**INTRODUCTION**

-**Background and Rationale**

* Anthropogenic changes to the environment are widespread and now affect all ecosystems on the planet (Bowler et al. 2020). The impacts on biodiversity of intentional changes to the environment, like habitat destruction for development, are relatively easy to detect and quantify (Aronson et al. 2014). However, we are still learning about the scope and impact on ecosystems of indirect anthropogenic effects such as chemical contamination from agricultural activities or pollution.
* **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**

Animal behavior is an important indicator of ecosystem stability and function. Changes to the environment, including pollutants and chemical contamination, affect animal populations by first forcing them to adapt their behavior in response to environmental change (Hellou 2011). Many behaviors are plastic such that animals can quickly adjust to accommodate changing environmental conditions. Even low concentrations of chemical contamination cause behavioral change, but not mortality (i.e., sublethal effects; Saaristo et al. 2018). Minor changes in animal behavior are concerning because they can result in disruptions to ecosystem services or lead to trophic cascades (Wong & Candolin 2015). For example, pesticides cause sublethal behavioral changes in honeybees to decrease the rate of foraging activity and ability to return to the hive, thus resulting in depressed pollination services (Bortolotti et al. 2003). Consequently, one way to evaluate and predict the impact of contamination on the natural environment is to determine the direction and magnitude of changes in animal behavior after exposure (Saaristo et al. 2018).

* **Concept of Animal Personality**

Growing evidence suggests significant constraints on behavioral plasticity across taxa (Pennisi 2016). In other words, individuals respond surprisingly consistently to different stimuli across time, even if the behavioral response may be maladaptive in a given context (Dall et al. 2012). For example, western bluebird males that are more aggressive in territorial defense, are also more aggressive towards their mate and less likely to contribute parental care (Duckworth & Badyaev 2007). These consistent individual differences (i.e., personality traits) in a given behavior are widespread within and across species. Behavioral plasticity can be further constrained through genetic correlations among personality traits. This idea, referred to as a “behavioral syndrome” means that traits have coevolved and should be considered as a unit, rather than individually (Sih et al. 2004; Reale et al. 2007). Research is beginning to show pervasive effects of environmental contaminants on animal personality traits. However, most studies evaluate the impact of contamination on one behavior in isolation, precluding inferences about the change in behavior in other contexts (Jacquin et al. 2020).

* **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.
    - Ecologically, the fathead minnow serves an important role in the middle of the trophic system. This was illustrated by a study that showed water contamination with birth control hormones decreased the reproductive success, and therefore population size, of the fathead minnow which led to significant indirect effects on the ecosystem. Lake trout, a species that predates the fathead minnow, sharply declined in the year after hormone additions, but emergence of water-associated insects (the prey of fathead minnows) increased (Kidd et al. 2014).

-**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) Relationship of personality traits - Are personality traits significantly correlated, do individuals differ in plasticity?
  + (3) How does BMAA exposure affect behavior in the open field test? To test this we will compare performance among treatment groups, look for differences in plasticity.
* **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 one of two treatment groups. 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 (Wiltsie et al. 2018; Carion et al. 2020), 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. To avoid developmental impediments due to social isolation5-7, we permitted visual contact among fish and introduced chemical cues from the home tanks of fish not used in this experiment. 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**

We assessed fish behavior 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, we gently introduced a focal larva 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.

**Behavioral analysis**

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). Next, we extracted a subset of behavior variables that previous literature suggests represent personality traits (e.g., Cote et al. 2010). We used the R package corrplot (CITE) to test the correlations among output variables to ensure that the performance behaviors we subsequently analyzed represented unique actions. Fish behavior variables were classified as one of three animal personality traits: boldness, exploration, and activity. Boldness was defined as the propensity to take risks (Toms et al. 2010) and the variable we used was the latency (s) of the center point of the fish to enter the risky zone. Exploration was defined as the propensity to investigate novelty and we measured it as the cumulative duration (%) of trial time spent in the risky zone. Activity (mobility) was defined as the distance covered in a set amount of time (Reale et al. 2007) and we quantified this behavior as the percentage (%) of pixel change detected in the subject from one time point to the next.

**Repeatability**

Personality traits are relatively fixed genetically or developmentally (Wolf & Weissing 2012). As such, these traits should result in performance in the open field test that is consistent across time and context, as quantified through repeatability (Reale et al. 2007). Also known as the intra-class correlation coefficient, repeatability is the proportion of variance attributable to differences among individuals and can be estimated as a ratio of the variance from the random effect of ID relative to total variance (Dingemanse and Dochtermann 2013). We first used the DHARMa package (Hartig 2019) to determine the best fitting model for each performance variable, then we used the rptR package (Stoffel et al. 2019) with each model to quantify the repeatability value, confidence interval and significance.

To assess repeatability of boldness (cumulative duration in the risky zone) and activity (percent pixel change over time), we used linear mixed-effects models with treatment (control, low or high), scaled age, fish ID and clutch ID as random effects, and the log-transformed cumulative duration as the response variable. We found that an interaction between treatment and age did not significantly add to the variance explained by the model, and so it was omitted.

The best fit model to assess repeatability of exploration (proportion of time spent in the risky zone) was a Poisson mixed-effects model with treatment, age, and the interaction between these two as fixed effects, as well as fish ID as a random effect. Clutch ID did not add significantly to the variance explained by the model, so it was omitted. We experienced some convergence problems with this model in the rptR package as the standard error was zero. Consequently, we instead ran the model using MCMCglmm (Hadfield 2010), with weak priors, to extract the variance components for the repeatability value. Then we used a permutation test to assess the significance of the repeatability value. We randomized the data by conducting 1000 iterations where we resampled the data within treatment without replacement. We reran the model on each randomized data set to compare the observed repeatability value to the distribution of values resulting from the randomized data.

**Treatment effects**

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.
  + BMAA exposure combined with other stressors (food competition, increased predations, other pollutants) to make it more ecologically valid
  + Assess the impact of BMAA exposure on fitness

**-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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