Development of a Reproducible Behavior Assay to Screen for Chemical Effects

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AIM:

To develop a reproducible and standardized behavior protocol to screen for chemical effects.

TEST SPECIES:

Pimephales promelas (fathead minnow) – 5 days post fertilization (dpf)

MAIN QUESTIONS:

1. Can we detect changes in behavior using movement data?

2. Does movement between light and dark photoperiods differ?

3. Is movement similar within photoperiods between trials?

4. Is change in behavior best represented by short-burst response to change in stimuli?

DISCLAIMER: The views expressed in this poster are that of the authors and do not necessarily represent the views of the U.S. Environmental Protection Agency.

BACKGROUND

Fathead minnows (FHM) are a native species widely-distributed in freshwater environments across North America. Because of their distribution, FHM are a good toxicological indicator of chemical exposure in the environment.[1] Fluoxetine (FLX) is a well-characterized Serotonin Selective Reuptake Inhibitor (SSRI) commonly found on surface waters.[1] FLX has been shown to influence innate behaviors of organisms including movement, feeding, and predator evasion.[1,2] Movement behavior can provide a sensitive sublethal measure of organismal response to exposure reflective of multiple levels of biological organization. However, additional research is needed to standardize movement behavior as a toxicological endpoint. Thus, we aimed to develop a reproducible behavior assay to examine changes in FHM movement behavior in response to environmentally-relevant FLX doses.

METHODS

1. **Egg Collections**: FHM eggs collected in one-hour window to ensure larvae at similar developmental ages
2. **Exposure Layout:** 8 5-dpf FHM exposed in beakers with 80mL media each (Figure 1). Repeated set of beaker exposures for 3 sets each trial. Data was collected over 2 trials. Doses are in Table 1. FHM transferred to 24-well plates for data collection (Figure 1). Data collection protocol in Table 2.

Label	Dose (ng/L)	Components
Control (C)	0	MHRW + DMSO
Low (L)	10	Control + Fluoxetine
Medium (M)	100	Control + Fluoxetine
High (H)	1000	Control + Fluoxetine

Table 1. List of doses for FLX exposures. MHRW+DMSO (1% v/v) was used as control. Doses were made using a 10-fold dilution. All doses are within environmentally relevant ranges[1,2,4].

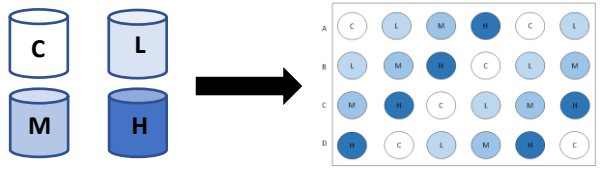

Figure 1. Trials consisted of 3 beaker sets where each set was 4 beakers for each dose (H=High, M=Medium, L=Low) and control (C). After 24 hours, FHM were transferred from each beaker set to plates in a diagonal layout[3] for behavioral observation.

Photoperiod	Minutes	Seconds	%Light
Acclimation	0-10	0-600	0%
Light 1	10-15	600-900	100%
Dark 1	15-20	900-1200	0%
Light 2	20-25	1200-1500	100%
Dark 2	25-30	1500-1800	0%
Light 3	30-35	1800-2100	100%
Dark 3	35-40	2100-2400	0%

Table 2. Protocol for data collection showing acclimation period and 6 photoperiod cycles. Movement data was collected every 30 seconds using ViewPoint ZebraBox system[5].

RESULTS

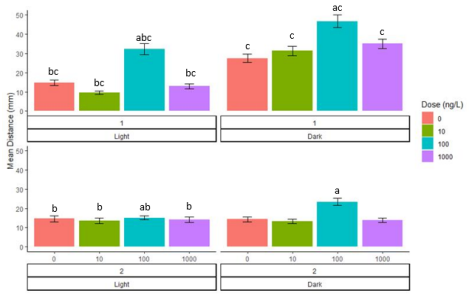

Figure 2. Bar graph showing mean distance (mm) aggregated by dose, trial (Trial 1: top, Trial 2: bottom) and photoperiod. (a) SSRI doses differed significantly from control between photoperiod and within trial ($F_{3,8634}$= 39.399, P<0.001). (b) SSRI doses and control differed significantly within trial and between photoperiods ($F_{1,8634}$= 112.890, P<0.001). (c) SSRI doses and control differed significantly within photoperiod and between trials ($F_{1,8634}$= 139.978, P<0.001). Results are from ANOVA and *post-hoc* tests.

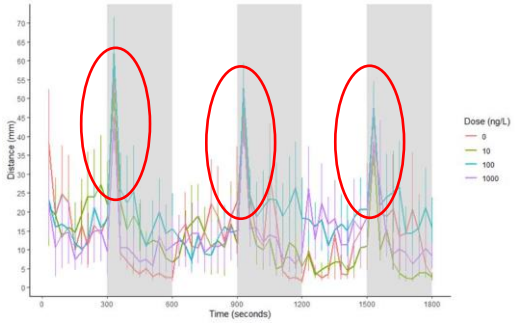

Figure 3. Example of movement data collected from FLX Exposure Trial 2. Within red circles are points of interest in changes of behavior in response to photoperiod stimulus (light to dark).

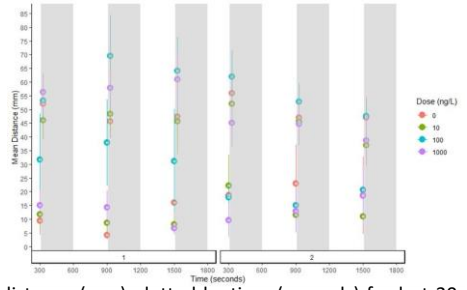

Figure 4. Mean distance (mm) plotted by time (seconds) for last 30 seconds of light changing to first 30 seconds of dark for Trials 1 (left) and Trial 2 (right).

Trial	n	Dose (ng/L)	Last 30 seconds of Light		First 30 seconds of Dark		Increase in Mean Distance (mm)	Increase in Mean Distance (%)
			Mean Distance (mm)	SE	Mean Distance (mm)	SE		
1	54	0	9.88	3.32	48.40	5.49	38.53	389.94
		10	9.46	2.10	46.66	4.19	37.20	393.36
		100	33.58	9.76	62.23	6.89	28.65	85.33
		1000	12.02	2.78	58.99	4.59	46.37	385.77
2	54	0	20.15	7.03	49.94	4.14	29.79	147.86
		10	14.83	4.27	44.92	4.58	30.09	202.85
		100	17.84	3.23	54.08	4.35	36.24	203.10
		1000	13.70	4.75	42.82	4.85	29.12	212.56

Table 3. Summary of mean distance (mm) for 30 seconds before and after photoperiod stimulus (see Figure 4). ANOVA and *post-hoc* tests indicated Mean distance (mm) differed significantly across all doses ($F_{3,858}$ = 4.94, P = 0.002) and between photoperiods ($F_{1,858}$ = 182.54, P<0.001), but did not differ between Trials ($F_{1,858}$ = 1.196, P=0.274).

DISCUSSION

Preliminary results indicate movement data may be a good candidate for detecting reproducible shifts in behavior in response to chemical exposure. However, additional research is needed to develop movement behavior as an endpoint for chemical screening. Our effort was exploratory, and the purpose of our poster was to solicit experimental and analytical feedback. We propose some additional questions that we believe need to be addressed to improve assay and movement behavior as an endpoint:

1. Are changes in short-burst behavioral responses better represent effect of chemical exposure?

2. What effect size should be considered for quantifying shifts in behavior?

3. Do other chemical groups provide similar evidence for behavior as a reliable endpoint?

4. Does FHM age or life stage at time of exposure influence behavior changes?

5. How can experimental protocol be improved to better characterize behavior as a screening tool while increasing throughput?

REFERENCES:

[1] Weinberger II J & R Klaper (2014) Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish *Pimephales promelas* (fathead minnow). *Aquat. Toxicol.* 151: 77-83 [2]Zinder F et al. (2020) Norfluoxetine is the only metabolite of fluoxetine in Zebrafish *(Danio rerio)* embryos that accumulates at environmentally relevant exposure scenarios. *Environ. Sci. Technol.* 54: 4200-4209. [3]Pohl J et al. (2019) Embryotoxicity of ozonated diclofenac, carbamazepine, and oxazepam in zebrafish *(Danio rerio)*. *Chemosphere*, 225: 191-199. [4]Nowakowska K, et al. (2020) Acute exposure of zebrafish *(Danio rerio)* larvae to environmental concentrations of selected antidepressants: Bioaccumulation, physiological and histological changes. *Comp. Biochem and Physiol.* Part C 229: 108670. [5]ZebraBox for Embryos of Larvae, ViewPoint Behavior Technology. Montreal, Canada.