ENHANCING THE FISH EMBRYO TOXICITY TEST: GROWTH, DEVELOPMENT ABNORMALITIES AND GENE EXPRESSION AS ADDITIONAL TEST ENDPOINTS

By

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ENHANCING THE FISH EMBRYO TOXICITY TEST: GROWTH, DEVELOPMENT ABNORMALITIES AND GENE EXPRESSION AS ADDITIONAL TEST ENDPOINTS

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ABSTRACT

In recent years, increased concern for animal welfare has resulted in the development of the fathead minnow fish embryo toxicity (FET) test as an alternative to the larval growth and survival (LGS) test. Thus far, the greatest limitation in developing and utilizing the fathead minnow FET test has been its inability to identify sublethal adverse effects or predict chronic toxicity, as the only endpoint currently utilized for the FET test is survival. The objectives of this study were to compare the sensitivities of the FET and LGS tests and to evaluate the utility of sublethal metrics as additional endpoints for improving the utility of the FET test. Fathead minnow FET and LGS tests were conducted using three reference toxicants (sodium chloride, ethanol and sodium dodecyl sulfate). Sensitivities for the FET and LGS tests were compared using the median lethal concentration (LC50) estimated by each test for each of the three reference toxicants. Estimated LC50s were not significantly different between FET and LGS tests, indicating a similar ability to assess acute toxicity. In addition to LC50, the FET tests allowed for the evaluation of the effects of sublethal concentrations of the reference toxicants on metrics such as growth, incidence of developmental abnormalities and gene expression. Reductions in fathead minnow mass, increased incidence of pericardial edema, reductions in their ability to hatch and alterations in gene expression for growth and stress at sublethal concentrations of reference toxicants suggest that the predictive power and utility of the FET test could be improved by including these sublethal metrics in toxicity assessments. These results also indicate that the fathead minnow FET test is a viable alternative to the fathead minnow LGS test.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Globally, extensive laws and regulations requiring the routine toxicity testing of chemicals and effluents released into the environment seek to protect human and environmental health [1-2]. In the United States, these tests have customarily been performed using the fathead minnow (*Pimephales promelas*) larval growth and survival (LGS) test, a method standardized by the US Environmental Protection Agency (USEPA) and determined to be valuable in estimating chemical and effluent toxicity in early life-stage fishes. However, increased legislative demand from worldwide agencies, such as the USEPA, EC (European Commission) and DEFRA (UK Department for Environment, Food and Rural Affairs) [3-5], and an enhanced public concern over the welfare of research animals during these tests has resulted in research efforts to develop alternative testing strategies that can account for non-lethal factors. One such effort is the development of embryo-based toxicity evaluation methods, such as the fish embryo toxicity (FET) test [6].

As an alternative to the LGS test, a 7-day toxicity test evaluating mortality and growth among larval fish exposed to toxicants, the FET test is a 5-day toxicity test that evaluates the mortality of fathead minnow embryos and eleutheroembryos exposed to the toxicant(s) of interest. The FET test is already standardized by the European Union, using zebrafish (*Danio rerio*) as the test organism [7]; however, the strong reliance of the USEPA on the fathead minnow as a model organism for toxicity testing makes adoption of the zebrafish FET test unlikely in the United States. While some research has been done investigating the viability of the fathead minnow FET test as a replacement for the fathead minnow LGS test [8-11], there are still some unresolved issues. First, in order for

the FET test to be considered an effective replacement for the LGS, its relative sensitivity must be determined to ensure that the FET test results are comparable to those from the LGS tests. Also, while the LGS test has the ability to evaluate both acute and chronic toxicity, with measurements of mortality and growth (mass and length) [12], the current FET test only allows for the evaluation of acute toxicity, as the only measured endpoint is mortality [7].

As a direct response to the aforementioned issues, the overall goal of the current study was to enhance the application of the fathead minnow FET test as an alternative toxicity testing strategy, with two experimental objectives in mind. The first objective was to compare the sensitivity of the FET test to that of the LGS test. Mortality data from both the FET and LGS tests (conducted using the same three toxicants) was used to estimate median lethal concentration (LC50) data (i.e., the concentration at which 50% of the test organisms die). The FET and LGS-determined LC50 were then compared to one another, allowing for comparison of sensitivity. The second objective was to enhance the FET test by evaluating the utility of sublethal metrics to increase test sensitivity and to allow for the estimation of chronic toxicity. Endpoints such as growth, developmental abnormalities (e.g., edema, inability to hatch, etc.) and both stress- and growth-related gene expression were evaluated for their ability to increase the sensitivity of the FET test, thereby allowing for the future possibility of evaluating chronic toxicity.

MATERIALS AND METHODS

Production of embryos and eleutheroembryos

Test organisms for the LGS and FET tests were collected from a sexually mature stock of adult fathead minnows housed at Texas Christian University. Embryos were

obtained by placing 10 cm long pieces of 10.2 cm diameter polyvinyl chloride schedule 40 pipe into individual aquariums to serve as spawning structures. Structures were checked daily for the presence of embryos. Once identified, embryos were collected and either transferred into aerated 1L beakers (incubated at 27° C) for use in LGS tests or transferred directly into test solutions for use in FET tests [7]. Embryos collected for use in LGS tests were maintained in the beakers for 3-4 days until hatch, utilizing both daily water changes and removal of unhealthy organisms to ensure survival.

Test chemicals

Fathead minnow LGS and FET tests were conducted using three common reference toxicants: sodium chloride (NaCl), sodium dodecyl sulfate (SDS) and ethanol (EtOH). FET tests were conducted three times per chemical, whereas LGS tests were conducted 4 times per chemical. Solutions for NaCl tests were made as 4-L stock solutions for use throughout the tests, while SDS and EtOH solutions were made daily as needed. All solutions were made by dissolving the associated toxicant in dechlorinated water, followed by a series of 1-to-1 dilutions using water warmed to 27° C. Solution concentrations varied between chemical and test type and are listed in Table 1. Additionally, a positive control of 3,4-dichloroaniline (DCA) (Table 1) and a negative control of dechlorinated water were used in each test.

Table 1. Concentrations of the chemical solutions used in the FET and LGS test exposures.

Chemical	FET Test Concentrations	LGS Test Concentrations
NaCl	16, 8, 4, 2, 1 (ppt)	16, 8, 4, 2, 1 (ppt)
EtOH	40, 20, 10, 5, 2.5, 1.25 (mL/L)	40, 20, 10, 5, 2.5, 1.25 (mL/L)
SDS	30, 15, 7.5, 3.75, 1.88 (mg/L)	60, 30, 15, 7.5, 3.75, 1.88 (mg/L)
DCA	16 (mg/L)	1.5 (mg/L)

Water quality

Water quality data was recorded on a daily basis to ensure a minimal effect of extraneous variables on the tests. Individual water quality traits (i.e., temperature, pH, conductivity, salinity, alkalinity and hardness) were measured in renewal test solutions and dilution water as well as the individual test chambers. Data was collected using certified water quality kits and calibrated water quality meters.

LGS protocol

The method used to conduct the fathead minnow LGS tests was based on a guideline previously set forth by the USEPA [12]. Ten eleutheroembryos (<five hours posthatch) were used for each concentration of test solution as well as the positive control and the negative control. The eleutheroembryos were transferred from the beakers they were being held in to their 300mL glass crystallizing dishes, which contained 250 milliliters of test solution. Each test chamber was placed in an incubator set to 27° C and then covered with a piece of acrylic sheeting to reduce evaporation and aerated. The light cycle inside the incubator was set to 16 hours of light and 8 hours of dark. To ensure proper water quality, test chambers were gently aerated and test solutions were renewed with ~90% volume solution changes on a daily basis. Beginning on the second day (24 to 48 hours post-hatch), each test chamber was administered approximately 0.03 g of *A. nauplii* twice daily (morning and evening). Mortality of the eleutheroembryos and larvae was recorded daily upon observation, and at the conclusion of the test (168 hours), surviving larvae were euthanized with tricaine methanesulfonate (MS-222), measured for their length and mass and then flash-frozen with liquid nitrogen for gene expression analysis in the future.

FET protocol

The method used to conduct the fathead minnow FET tests was based on a guideline previously set forth by the OECD (Organisation for Economic Co-operation and Development) [7]. Twenty embryos (≤32 cell stage) were used for each concentration of test solution as well as the positive control (DCA) and negative control (dechlorinated H_2O). Each set of twenty embryos was examined under a microscope in petri dishes containing test solution in order to confirm their cell stage and then transferred to their respective polystyrene 24-well culture plates (pre-soaked for 24 hours), in which twenty of the wells were filled with two milliliters of test solution and the remaining four wells were filled with dechlorinated water to serve as an internal controls for the test chamber. Each test chamber was then covered with the pre-packaged lid and set in an incubator maintained at 27° C under a light cycle of 16 hours of light to 8 hours of dark. To ensure proper water quality, test solutions were replaced in each test chamber at a volume minimum of 90% on a daily basis. Both mortality and the presence of developmental abnormalities were recorded daily upon observation under an inverted microscope, and at the conclusion of the test (120 hours) surviving embryos and eleutheroembryos (unaltered embryos tend to hatch around 96 hours post-fertilization) were euthanized with MS-222, measured for their mass and then flash-frozen in liquid nitrogen for subsequent gene expression analysis.

Evaluation of sublethal metrics

In addition to survival, sublethal endpoints were also evaluated. These endpoints included growth, developmental abnormalities and the expression of genes associated with growth and toxicity. Growth was evaluated using calipers and an analytical balance at the conclusion of LGS tests by measuring both length and mass of individual larvae,

and at the conclusion of FET tests by measuring a pooled larval mass of two embryos or eleutheroembryos. Developmental abnormalities (e.g., edema, inability to hatch, etc.) observed in FET tests were recorded daily upon observation with a microscope. Gene expression analysis of samples from both LGS and FET tests was completed via qPCR as described in Jeffries et al. 2015 [8]. RNA was extracted from the tissues using a Maxwell Research System, followed by conversion of the RNA to cDNA via the iScript cDNA synthesis, followed by real-time qPCR using SyberGreen with a CFX Connect real-time PCR detection. The expression of the target genes (Table 2) was calculated via the standard curve method using ribosomal L8 as a reference gene.

Table 2. Target genes analyzed in this study and their respective function

Target Gene	Gene Function
Insulin-like growth factor (*igf1*)	Primary growth factor during early development
Growth hormone (*gh*)	Induces the production of igf; Associated with growth and development
Growth hormone receptor (*ghr*)	Receptor for growth hormone
Heat shock protein 70 (*hsp70*)	A biomarker of stress; associated with the generalized stress response
Corticotropin-releasing factor (*crf*)	Hormone that stimulates the production of stress hormones
Glucocorticoid receptor (*gcr*)	Receptor for stress hormones; involved in generalized stress response

Data analysis

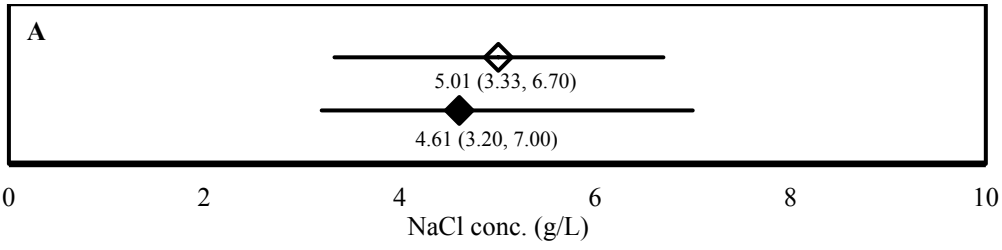
The data from this study was analyzed using the statistical software package JMP 11.0 (SAS Institute). Mortality data from each test replicate was used to calculate LC50 values for each test and associated toxicant via a log-logistic regression. To compare the LC50s determined via LGS and FET tests, the overlap (or lack thereof) between the LC50 95% confidence intervals was examined. If an overlap in confidence intervals was observed, then no significant difference was detected between tests with regard to LC50 data. However, if there was not an overlap in confidence intervals, the difference was considered to be significant. Developmental abnormality data was used to calculate

effective concentrations to induce an abnormality in 25% of the population (EC25) and the concentrations of toxicants capable of impeding developmental markers in 25% of the population (IC25) via a log-logistic regression, which could then be compared to LC50 data to help determine its usefulness in increasing the sensitivity of the FET test. Growth data was analyzed by one-way analysis of variance (ANOVA) and a post hoc Dunnett's multiple comparisons test to determine which groups differed significantly from the control. A nonparametric comparison using the Wilcoxon method was used to determine differences between groups when it was established that the variances were not equal among groups. Relative expression of messenger RNA (mRNA) of target genes was analyzed by ANOVA and a post hoc Dunnett's multiple comparisons test to determine which groups differed significantly from the control. A nonparametric comparison using the Wilcoxon method was used to determine differences between groups when it was established that the variances were not equal among groups.

RESULTS

Toxicity of reference toxicants

Median lethal concentration (LC50) values for FET tests were not found to be significantly different from those calculated from the LGS tests for any of the reference toxicants (Figure 1).



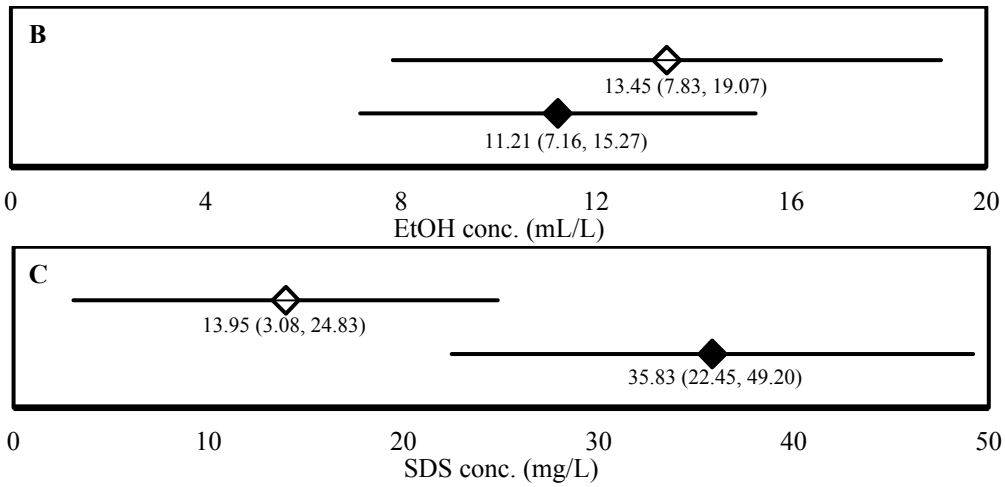


Figure 1. The median lethal concentrations (LC50s) of NaCl (**A**), EtOH (**B**) and SDS (**C**) as determined via the fathead minnow fish embryo toxicity (FET) test (open diamond) and larval growth and survival (LGS) test (closed diamond). The lines to the left and right of the LC50 indicate the 95% upper and lower confidence intervals. The LC50 and 95% confidence intervals are also numerically represented under each line.

Growth as a FET test endpoint

Significant reductions in mass were observed in FET tests conducted with NaCl (ANOVA, $p = 0.03$; Figure 2) and EtOH (ANOVA, $p < 0.01$; Figure 2), but not in those conducted with SDS (ANOVA, $p = 0.57$; Figure 2).

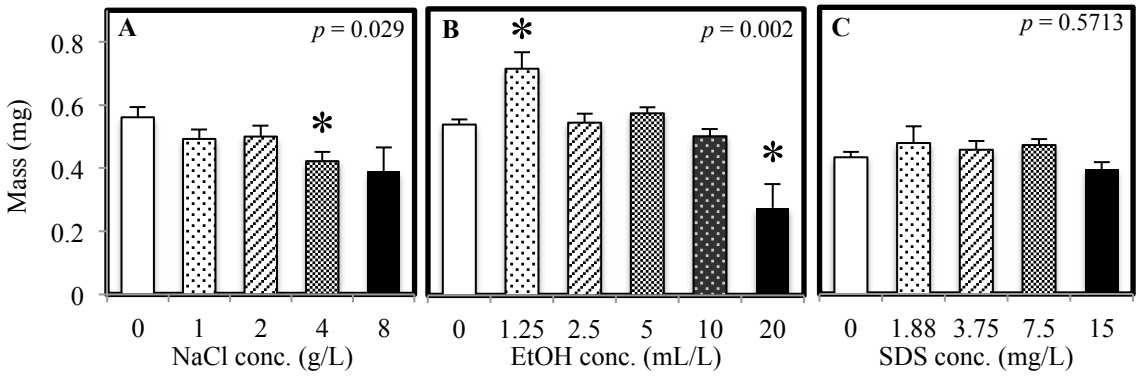


Figure 2. The mass of minnows from FET tests with NaCl (**A**), EtOH (**B**) and SDS (**C**). * indicate significant differences from the control group. Error bars represent standard error.

Development abnormalities as FET test endpoints

The most commonly observed developmental abnormalities identified during the FET tests were increased presence of pericardial edema and increased inability to hatch.

Edema was observed in minnows from FET tests conducted with NaCl, EtOH and SDS in a dose-dependent manner; however, significant differences from the control group were only detected in the 20 mL/L EtOH exposure group. The frequency of edema occurrence, as well as the associated EC25, is shown in Figure 3.

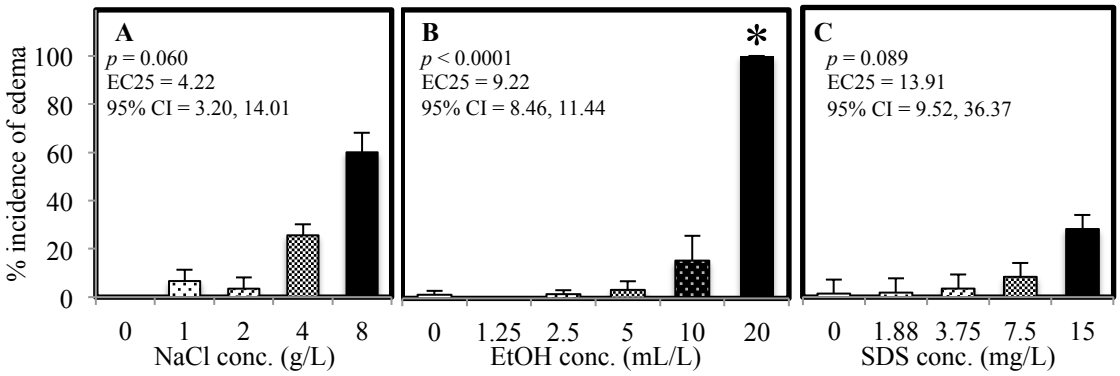


Figure 3. Percentage of minnows displaying edema following FET tests with NaCl (**A**), EtOH (**B**) and SDS (**C**). * indicate significant differences from the control group. Error bars represent standard error.

Inhibition of hatch was observed with minnows in FET tests conducted with EtOH and SDS; however, significant differences from the control group were only detected in the 20 mL/L EtOH exposure group. The frequency of test organisms that did not hatch, as well as the associated IC25, is shown in Figures 4.

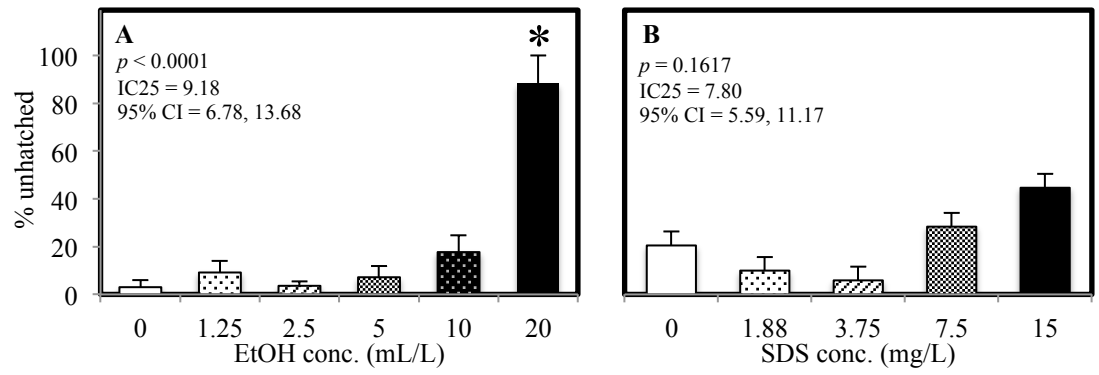


Figure 4. Percentage of unhatched minnows at the end of FET tests with NaCl (**A**), EtOH (**B**) and SDS (**C**). * indicate significant differences from the control group. Error bars represent standard error.

Gene expression as a FET test endpoint

Among fathead minnows exposed to EtOH during the FET tests, significant differences in the mRNA expression of *igf1* were observed (ANOVA, $p < 0.0001$; Table 3), with minnows in both the 10 and 20 mL/L exposure groups having significantly higher expression than the controls. Significant differences were also noted in the mRNA expression of *gh* (ANOVA, $p = 0.0015$; Table 3), with minnows in the 20 mL/L exposure group having significantly lower expression than those from the control group. Finally, significant differences in the mRNA expression of *hsp70* were detected (Welsh ANOVA, $p = 0.0239$; Table 3), with minnows in the 20 mL/L exposure group having significantly higher expression than the controls. Significant differences were not found in mRNA levels of *ghr*, *crf*, or *gcr* among minnows exposed to EtOH (ANOVA, $p > 0.19$; Table 3).

Among fathead minnows exposed to SDS during the FET tests, statistical analysis revealed significant differences in the mRNA expression of *igf1* (ANOVA, $p = 0.0254$; Table 3); however, post hoc multiple comparisons (Dunnett's, Tukey-Kramer) revealed that none of the exposure groups significantly differed from the control group. Significant differences were not found in mRNA levels of *gh*, *ghr*, *hsp70*, *crf* and *gcr* among minnows exposed to SDS (ANOVA, $p > 0.07$; Table 3).

No significant differences were found in gene expression among the minnows exposed to NaCl (ANOVA, $p > 0.20$; Table 3).

Table 3. The mean (± standard error) relative mRNA expression of target genes associated with growth and stress in fathead minnows subjected to fish embryo toxicity tests with exposures to NaCl, EtOH and SDS.

	igf1	*Gh*	*ghr*	*hsp70*	*crf*	*Gcr*
NaCl FET (g/L)						
Control	3.97 (1.47)	--	0.70 (0.23)	0.84 (0.19)	0.82 (0.10)	--
1	4.05 (0.39)	--	0.55 (0.13)	0.75 (0.12)	0.86 (0.18)	--
2	6.10 (2.49)	--	0.48 (0.12)	0.52 (0.07)	1.05 (0.30)	--
4	2.93 (1.21)	--	0.68 (0.22)	1.23 (0.35)	0.81 (0.17)	--
8	2.47 (1.49)	--	0.50 (0.18)	2.63 (1.39)	1.08 (0.17)	--
16	NS	--	NS	NS	NS	--

EtOH FET (mL/L)						
Control	0.46 (0.03)	0.76 (0.09)	0.59 (0.07)	0.22 (0.09)	1.05 (0.12)	2.68 (0.26)
1.25	0.52 (0.05)	0.88 (0.09)	0.54 (0.07)	0.42 (0.10)	0.95 (0.17)	2.78 (0.40)
2.5	0.50 (0.03)	0.70 (0.09)	0.57 (0.08)	0.21 (0.09)	0.94 (0.09)	2.85 (0.19)
5	0.51 (0.08)	0.72 (0.09)	0.66 (0.09)	0.18 (0.10)	1.19 (0.15)	2.94 (0.21)
10	0.62 (0.04)*	0.64 (0.09)	0.66 (0.09)	0.27 (0.09)	1.13 (0.10)	3.01 (0.23)
20	0.87 (0.10)*	0.12 (0.13)*	0.70 (0.16)	1.08 (0.14)*	1.54 (0.80)	3.99 (0.67)
40	NS	NS	NS	NS	NS	NS
SDS FET (mg/L)						
Control	0.44 (0.04)	0.77 (0.12)	0.75 (0.01)	0.17 (0.07)	0.70 (0.13)	2.62 (0.21)
1.88	0.56 (0.06)	0.88 (0.17)	0.77 (0.08)	0.11 (0.01)	0.97 (0.13)	2.95 (0.30)
3.75	0.48 (0.03)	0.59 (0.10)	0.71 (0.06)	0.14 (0.03)	0.90 (0.10)	2.61 (0.32)
7.5	0.42 (0.03)	0.44 (0.06)	0.71 (0.06)	0.17 (0.03)	0.79 (0.04)	2.63 (0.27)
15	0.37 (0.02)	0.52 (0.12)	0.65 (0.06)	0.29 (0.09)	0.91 (0.08)	2.55 (0.31)
30	NS	NS	NS	NS	NS	NS

* Significant difference in the expression of the target gene relative to organisms in the control group
NS = no survivors

DISCUSSION

Comparison of test sensitivity

In order for the FET test to be considered an effective replacement for the LGS, the sensitivity of both the FET and LGS test must be evaluated and compared to ensure that the results of the FET are predictive of those of the LGS. To compare the sensitivity of the FET and LGS tests, LC50 values (i.e., the concentration of a toxicant at which 50% of the test organisms die) generated via the FET tests were compared to those generated via the LGS tests. The data from tests conducted in this study indicates that the fathead minnow FET and LGS are similar in regard to their ability to predict the acute toxicity of NaCl, EtOH and SDS. Previous studies have found this to be true for other test chemicals including NH_3 [9, 13]. However, this is not always the case as some previous research has shown that exposure to other chemicals, such as DCA, has resulted in differences in test sensitivity [8-9]. To increase the fathead minnow FET test's viability as an alternative to the LGS, additional research using an increased number and variety of

toxicants is required to further provide evidence (in the form of LC50 values) of the ability of the FET to effectively assess acute toxicity.

Utility of growth as a FET test endpoint

The first sublethal metric evaluated for its usefulness as an additional endpoint for the FET test was growth. Given that growth is a standardized and useful metric used in the LGS test [12, 14], it seemed plausible that it could enhance the FET test as well. Measurements of wet weight (previously determined to be the most reliable method of measurement [15]) recorded upon conclusion of the FET tests revealed significant reductions in mass among organisms subject to the NaCl and EtOH FET tests. Similar results have indicated the ability to detect alterations in mass of fathead minnows as a result of early life stage exposures, such as Mager et al. 2010 [16] in which fathead minnow embryos exposed to lead experienced significant alterations in growth after as early as four days of exposure [8-9, 17]. Although there are a large number of factors that influence the growth of an embryo before hatch (especially in poor environmental conditions)[18-19], this data provides promising evidence of the utility of growth data as an endpoint in the evaluation of the chronic toxicity of a chemical or effluent. Future studies should seek to continually establish reductions in growth as a common observation upon chemical exposure, and to calculate IC25 values (i.e., the concentration of a toxicant required to inhibit a developmental marker in 25% of the test organism population) for a variety of chemicals. Comparison of IC25 values with the known LC50 values would give insight to the sensitivity of growth as an endpoint in relation to mortality. It would also be beneficial to conduct studies on the delayed effects of stunted

growth that would not be detected until months after the initial exposure (e.g., reproduction, development, selection by prey).

Utility of development abnormalities as a FET test endpoint

The next sublethal metric to be evaluated as a possible additional endpoint for the FET test was the incidence of physical irregularities. The two most commonly observed developmental abnormalities in this study were the presence of pericardial edema and altered ability to hatch. Pericardial edema is a well-documented abnormality that has routinely been shown to present itself following exposures to a variety of chemicals including antifoulants, flame retardants, heavy metals, pesticides, polycyclic aromatic hydrocarbons, etc. [20-24]. In the present study, pericardial edema was observed among organisms from FET tests with all three toxicants. It is important to note that the prevalence of the edema was dose-dependent, with increasing incidence correlating with increasing exposure doses. The other commonly observed abnormality was an increased inhibition of the ability to hatch on the final day of the FET test, which was seen in tests exposed to EtOH and SDS. Although the FET test is only 120-hours long, fathead minnow embryos are expected to hatch within 96 to 120 hours post-fertilization under the FET test conditions [25]. Interestingly, recent studies have found that significant reductions in the ability to hatch is a common response to embryonic exposures with chemicals, such as fungicides, antifoulants, chitosan nanoparticles, etc. [20, 26-27].

Although these results provide promising evidence of the utility of development abnormalities to be used as an endpoint in the evaluation of the toxicity of a chemical or effluent, caution must be taken when discussing its ability to increase the sensitivity of the FET test. The EC25 (i.e., the concentration of a toxicant required to induce an

abnormality in 25% of the test organism population) and IC25 values calculated from the edema and hatch data for each test chemical fall within the 95% confidence interval of the LC50 values, indicating that survival is just as sensitive of a parameter as the presence of edema or the inhibition of hatch. However, other recent studies have produced data in support of these metrics improving FET test sensitivity [8], and regardless, the inclusion of metrics such as these can provide other information about the potential hazards of chronic exposure associated with the chemical or effluent tested. Research such as that done by Hicken et al. 2011 [28], in which embryos were exposed to low concentrations of oil and experienced sublethal and delayed effects on heart development and function which were identified a year after the initial exposure, suggests that these development abnormalities serve as potential markers of negative future consequences that can negatively impact an organisms ability to survive. Therefore, it is recommended that future studies continue to explore the inclusion of these two development abnormalities as endpoints for the fathead minnow FET test by evaluating the negative repercussions of these abnormalities months after the exposure, thereby illustrating the detrimental consequences such exposures can have on the survival of the organism apart from mortality directly.

Utility of gene expression as a FET test endpoint

The final sublethal metric assessed for utilization as an endpoint for the FET test was the expression of growth- and stress-related genes in the eleutheroembryos sampled upon conclusion of the FET tests. Although previously utilized [8], the inclusion of molecular endpoints, such as the expression of genes, is a relatively new approach to enhancing the FET test. In the present study, significant alterations in gene expression

were only seen with groups exposed to EtOH. *Igf1*, a gene that encodes for insulin-like growth factors involved in physiological processes such as metabolism and growth [29], was significantly downregulated. EtOH exposure groups displaying this alteration also displayed significant reductions in growth, suggesting that *igf1* expression could possibly be an indicator of impaired growth. However, *gh*, also associated with metabolism and growth [29], was found to be significantly upregulated in the same EtOH exposure groups that experienced significant reductions in mass. This seems counterintuitive based on the altered *igf1* expression, although it is hypothesized that the upregulation of *gh* is functioning in a compensatory manner for the lack of growth or *igf1* expression. *Hsp70*, most often expressed in response to environmental stress [30], was the final gene to exhibit altered expression. Therefore, it should not come as a surprise that the fathead minnows exposed with EtOH during the FET tests would exhibit increased expression of *hsp70*, as exposure to chemicals is stressful to any organism. Given this line of thinking, significant alterations in other stress-related genes would be expected. However, this was not the case, as *crf* and *gcr* were not altered. Given that *crf* and *gcr* are part of the same pathway, as *crf* regulates production of glucocorticoids and *gcr* regulates the binding of glucocorticoids [31], it is not surprising that they showed similar expression results. While it is possible that *hsp70* was simply more sensitive to stress with regard to the short-term exposure, it is hypothesized that differences in expression could be due to tissue specificity, as gene expression data can only be collected from whole organisms and not individual tissues. It is also important to note that previous gene expression research with the FET test detected similar significant alterations in *igf1* and *hsp70* [8].

Although alterations were seen in the expression of multiple genes, several issues were identified with utilization of this sublethal metric as an additional FET test endpoint. First, significant alterations in gene expression were only observed in the embryos from the EtOH FET tests, just one of the three toxicants used in this study. This suggests that gene expression cannot be used as a stand alone sublethal endpoint, as not all chemicals produce alterations indicative of the toxic effects on the organism displayed through alterations in growth and development. Additionally, the difference in the expression of genes associated with the same function (i.e., growth, stress) suggests that gene expression data is less sensitive than whole organism data. Given the lack of results from both this and prior studies [8], future studies should focus on developing ontogenetic expression profiles of genes related to growth and stress with the hope of identifying genes that most consistently result in altered expression, and the time at which measuring the expression of these genes would be most valuable.

CONCLUSION

The results from the fathead minnow FET and LGS tests allow for several conclusions to be drawn. First, this study provided evidence that the FET and LGS were similarly sensitive to the effects of three reference toxicants. However, due to the limited number of chemicals tested, and previous research suggesting that the tests are not similarly sensitive to every chemical, the results of this study should serve as a stepping-stone, along with future studies involving a larger range and number of chemicals, towards building the relationship between the tests. Second, this study identified potential avenues by which to increase the utility of the FET test using sublethal metrics. Specifically, endpoints including growth, edema and hatch rates and expression of genes such as *igf1* and hsp70, which experienced significant alterations and were backed by previous research, were identified as potentially being useful in updating this test. Future research exploring these and other alternative endpoints will be key in establishing this test as useful method by which to assess both acute and chronic toxicity.

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