Determination of an optimal age and duration of exposure of larval fathead minnows for gene expression-based studies

Robert Flick[1], David Bencic[1], Mitchell Kostich[2], Adam Biales[1]

[1]Center for Computational Toxicology and Exposure, Office of Research and Development, US EPA, Cincinnati, OH 45268 USA
[2]The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut

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Introduction

Genomic-based methods (microarrays, and RNA sequencing) continue to expand their role in the field of ecotoxicology. The exposure designs used in such experiments may be based on acute and chronic testing methods, which were designed for entirely different classes of endpoints. Very little work has been done to systematically evaluate the effect of exposure regime on genomic endpoints. In order to undertake such a systematic effort, we exposed fathead minnow larvae at three different ages (48, 72 and 96 hours post fertilization) for three different durations (6, 24 and 48 hours) to several compounds under static renewal conditions. Using RNA-sequencing, we evaluated the global transcriptional profiles of the fish to determine the age and duration that provided the strongest transcriptional response signal.

Materials and Methods

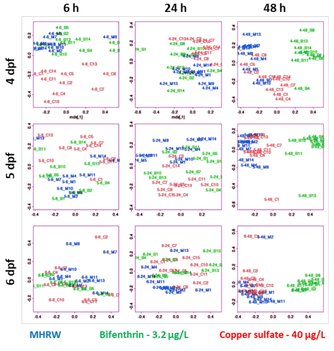
Exposure organisms
Fathead minnows (*Pimephales promelas*) were obtained from the on-site culture at the Andrew W. Breidenbach Environmental Research Center in Cincinnati, OH.
To ensure that larvae were closely synchronized in their development, all larvae used for an experiment were from eggs that had been fertilized within a one-hour window.
Exposures
All exposures were performed in moderately hard reconstituted water (MHRW).
For each treatment, ten larvae were placed in each of five replicate 150 mL beakers containing 100 mL exposure solution.
Exposures were conducted in an incubator at 25 °C, and exposure solutions were replenished at 24 h.
After 6, 24 h, and 48 h exposure, the beakers were checked, and any dead larvae were removed. Three larvae from each beaker were removed at each time point. Each larva was placed into a 1.5 ml microcentrifuge tube and snap-frozen in liquid nitrogen.
RNA isolation and sequencing libraries
RNA was extracted from larvae using MagMAX™-96 Total RNA Isolation Kit (Thermo Fisher Scientific).
Sequencing libraries were prepared using the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen). Ten or eleven larvae (at least two from each beaker) from each treatment were used.
Libraries were pooled into groups of 16 and sequenced by the Genomics Core at Michigan State University.

Materials and Methods (continued)

Phase 1
Larvae were exposed to either bifenthrin (B) at 3.2 µg/L, copper sulfate (C) at 40 µg/L or moderately hard water (M).
Two-way classification was performed on the copper and bifenthrin treatments within each age-duration level. Raw counts for each selected feature were normalized using upper quartile method, sqrt(x+0.5) transformed, and centered and re-scaled based on negative control mean and standard deviation. Transformed features used for random forest 2-class classification. Evaluated by wrapping feature selection and classification steps in 9-fold cross-validation loop, as well as using C vs M classifier to classify B samples, and B vs M classifier to classify C samples.

Phase 2
Larvae were exposed to three chemicals at two concentrations each: propranolol (P) at 0.66 and 3.3 mg/L; sertraline (S) at 0.066 and 0.33 mg/L; and 17α-ethynylestradiol (E) at 2 and 10 ng/L. MHRW was used for controls. For each combination of chemical, concentration, age and duration, the average (per observation) brier score, the lower 95% bound on AUC, and misclassification rate were calculated. For each age and duration, the mean, median and minimum (for brier and misclassification) or maximum (for AUC) values were tabulated across chemicals and concentrations.

Results – Phase 1



Left: MDS plot of the top 1000 features indicate that effects of copper and bifenthrin are fairly discernible using highly expressed genes.

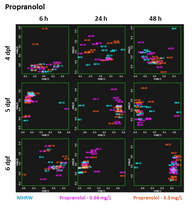
Right: Best age-duration for bifenthrin and copper, based on the classifier developed independently for each chemical.

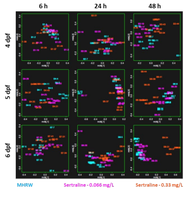
trt	dpf	hrs	AUC	Brier score
Bifenthrin	4	6	0.827	0.357
		24	1.000	0.202
		48	1.000	0.005
	5	6	0.605	0.475
		24	1.000	0.073
		48	1.000	0.001
	6	6	0.988	0.258
		24	1.000	0.110
		48	1.000	0.005
Copper	4	6	1.000	0.057
		24	1.000	0.161
		48	1.000	0.080
	5	6	0.988	0.108
		24	1.000	0.123
		48	1.000	0.117
	6	6	1.000	0.195
		24	0.975	0.213
		48	0.901	0.266

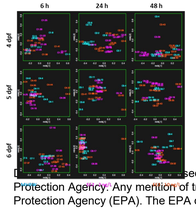
Right: Best single age-duration regime for both bifenthrin and copper exposures. Classifiers were developed for each chemical independently, then tested against samples in the other exposure.

dpf	hrs	trt	AUC	Brier score
4	6	B	0.698	0.493
		C	1.000	0.100
	24	B	0.988	0.290
		C	0.988	0.196
	48	B	1.000	0.039
		C	0.975	0.300
5	6	B	0.889	0.605
		C	0.938	0.186
	24	B	1.000	0.192
		C	0.858	0.393
	48	B	1.000	0.025
		C	0.667	0.576
6	6	B	0.698	0.483
		C	1.000	0.244
	24	B	0.963	0.220
		C	0.728	0.478
	48	B	1.000	0.003
		C	0.802	0.428

Results – Phase 2







MDS plot of the top 1000 features. At the global gene expression level, sertraline (high conc.) shows some differentiation from controls, but to a lesser extent than in bifenthrin and copper exposures. For both propranolol and EE2, differences between exposed larvae and controls is not obvious.

A

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
bifen	0.127	0.097	0.003	0.109	0.042	0.003	0.087	0.023	0.008
copper	0.017	0.026	0.029	0.031	0.055	0.096	0.025	0.055	0.068
EE2 lo	0.126	0.088	0.094	0.111	0.051	0.053	0.068	0.109	0.064
EE2 hi	0.116	0.079	0.070	0.091	0.126	0.072	0.027	0.078	0.063
prop lo	0.103	0.100	0.112	0.115	0.150	0.117	0.137	0.057	0.064
prop hi	0.100	0.114	0.179	0.087	0.108	0.172	0.107	0.082	0.131
sert lo	0.123	0.087	0.144	0.123	0.128	0.113	0.135	0.088	0.111
sert hi	0.111	0.081	0.098	0.105	0.079	0.102	0.107	0.064	0.102

B

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
bifen	0.125	0.133	0.000	0.117	0.042	0.000	0.108	0.017	0.017
copper	0.000	0.008	0.033	0.017	0.042	0.100	0.025	0.076	0.083
EE2 lo	0.167	0.083	0.167	0.133	0.017	0.042	0.058	0.118	0.050
EE2 hi	0.142	0.050	0.058	0.100	0.126	0.083	0.042	0.050	0.083
prop lo	0.092	0.100	0.158	0.142	0.218	0.158	0.200	0.084	0.058
prop hi	0.108	0.117	0.183	0.142	0.134	0.267	0.117	0.067	0.192
sert lo	0.150	0.058	0.192	0.108	0.218	0.142	0.175	0.101	0.125
sert hi	0.092	0.117	0.125	0.117	0.101	0.125	0.133	0.084	0.108

C

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
bifen	0.644	0.912	1.000	0.895	0.994	1.000	0.917	0.983	1.000
copper	1.000	0.997	0.994	0.973	0.978	0.900	0.992	0.988	0.912
EE2 lo	0.816	0.811	0.786	0.787	0.993	0.972	0.917	0.863	0.962
EE2 hi	0.877	0.888	0.753	0.888	0.548	0.826	0.992	0.941	0.897
prop lo	0.822	0.910	0.835	0.889	0.844	0.726	0.738	0.935	0.978
prop hi	0.819	0.827	0.512	0.895	0.899	0.529	0.904	0.814	0.723
sert lo	0.803	0.978	0.785	0.860	0.880	0.940	0.763	0.934	0.921
sert hi	0.888	0.846	0.738	0.836	0.836	0.775	0.725	0.895	0.747

Left. The optimum age and duration exposure regime for each chemical/concentration, based on three scoring metrics: the lowest Brier score (A); the lowest misclassification rate (B); and the highest lower 95% bound on confidence interval (C).

Right. The top three exposure regimes for each chemical/concentration, based on the ranks of the three scoring metrics in the previous table: the best Brier scores (A), misclassification rates (B), and lower 95% bound on confidence intervals (C)

A

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
bifen			1			2			3
copper	1	3					2		
EE2 lo					1	2			3
EE2 hi			3				1		2
prop lo		3						1	2
prop hi	3			2				1	
sert lo		1						2	3
sert hi		3			2			1	

B

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
bifen			1			1			3
copper	1	2		3					
EE2 lo					1	2			3
EE2 hi		2					1	3	
prop lo	3						2	1	
prop hi	2	3					3	1	
sert lo		1		3				2	
sert hi	2				3			1	

C

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
bifen			1			1			1
copper	1	2	3						
EE2 lo					1	2			3
EE2 hi							1	2	3
prop lo		3						2	1
prop hi				3	2		1		
sert lo		1				2		3	
sert hi	2	3						1	

Counts of the number of times each age-duration combination ranks as the best, second-best, and third-best exposure regime for each chemical/concentration for all three scoring metrics,
The 6-dpf, 24-h exposure regime is the best based on having the largest number of first- and second-place wins.

	4 dpf			5 dpf			6 dpf		
	6	24	48	6	24	48	6	24	48
1s	3	3	3	0	3	2	4	6	3
2s	3	3	0	1	2	5	1	5	2
3s	2	6	2	3	1	0	1	2	7
Total	8	12	5	4	6	7	6	13	12

Based upon this diverse set of chemical exposures, we found an optimal exposure regime of 6 dpf, 24 h, based on preliminary analysis. We have not yet tested how each of the classifiers developed in Phase 2 works with samples from the other chemical exposures. Further analysis must be done to investigate the sensitivity and specificity of these classifiers.