank in an Aquaneering Flow Through System at 77 dpf, where they remained for the rest of the experiment.

**Behavioral tests**

Fish behavior was assessed via an open field assay eight times throughout development; once during exposure, once at the completion of the exposure period, and an additional six tests every 28 days following exposure to measure the effects of the chemical at sequential points of development. Therefore, every fish was tested on 14, 21, 49, 77, 105, 133, 161, and 189 dpf ( 2 d; Fig XX). Tank size has the potential to alter risky behaviors in fish11–13 so arena size increased as the fish did to account for growth; average fish total length was approximately one quarter of the arena diameter (Table XX).

Trials were conducted in clean, conditioned water under differential lighting in a circular arena placed on a no-heat, LED light pad (Tiktek/A4-DWT) (Fig XX, Table XX). To begin a trial, a focal larva was gently introduced to the arena via a glass dropper and the trial was started immediately. The free swimming behavior of focal fish was recorded for 6 min using a monochrome GigE camera (Basler AG, Ahrensburg, Germany) mounted above the arena.

We analyzed the behavior of each fish in each trial using Ethovision XT software (version 13; Noldus Information Technologies, Inc., Wageningen, Netherlands). First, we divided the arena into two zones; the inner zone, classified as the “risky” area, was approximately half the diameter of the outer zone (Fig XX). Fish behavior variables were classified as one of three animal personality characteristics: boldness, exploration, and activity. Boldness was defined as the latency (s) of the center point of the fish to enter the risky zone. Exploration was measured as the cumulative duration (%) of trial time spent in the risky zone. Mobility, or the percentage (%) of pixel change detected in the subject, was used as a proxy for activity.

**Behavioral repeatability analysis**

Output provided by Ethovision software includes many variables; the R package corrplot was used to find correlation among output variables to ensure the personality behaviors analyzed represented unique actions. Repeatability analyses for cumulative duration were conducted using a multivariate generalized linear mixed model (Hadfield 2010; MCMCglmm package in R 3.5.1, R Development Core Team 2018) with treatment, scaled age, and a treatment by age interaction as fixed effects, fish ID as random effects, and behavior as the response variable with 63000 MCMC iterations, a thinning interval of 20, and a burnin of 5000. Cumulative duration in the risky zone was rounded and fit a Poisson distribution. The data were permutated 1000 times to test the significance of the repeatability value; when the observed repeatability is greater than the permutated repeatability value, there are significant individual differences in behavior. Linear mixed models using the nlme package in R (CITE) were used to find the model of best fit for latency and mobility. The best fitted model was then used to assess if there are treatment effects on the behavior. Next, the same model was put into the rpt function in the rptR package (Stoffel et al. 2019) to extract a repeatability value. Latency was log transformed and put in a linear mixed model with treatment and scaled age as fixed effects and fish ID and clutch as random effects. Similarly, mobility fit a Gaussian distribution and was used as a response variable to a model with treatment and scaled age as fixed effects and fish ID and clutch as random effects. Both models had 500 permutations and bootstrapped 500 times. Behavioral syndromes were assessed using a multivariate model…

**RESULTS**

**DISCUSSION**

-**Summary of Key Findings**

* **Behavioral Traits and Repeatability**
  + Summarize the repeatability of behaviors recorded in the open field test, indicating underlying personality traits.
* **Effects of BMAA on Behavior**
  + Discuss the impact of BMAA exposure on the recorded behaviors across treatment groups.

**-Comparison with Previous Studies**

* **Alignment with Existing Research**
  + Compare your findings with those of previous studies on the behavioral effects of BMAA and personality traits in fish.
* **Novel Contributions**
  + Highlight any novel findings or contributions your study makes to the field of behavioral ecology.

**-Mechanisms of Action**

* **Potential Mechanisms**
  + Discuss potential biological mechanisms through which BMAA might affect behavior and personality traits.
* **Literature Support**
  + Cite relevant studies that support the proposed mechanisms.

**-Implications for Behavioral Ecology**

* **Ecological and Evolutionary Implications**
  + Discuss the broader ecological and evolutionary implications of altered behavior and personality traits due to BMAA exposure.

**-Limitations and Future Directions**

* **Study Limitations**
  + Acknowledge the limitations of your study, such as sample size, duration, or methodological constraints.
    - While sex often influences personality (CITE), we were unable to evaluate sex differences in behavior because sex determination is difficult in fish larvae.
    - Drop off of individuals throughout the 6 month trial period limited sample size.
* **Suggestions for Future Research**
  + Propose future research directions to address unanswered questions and build on your findings.

**-Conclusions**

* **Key Takeaways**
  + Summarize the key conclusions drawn from your study, focusing on the repeatability of behaviors and the effects of BMAA.
* **Final Remarks**
  + Emphasize the importance of continued research on environmental toxins and their effects on animal behavior and personality traits.

**Figures and Tables**

Table XX. Arena sizes (mm) used corresponding to the age of fish on the day of larval testing.



A computer screen shot of a drawing

Description automatically generated

Fig. XX. Arena characteristics during the free swimming, larval testing.

A computer screen with a picture of a circle

Description automatically generated

Fig. YY. Arena characteristics during the free swimming, larval testing. **\*this will not be included in the final publication, just including now to show what the setup was\***

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