DEVELOPMENT OF CARDIOVASCULAR AND NEURODEVELOPMENTAL METRICS AS SUBLETHAL ENDPOINTS FOR THE FISH EMBRYO TOXICITY TEST

By

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Master of Science, 2017

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Fort Worth, Texas

Submitted to the Graduate Faculty of the

College of Science and Engineering

Texas Christian University

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

May 2020

ACKNOWLEDGEMENTS

I would like to first thank my advisor Dr. Marlo Jeffries. The undertaking of this project has been a rollercoaster from start to finish and she has been there to support me throughout it all. I could not have completed this project without her continuous support. I would also like to thank my lab mates for all their help. Every single one of them has helped with my project over the past 3 years and their help has been invaluable. I would specifically like to thank Leah Thornton Hampton for being a wonderful desk mate and crucial emotional support. I would also like to thank my partner Christian Olinger for his unconditional support. Finally, I would also like to thank my parents, Doris and Jim Krzykwa, for their endless encouragement of my interest in science. I might not have done this if they had not sent me to every science camp possible when I was a child and if they had not taken a chance by sending me to an undergraduate institution that emphasized research over having a GPA. While my father is not here to see me finally graduate and get a real job, I know he would have been proud that I stuck with it.

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Chapter 1: Introduction

In the 1960s and 70s, there was an increase in public concern about environmental protection and quality stemming from the publication of Silent Spring (Carson 1962) and from key adverse environmental events such as the burning of the industrial-effluent contaminated Cuyahoga River in 1969 (Adler 2002). This book and environmental events brought to light the adverse impacts widespread pesticide use was having on non-target species. In response to public outcry, the United States Environmental Protection Agency (US EPA) was created in 1970 and tasked with developing and implementing policies aimed at improving both air and water quality. To aid the agency in doing so, Congress passed the Clean Water Act (CWA, aka the Federal Water Pollution Control Act) in 1972 and the Toxic Substances Control Act (TSCA) in 1976. Under the CWA, point sources of pollution such as industrial facilities and sewage treatment plants, must routinely verify that any effluent released by their facility is within water quality standards (US Congress 1972). The TSCA targets specific compounds (e.g., formaldehyde, polymers, and nanoparticles) and enables the US EPA to assess and regulate new compounds before they enter commercial markets (US Congress 1976). The TSCA was recently amended via the Frank R. Lautenberg Chemical Safety for the 21st Century Act in 2016, which allows the US EPA to request that industrial producers generate the toxicity data needed to determine if their compounds are a potential threat to the environment (US Congress 2016). In 2006, similar legislation titled Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) was passed in the European Union. Under REACH, all chemicals sold on the consumer market must be evaluated for their toxicity by industrial producers (EU 2006).

Legislative efforts to minimize surface water contamination have been largely successful, as evidenced by a decrease in the presence of industrial contaminants, such as heavy metals and organochlorines (Santschi et al. 2001), reduced impacts of municipal water treatment plants on nearby water bodies (Stein and Cadien 2009) and by the population recoveries of species that had been adversely impacted by contamination of water ways such as ospreys (Bierregaard et al. 2014) and bald eagles (Watts et al. 2008). However, such efforts to improve environmental quality are not without their costs. When REACH was passed in 2006, it was estimated that it would cost the chemical industry between €3.2–9.5 billion ($3.4–10.1 billion) to

complete all necessary chemical evaluations (Breithaupt 2006; Rovida and Hartung 2009). In addition to the monetary cost of running extensive toxicity assessments, it was also estimated that 10–54 million vertebrate animals, including 1 million fish, would be required to carry out all necessary toxicity tests (Höfer et al. 2004; Rovida and Hartung 2009).

Fish have long been used as model organisms in aquatic toxicology, and there are several standardized toxicity test methods utilizing fish. These testing methods cover a wide range of endpoints including 96-h acute toxicity tests using juvenile and/or adult fish (OECD 203, US EPA OCSPP 850.1075), early-life-stage chronic toxicity studies (OECD 210, 212, US EPA methods 1000.0 and OPPTS 850.1400), and tests that focus on more specific adverse impacts such as the 21-d fathead minnow (*Pimephales promelas*) reproductive toxicity test (US EPA 2002a). One of the common test methods used in the US is the fathead minnow larval growth and survival (LGS) test (US EPA 2002b), which is used for estimating the toxicity of effluents to ensure compliance with water quality guidelines as mandated by the CWA. In the LGS test, newly hatched (< 24 h post hatch) larval fathead minnows are exposed to a minimum of 5 concentrations of effluent (Fig. 1). After a 7-d exposure period, mortality and growth are assessed to estimate acute and chronic toxicity, respectively.

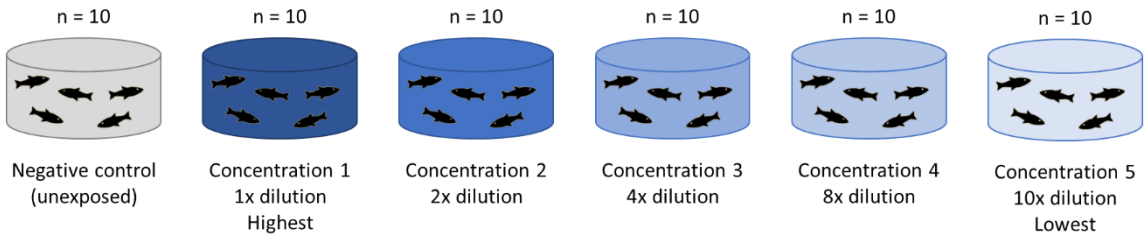

Figure 1. Schematic of general design of the larval growth and survival test. Each test is repeated four times. Mortality is evaluated daily and growth is measured at the end of the test at 7 d.

These tests have been well validated and are widely used (Pickering 1974; DeGraeve et al. 1991); however, there is a need to re-evaluate their use given legislative demands calling for a reduction in the number of vertebrate animals used in toxicity testing. Both REACH and the Frank R. Lautenberger Act, which call for increased toxicity testing, also mandate that alternatives to vertebrate testing methods be used whenever possible (EU 2006; US Congress 2016) to further comply with the 3Rs of animal testing: replacement of methods that use vertebrate animals, reduction in the number vertebrate animals used, and

refinement of methods to reduce pain and distress (Russell and Burch, 1959). In addition to enhancing animal welfare, it has been suggested that embracing alternatives to animal testing methods may be economically advantageous by reducing the financial burden of REACH on the chemical industry (Combes et al. 2004). While a wide variety of potential toxicity testing methods that do not use vertebrate animals as model organisms are being developed, including *in vitro* methods and computational modeling of compounds (Höfer et al. 2004; Tanneberger et al. 2013), one proposed alternative is the use of fish embryos in place of older, more-developed organisms (Lammer et al. 2009; Embry et al. 2010; Knöbel et al. 2012; Strähle et al. 2012; Belanger et al. 2013). Using fish embryos in place of more-developed organisms is considered a refinement to current fish-based testing methods per US guidelines, because it is thought that embryos experience less distress during toxicant exposures than older fish (EFSA 2005; Strähle et al. 2012). In addition, under current EU regulations embryonic fish are not regulated as protected vertebrate organisms until they have hatched and are free feeding (EFSA 2005), making fish embryo testing methods a replacement per the 3Rs.

Because of the interest in using fish embryos as an alternative to standard fish-based toxicity testing methods, a fish embryo toxicity (FET) test using zebrafish (*Danio rerio*) was developed as an alternative to the OECD 203 fish acute toxicity test (Schulte and Nagel 1994; OECD 2013). In this test, zebrafish embryos < 1.5 h post fertilization (hpf) are exposed to a minimum of 5 concentrations of effluent or chemical, as well as a positive control (Fig. 2). At the conclusion of the 4-d exposure period, mortality—as indicated by coagulation, lack of somite development, lack of tail bud detachment, or lack of heartbeat—is used to estimate acute toxicity (OECD 2013). Since the original proposal of the FET test in 1994 (Schulte and Nagel 1994), it has undergone extensive validation, has been approved as an OECD test method (OECD 236; OECD 2013), and has become the standard test method for the assessment of whole effluent in Germany (DIN 2003). Despite the approval of the FET test as a method for assessing whole effluent toxicity, US

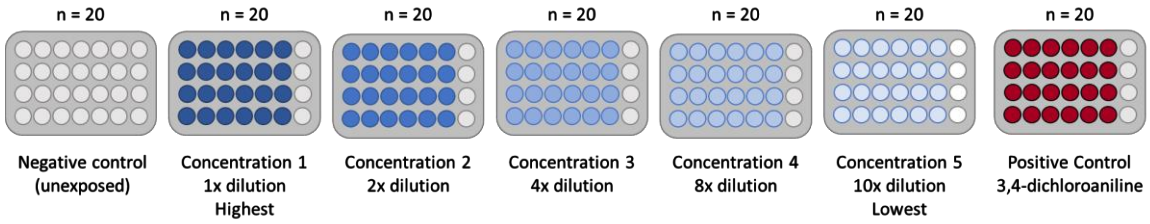

Figure 2. Schematic of the general design of the fish embryo toxicity test. Each test is repeated three times. Mortality is evaluated daily.

regulatory agencies have not yet approved this test method as an alternative to the LGS test. This is likely due to 3 factors: 1) the FET test utilizes zebrafish rather than the US EPA's favored fathead minnow (Ankley and Villeneuve 2006), 2) the FET test lacks endpoints capable of estimating chronic toxicity and sublethal adverse effects (US EPA 2002b; OECD 2013), and 3) the FET test has been found to be less sensitive than the LGS test for some chemicals, particularly those classified as neurotoxicants (Klüver et al. 2015).

To broaden the appeal of the FET test, the FET test protocol has been modified for use with other model fish species, including the fathead minnow (Braunbeck et al. 2005; Jeffries et al. 2014). This work has demonstrated that zebrafish FET test methods can be easily transferred to the fathead minnow with only minor modifications, such as increasing the test duration to 120 h for fathead minnow FET tests. There have also been investigations aimed at identifying additional sublethal metrics for use as FET test endpoints to expand the predictive power of the FET test beyond the estimation of acute toxicity. Proposed endpoints include the expression of genes related to development (Voelker et al. 2008; Adeyemo et al. 2015; Jeffries et al. 2015; Krzykwa et al. 2018; Roush et al. 2018), the presence of developmental abnormalities, such as pericardial edema (Selderslaghs et al. 2012; Jeffries et al. 2015; Krzykwa et al. 2018; Roush et al. 2018; Krzykwa et al. 2019), and embryonic behaviors such as spontaneous contraction (Selderslaghs et al. 2013). The inclusion of sublethal endpoints may also improve the sensitivity of the FET test for compounds that do not show a strong correlation between the FET test and other test types (Klüver et al. 2015; Glaberman et al. 2017). For example, two easily-measurable and cost-effective parameters that have been demonstrated to have potential for inclusion as sublethal FET test endpoints are eye size and pericardial area (Krzykwa et al. 2018; Krzykwa et al. 2019).

Alterations in eye size, which have been observed in response to a wide variety of neurotoxicants including heavy metals (Kim et al. 2013; Wold et al. 2017), pesticides (Cook et al. 2005), and polycyclic aromatic hydrocarbons (Sørhus et al. 2017) are linked to altered neurological function (Cheng et al. 2000; Kim et al. 2013; Wold et al. 2017). Specifically, embryonic alterations in eye size have been associated with the altered development of neural tissues such as reduced brain size (Cheng et al. 2000; Wold et al. 2017), alterations in embryonic behavior (Kim et al. 2013; Wold et al. 2017), and reduced visual acuity (Bilotta et al. 2002) in zebrafish.

Pericardial edema is of particular interest as a potential FET test endpoint because it occurs in response to a wide range of chemicals with different modes of action, including pharmaceuticals (Carlsson and Norrgren 2004; Akande et al. 2010), dioxins (Henry et al. 1997; Belair et al. 2001; Hill 2004; Antkiewicz et al. 2005), polycyclic aromatic hydrocarbons (Colavecchia et al. 2006; Ana dos Anjos et al. 2011; He et al. 2012), and heavy metals (Cheng et al. 2000; Chen et al. 2015). In addition to pericardial edema being associated with abnormal cardiovascular development, these observed alterations to cardiovascular development can have lasting effects. For example, fish exposed as embryos to polycyclic aromatic hydrocarbons at concentrations that induced pericardial edema also displayed reductions in swimming performance and respiration as juveniles and adults, indicating that pericardial edema may be an early indicator of long-term cardiovascular dysfunction (Hicken et al. 2011; Mager et al. 2014; Incardona et al. 2015).

While a wide variety of metrics are being investigated as additional FET test endpoints, developmental abnormalities such as eye size and pericardial edema may be particularly useful, because they occur in response to a wide variety of toxicants including, but not limited to, heavy metals (Cheng et al. 2000; Kienle et al. 2009; Cheng et al. 2013; Zhu et al. 2014; Chen et al. 2015; Wold et al. 2017), pesticides (Henry et al. 1997; Cook et al. 2005; Weichert et al. 2017), and polycyclic aromatic hydrocarbons (Incardona et al. 2004; Colavecchia et al. 2006; Ana dos Anjos et al. 2011; He et al. 2012; Sørhus et al. 2017). In addition, the quantification of developmental abnormalities is relatively easy and does not require specialized equipment beyond what is already needed to conduct FET tests, making inclusion of such endpoints feasible and cost

effective. One of the main limitations preventing the inclusion of these sublethal metrics as FET test endpoints is the lack of information regarding their ability to predict longer-term adverse effects or chronic toxicity (Krzykwa et al. 2018; Krzykwa et al. 2019).

This project will address this gap in knowledge by investigating whether alterations in these potential sublethal endpoints for the FET test (eye size and pericardial edema, quantified as pericardial area) are predictive of sublethal adverse effects later in life as measured by a variety of assays (i.e., feeding, optomotor response, C-start, and swimming performance assay). To achieve this goal three major objectives were met:

1. Develop and validate methods for assessing neurological function and behavior in fathead minnow larvae. Many of the assays available for assessing neurological function have not been standardized and are used primarily in zebrafish larvae (Peyster and Long 1993; Orger et al. 2004; Abdel-moneim et al. 2015). Therefore, it was first necessary to identify methods that could be transferred to larval fathead minnows. To that end, two subobjectives were met:
    a. Evaluate the influence of various testing parameters (e.g., acclimation time, volume of water in chambers, necessary sample sizes, etc.) on the optomotor response of larval fathead minnows and develop standardized methods for evaluating optomotor response in larval fathead minnows. (Chapter 2)
    b. Identify sensitive behavioral assays by comparing the responsiveness of three common assays (i.e., feeding, optomotor response, and C-start assay) in larval fathead minnows exposed to the known neurotoxicant, chlorpyrifos. (Chapter 3)
2. Identify and validate methods for assessing cardiovascular function in early life stage fathead minnows. Methods for assessing cardiotoxic impacts on fish typically utilize either juveniles from larger species, such as salmonids (Wilson and Wood 1992), or adult fathead minnows (Kolok et al. 2004). Therefore, it was necessary to identify a suitable method for assessing cardiovascular function in larval fathead minnows. Two subobjectives were met:

a. Modify two methods for assessing cardiovascular function, the spinning task assay and the laminar flow assay, for use with larval fathead minnows and determine if the spinning task assay is a suitable alternative to the laminar flow assay. (Chapter 4)
b. Compare the ability of the spinning task assay and the laminar flow assay to detect chemically induced alterations in the cardiovascular performance of larval fathead minnows. (Chapter 5)

3. Determine if chemically induced alterations in eye size and/or pericardial area were predictive of longer-term sublethal adverse effects, indicated by altered performance in the validated neurological and cardiovascular function assays. (Chapter 6)

Chapter 2: Development of A Larval Fathead Minnow Optomotor Response Assay for Assessing Visual Function

INTRODUCTION

The optomotor response (OMR) is a position stabilizing reflex to whole field visual motion, demonstrated by fish instinctually moving to follow alternating black and white stripes (Rock and Smith 1986). The OMR assay is used to assess the visual function of a fish. Typically, the fish is given a set amount of time in the OMR chamber and the amount of time the fish spends swimming in the same direction as the stripes, or following time, is quantified. Decreases in following time are thought to indicate reduced visual acuity (Orger et al. 2004). Another metric used for assessing OMR is the measurement of response latency—the amount of time it takes the fish to respond to a change in the direction of the rotating stripes (Portugues et al. 2015). While researchers have been using the OMR assay for years, no standardized methods exist, which limits the utility of OMR data for the purpose of environmental risk assessment (Little 1990; Legradi et al. 2018). The lack of method standardization is exemplified by variability in the age of fish used, the amount of time a fish is given to acclimate to the OMR chamber, the amount of time the fish spends performing the assay, and in the approaches utilized to quantify the response (Dutta et al. 1994; Bilotta 2000; Floyd et al. 2008; LeFauve and Connaughton 2017; Magnuson et al. 2018). The absence of standardization makes it difficult to compare results across multiple studies and could lead to the use of insufficient methods. As such, there is a need to develop standardized OMR methods to address these issues. The goal of the present study was to develop a standardized protocol for the measurement of OMR in larval fathead minnows, a common model species in ecotoxicity testing (Ankley and Villeneuve 2006). Specifically this study sought to determine: 1) the age at which OMR could be quantified in fathead minnows, 2) the amount of acclimation time needed before recording OMR, 3) whether OMR performance was reproducible between different replicates of fish, 4) what volume of water to use in the OMR chamber, 5) if there were differences between two different OMR chambers, and 6) whether response latency was a useful endpoint for the OMR assay.

METHOD DETAILS

On mornings when OMR was measured, fish were given 1 h to feed before the initiation of measurements. Measurements were limited to the number of videos that could be recorded within 6 h of the morning feeding (McGee et al. 2009). The OMR chambers were kept in an incubator (Panasonic MIR-254) at 27 °C during the measurement of OMR. Each chamber consisted of a rotating drum with alternating black and white stripes, a motor to turn the drum, and a GoPro Camera mounted above to record the fish behavior (Fig. 3). All components were mounted on plywood that had an opening to allow light to enter the recording arena of the OMR chamber (Fig. 4). LED clamp lamps (Ikea) were used as the light source as they emitted no heat and were flexible for positioning. Light levels were dimmed by placing a piece of blank, white printer paper under the OMR chamber. This ensured that the light level was not so bright that the image of the larval fish in the chamber was washed out in recordings. Fish were placed in a custom-built glass vessel (36 mm wide x 75 mm tall) inside the drum and the stripes rotated at a speed of 10 rpm. Cards with identifying information were used to keep track of fish and recorded at the beginning of each video to identify the fish in the recording. Individual larvae were transferred to a vessel containing 10 mL of fresh, warmed dechlorinated municipal water and placed in the recording arena of

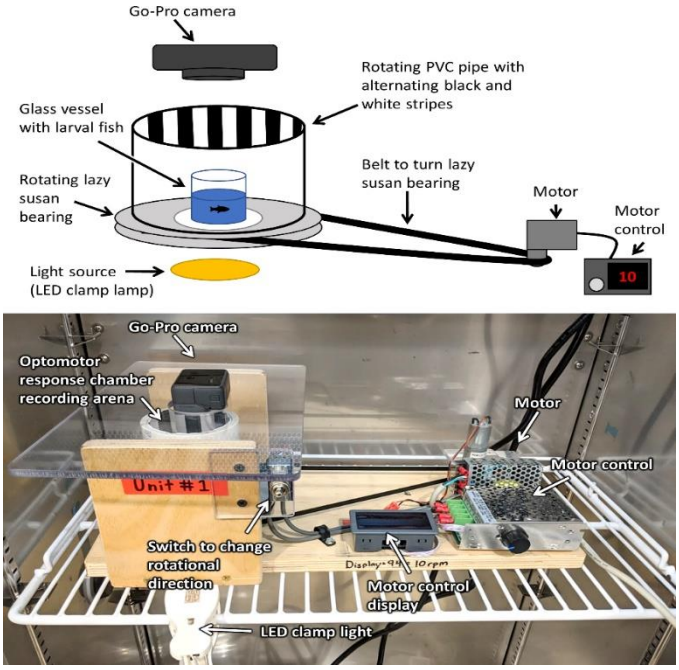

Figure 3. Detailed schematic of the OMR chamber, with the different components indicated.

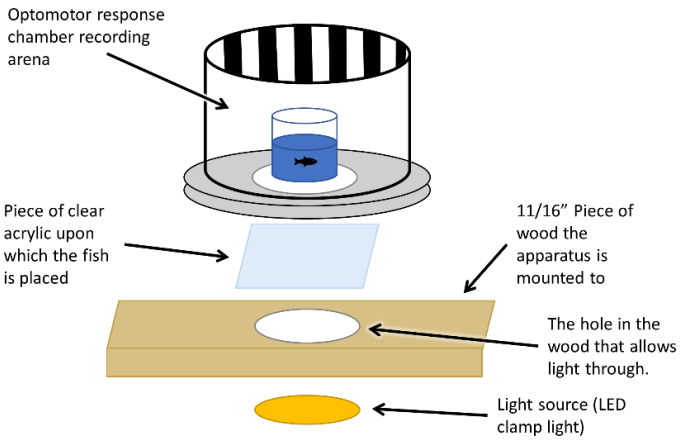

Figure 4. Detailed schematic of the OMR chamber recording arena and light source.

the optomotor chamber. Larvae were given 14 min in the chamber before being removed and transferred back to their original container. For the first 9 min the fish were in the OMR chamber there were no direction changes, with the drum turning in the same direction the whole time, allowing for 4 min of acclimation and 5 min of following time measurement. After the first 9 min, the rotational direction of the drum was altered every minute for 5 min for the measurement of response latency.

All videos were recorded as MP4 video files via a GoPro Camera and then converted to 10 fps TIFF stacks using ffmpeg (https://www.ffmpeg.org/) via a batch file with the following code:

```
@echo offsetlocal enabledelayedexpansion
for %%f in (*.MP4) do ffmpeg -y -ss 00:04:00 -i "%%f" -r 10 -
qscale 0 -vf scale=825:464 "%%~nf_10fps.avi"
for %%f in (*.AVI) do ffmpeg -i "%%f" -pix_fmt rgba -
compression_algo deflate %%~nf_%%05d.tiff
for %%a in (*.TIFF) do (
    set f=%%a
    set g=!f:~0,8!
    md "!g!" 2>nul
    move "%%a" "!g!"
)
pause
```

This code only converted the video after the 4 min mark to account for acclimation time. It also moved all the TIFF files into folders named according to the original MP4 so that TIFF stacks were easily navigable and could be tracked back to the original files. Because the first 4 min were not included in the TIFF stack—which included the segment with the fish ID—the video analysis was done blind and the fish ID was later determined from the original MP4 file. To quantify OMR, the TIFF stacks were first imported into ImageJ (Rasband 1997) as virtual stacks. The slice numbers of direction changes by the fish were then recorded in an excel document, with the swimming direction of the fish (following the stripes or opposite of the stripes) also being recorded. For videos in which response latency was measured, the slice number at which the stripes changed direction was recorded and the slice number at which the fish initially changed directions was recorded to determine how long it took the fish to notice the direction change. To account for the 10-fps speed of the videos, the number of slices that the fish spent swimming was divided by 10 to convert to seconds. This method of using TIFF stacks in ImageJ allowed for finer resolution of direction changes by the

fish. An alternative method of watching the video play at a reduced speed while timing fish direction changes with a stopwatch was also attempted and was found to be stressful for the technician quantifying OMR and slower than the TIFF stack method.

METHOD DEVELOPMENT AND VALIDATION

All procedures involving fathead minnows were conducted per Texas Christian University (TCU) Institutional Animal Care and Use Committee approved methods (Protocols 1707, 1708). Fathead minnow embryos used in the present study were produced by the TCU fathead minnow colony which was originally, obtained from Hydrosphere and Aquatic Research Organisms. Fathead minnow embryos at < 24 hours post fertilization were placed in an aerated beaker and kept in an incubation chamber (Panasonic, MIR-254) at 27°C with a 16 h light: 8 h dark photoperiod until hatch, at which time point larvae were transferred to crystalizing dishes containing 250 mL of dechlorinated municipal water (n = 10/dish). Dishes were subject to 80% water changes daily, and larvae were fed 1.06 mg *Artemia* nauplii/larva twice daily starting at 6 days post fertilization (dpf).

1) When do larval fathead minnows begin displaying quantifiable OMR?

To determine when fathead minnow larvae begin displaying OMR, measurements were made on 7, 9, 10, and 11 dpf. Optomotor response could not be quantified until 9 dpf in FHM larvae. Once the larvae did show a quantifiable response, there were no significant differences in the performance of larvae between days 9, 10 and 11 (Fig. 5, ANOVA, p = 0.55).

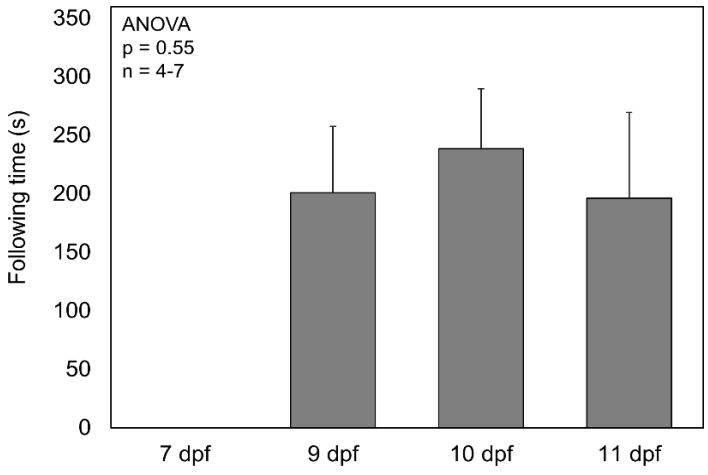

Figure 5. Average following time of larval fathead minnows in the optomotor response assay. Error bars indicate standard deviation. Dpf = days post fertilization.

2) How long of an acclimation time is needed?

As a result of inconsistent acclimation times reported in the literature, and a desire to use as short of a time as possible to increase the number of fish that could be sampled/day, it was necessary to determine how long fish needed to acclimate to the OMR chamber before quantifying OMR response. To do this, following time was measured in 2 min intervals from the time the larvae was placed in the OMR chamber through 14 min of rotation for a total of seven 2-min intervals. The mean duration of following time was then compared between each interval at 10 and 11 dpf. There were significant differences between intervals at 10 dpf (Fig. 6, ANOVA, $p < 0.01$), but not at 11 dpf (Fig. 6, ANOVA, $p = 0.26$). At 10 dpf, the mean following time over 0 to 2 min and 2 to 4 min was significantly lower than the mean response time at 12 to 14 min. There were no significant differences in following time between intervals at any age after 4 min. The lack of significant differences between groups after 4 min indicates that at least a 4 min acclimation should be implemented when measuring OMR.

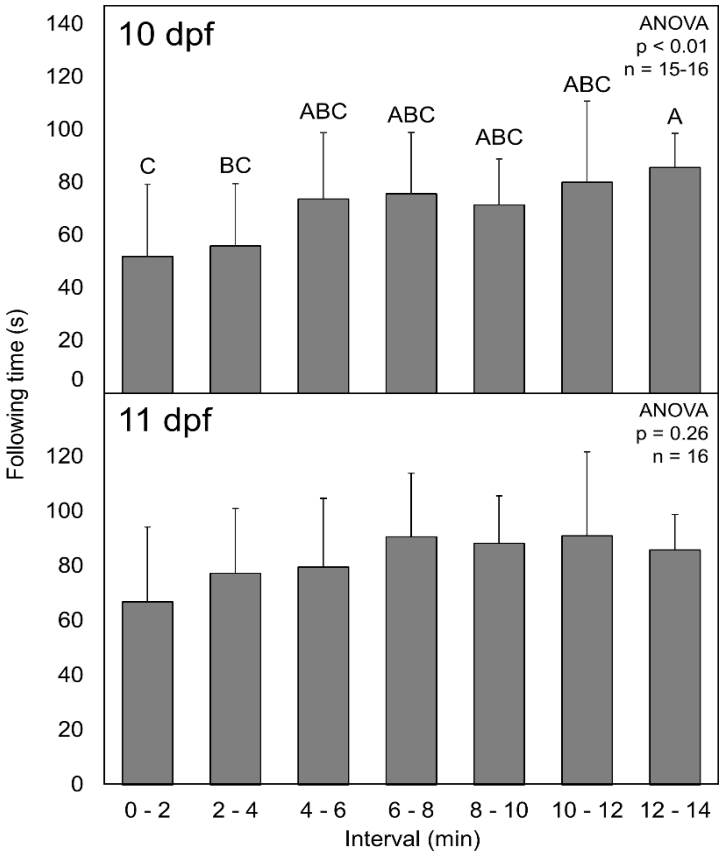

Figure 6. Average following time of larval fathead minnows in the optomotor response assay over 2-min intervals at 10 and 11 days post fertilization (dpf). Error bars indicate standard deviation. Different letters above bars indicate significant differences between groups.

3) Are there differences between different replicates?

The time spent following for 3 different replicates of fish was also compared at 10 and 11 dpf to make sure OMR was a reproducible response. There were no significant differences in following time

between the three different replicates of fish at 10 or 11 dpf (Fig. 7, ANOVA, $p > 0.12$). These results show that OMR is a reproducible response.

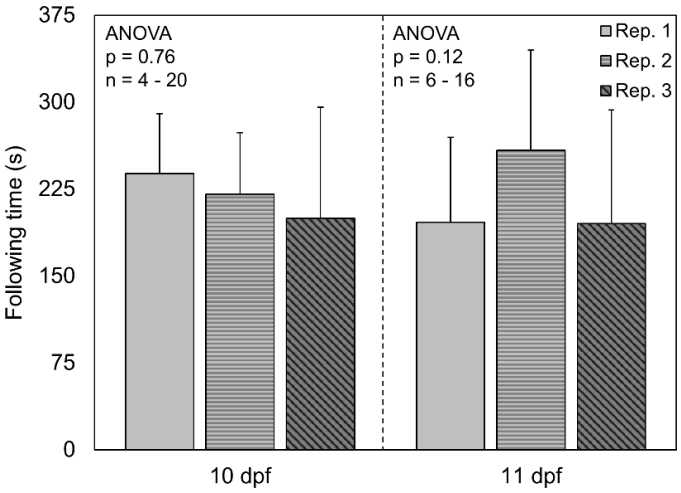

Figure 7. Average following time of larval fathead minnows in the optomotor response assay from different replicates of fish. Error bars indicate standard deviation. dpf = days post fertilization.

4) Does the volume of water in the vessel influence the results?

To determine if the volume of water in the glass OMR vessel influenced the performance of fish in the OMR assay, two volumes were evaluated. Fish were placed into 10 or 20 mL of dechlorinated tap water in the vessel. The mean duration of time spent following was compared for the two volumes at 10 and 11 dpf. No significant differences were found in the following time between the two volumes at either day (Fig. 8, t-test, $p > 0.52$). The lack of differences shows that, for at least the volumes evaluated, performance of fish in the OMR assay is not impacted by the volume of water in the vessel.

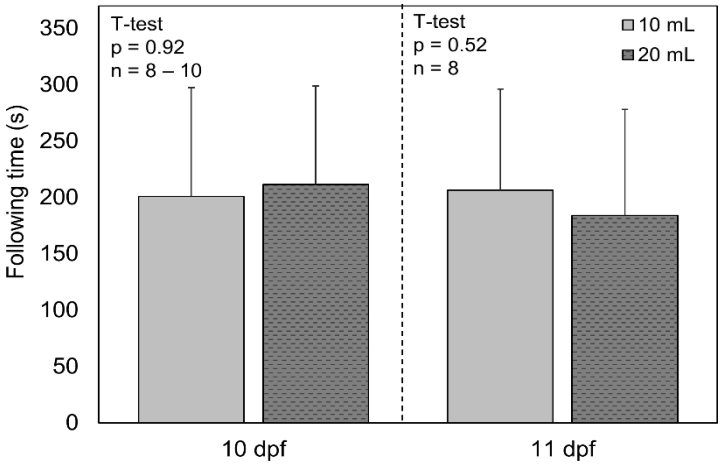

Figure 8. Average following time of larval fathead minnows in the optomotor response assay with different volumes of water in the OMR vessel. Error bars indicate standard deviation. dpf = days post fertilization.

5) Are there differences between the two OMR chambers?

The two OMR chambers being used were on different shelves in the incubator, potentially causing a shelf-effect. The performance of fish in the two OMR chambers was compared to ensure that there was no impact of the OMR chamber and/or where it was located in the incubator. There were no significant differences

between the average performance of fish in the OMR chamber on the top shelf and the OMR chamber on the bottom shelf at 10 dpf (Fig. 9, t-test, $p = 0.65$) or at 11 dpf (Fig. 9, Welch's t-test, $p = 0.49$).

6) Is response latency a useful OMR endpoint?

In addition to developing standardized methods for measuring OMR via following time, the utility of including response latency as a secondary OMR endpoint was investigated. Due to concerns of a learning component to response latency, response latency was measured 5 times with a 1-min interval between direction changes. The mean time until direction change was compared for each interval at 10 and 11 dpf. There were no significant differences in response latency over the 5 trials on either day (Fig. 10, ANOVA or Wilcoxon, $p > 0.08$.)

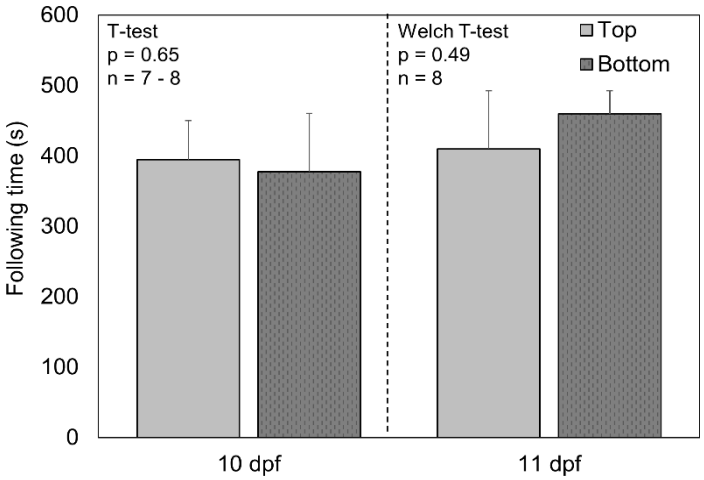

Figure 9. Average following time of larval fathead minnows in the optomotor response assay from optomotor response chamber on the top and bottom shelves of the incubator. Error bars indicate standard deviation. dpf = days post fertilization.

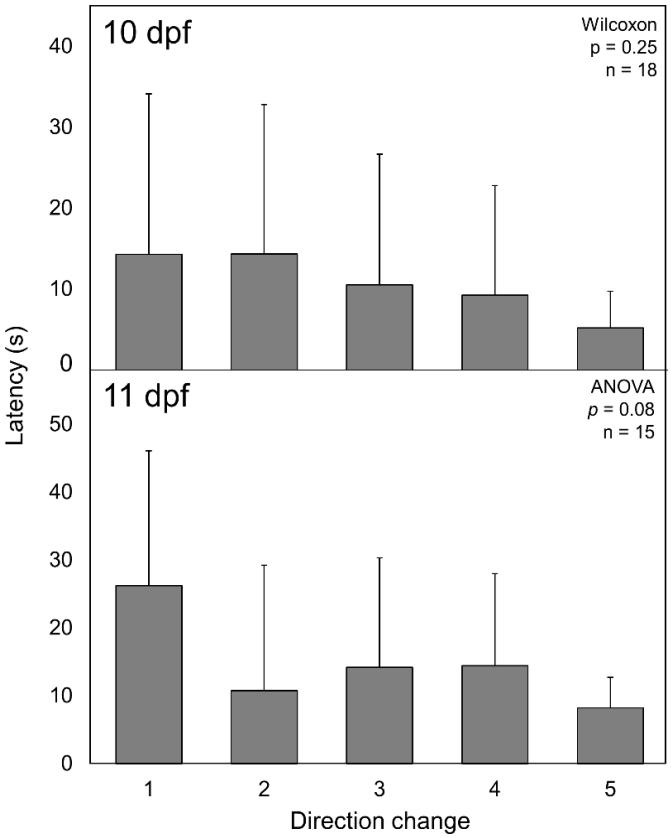

Figure 10. Average response latency of larval fathead minnows in the optomotor response assay over five directional changes. Error bars indicate standard deviation. dpf = days post fertilization.

CONCLUSIONS

Overall, the results of this study suggest that larval age and acclimation time can impact OMR results, while the volume of water fish are housed in during OMR assessment and the position of the OMR chambers in an incubator

are less likely to influence assay outcome. Specifically, this study indicated that larval fathead minnows utilized in OMR assays should be a minimum of 9 dpf and that an acclimation period of at least 4 min should be employed. Although the volume of water in test vessels (in this study 10 or 20 mL) did not affect OMR performance, deviations from these volumes are not recommended without confirmation that they do not impact OMR. There did not appear to be a significant learning component to response latency; however, the high variation in response latency likely impede the ability to detect significant differences between groups. A post-hoc power analysis (given the variance from the 5th direction change at 11 dpf, $\alpha = 0.05$ and a $\beta = 0.2$) revealed that the minimum total sample size needed to detect a 20% change in response latency is approximately 828 fish, well beyond the sample size used during methods development ($n = 15 – 18$). In comparison, a post-hoc power analysis for following time (given the variance from the replicate 2 at 10 dpf, $\alpha = 0.05$ and a $\beta = 0.2$) revealed that the minimum total sample size needed to detect a 20% change is approximately 14 fish, a more achievable sample size. The need for large sample sizes to detect differences between groups limits the utility of response latency as an endpoint in the OMR assay.

Chapter 3: Comparison of Behavioral Assays for Assessing Toxicant-Induced Alterations in Neurological Function in Larval Fathead Minnows

INTRODUCTION

Historically, the assessment of compounds for neurotoxicity has been focused on potential impacts to human health. However, neuroactive compounds have been found in the environment at concentrations that may pose a threat to wildlife (Deo 2014). Compounds known to impact the nervous system, such as heavy metals (Wright and Baccarelli 2007), pharmaceuticals (Brodin et al. 2014), and insecticides (Grandjean and Landrigan 2006), are routinely found in surface waters (Kim et al. 2007; Yi et al. 2011; Wolfram et al. 2018). In an analysis of micropollutants in German waterways, 13% of the detected compounds were identified as having a neurotoxic mode of action (Busch et al. 2016) and it has been estimated that up to 28% of industrial chemicals may have neurotoxic effects (Tilson et al. 1995). Exposure to neuroactive compounds is most often associated with alterations in behavior (e.g., reduced prey capture or predator response) that could impact the fitness of an organism, leading to population level effects (Hellou 2011). Because neurotoxic compounds are present in the environment and are known to affect ecologically important behaviors in aquatic organisms, there is a need to develop and validate methods to assess the potential environmental impacts of these compounds.

Behavioral alterations often occur at concentrations much lower than those that would induce overt toxicity as indicated by mortality or decreased growth (Clotfelter et al. 2004; Scott and Sloman 2004; Hellou 2011; Melvin and Wilson 2013). However, assays aimed at detecting behavioral alterations caused by exposure to neurotoxicants can be time consuming, expensive, and subject to high variation due to inherent differences in the behavior of individual organisms (Zala and Penn 2004). Although high-throughput methods (e.g., the zebrafish photomotor assay) have been developed to address some of these issues, their ties to population-level impacts are still being evaluated, limiting their usefulness for environmental risk assessment (Ågerstrand et al. 2020).

Three of the behavioral assays commonly used to assess the neurological function of fish are: feeding, optomotor response (OMR), and C-start. Feeding (also known as prey capture) is a holistic endpoint for evaluating the wellbeing of an organism, as it involves the coordination of multiple physiological systems to both identify and capture prey (Floyd et al. 2008; Portugues and Engert 2009; Moreira et al. 2010; Abdel-moneim et al. 2015). Decreases in feeding can lead to reductions in growth, potentially causing decreases in reproductive output and recruitment (Houde 1989; Groh et al. 2015). Optomotor response is a position-stabilizing reflex to whole-field visual motion (Rock and Smith 1986) that can be used to assess the visual function of fish. Because the development of the central nervous system and optic systems are closely related (Langenberg et al. 2008), and central nervous system alterations can manifest as alterations to the optic system (Zhang et al. 2015), decreased OMR may also be indicative of general neurotoxicity. Decreased visual function, as indicated by altered OMR, can result in altered prey capture (Gahtan 2005) and predator response (Fuiman et al. 2006; Preuss 2006), two factors important in recruitment of larval fishes (Houde 1989). The C-start response is a fast-start predator escape response, during which the fish makes a C-shape, mediated by Mauthner cells located in the hindbrain of fish (Domenici and Blake 1997; Eaton et al. 2001). Abnormal neurodevelopment can result in alterations in C-start response (Shan et al. 2015), which are thought to be indicative of decreased predator response (Liu and Fetcho 1999; Eaton et al. 2001), which could again lead to decreased larval recruitment (Houde 1989).

Though each of these methods have been utilized to assess the behaviors of larval fish, to our knowledge, there have been no comparisons of the sensitivity and practicality of these three behavioral assays. Such a comparison would allow for more informed decisions when selecting assay(s) to be included in future studies investigating neurotoxicity and/or behavioral alterations resulting from toxicant exposure. Therefore, the goal of the present study was to promote the use of behavioral assays for the assessment of neurotoxic compounds by comparing the sensitivity and practicality of three different behavioral assays in larval fish. To achieve this goal, the sensitivity and practicality of three behavioral assays (feeding, OMR, and C-start assay) were compared in larval fathead minnows (*Pimephales promelas*) exposed to the known neurotoxicant chlorpyrifos (CPF). Larval fathead minnows were selected as the model organism, as the developing nervous

system is particularly sensitive to neurotoxic effects (Rice and Barone 2000), and the fathead minnow is the US EPA's fish model of choice (Ankley and Villeneuve 2006). Chlorpyrifos was selected due to its use as a model neurotoxicant (Selderslaghs et al. 2012; Crosby et al. 2015) and known mode of action as an acetylcholinesterase (AChE) inhibitor (Fukuto 1990). In addition to comparing these three assays to one another, the impacts of a 5-d embryonic exposure versus a 12-d embryo-larval exposure were also compared to determine if an embryonic-only exposure would induce alterations in the behavioral assays, as timing of exposure can alter the impacts of neurotoxicant exposure (Rice and Barone 2000; Hellou 2011; Klüver et al. 2015) and there is currently a movement towards using fish embryos in place of more developed organisms (Braunbeck et al. 2014).

METHODS

Maintenance of Brood Stock

All procedures involving fathead minnows were conducted per Texas Christian University (TCU) Institutional Animal Care and Use Committee approved methods (Protocol 1809). Fathead minnow embryos and larvae used in the present study were produced by the TCU fathead minnow colony which was originally obtained from Hydrosphere and Aquatic Research Organisms. To obtain embryos and larvae, 10 – 20 breeding colonies, each consisting of 1 male and 4 females or 1 male and 8 females, were housed in 30 L glass aquaria. These ratios were selected based on the results of Roush et al. (2018). For colonies with 1 male and 4 females, aquaria were divided by a plastic tank partition, with two colonies per aquarium. Fish were kept under optimal breeding conditions (26 °C, 16 h light: 8 h dark photoperiod). Aquaria were subject to daily 1/3 water changes and fish were fed commercially available flake food (Tetramin) to satiation twice daily. Breeding structures (10-cm-long section of 7.6-cm diameter polyvinyl chloride schedule 40 pipe halved lengthwise) were examined at least once daily for the presence of eggs. Eggs were gently removed from the structures using a metal spatula, transferred into petri dishes containing the appropriate test solution, and assessed for viability using a Leica DMi1 inverted microscope with a heated stage.

Chemicals

A CPF (Sigma-Aldrich, analytical standard grade) stock solution was made in acetone to achieve a nominal concentration of 1.2 mg CPF/mL and stored in the dark at -20 °C. Test solutions were made via serial dilution using dechlorinated municipal water. Nominal concentrations were 0 (solvent control, SC), 30, 60, and 120 μg CPF/L. The highest concentration (120 μg CPF/L) was the calculated 12-d LC20 value from range finding studies. Solvent concentrations were adjusted to 0.01% for all groups (OECD 2013). A clean water control was run alongside all CPF exposures to ensure that there were no effects resulting from the solvent exposure. Water samples (200 mL) were collected from the highest concentration (120 μg CPF/L) of renewal solution at three time points (days 1, 6, and 11), and from dilution water at one time point (day 6) during each exposure. Samples were stored in glass containers, in the dark, at 4 °C for ≤ 24 h prior to extraction via solid phase extraction on HyperSep C18 (500 mg/6 mL) cartridges using EPA method 525.2 (US EPA 1995). Cartridges were then wrapped in foil and stored at -20 °C until being sent to the Agricultural and Environmental Services Laboratories at the University of Georgia for elution and analysis via gas chromatography with a nitrogen phosphorous detector. Water concentrations were 61% of nominal and CPF was below the detection limit in the dilution water.

Experimental Exposure Procedures

To investigate the sensitivity/practicality of the chosen behavioral assays, 3 independent CPF exposures were performed. Until 5 dpf, exposures were conducted using modified OECD protocols and previously published fathead minnow fish embryo toxicity (FET) test methods (Braunbeck et al. 2005; Jeffries et al. 2015, 2014; OECD 2013). Briefly, fathead minnow embryos at ≤ 32 cell stage were randomly sorted into 24-well plates after staging, with one embryo and 2.5 mL of the appropriate test solution per well. Plates were covered with manufacturer-supplied lids and kept in an incubation chamber (Panasonic, MIR-254) at ~27 °C under a photoperiod 16 h light: 8 h dark. At 5 dpf, individual larvae were randomly sorted into gently aerated 30 mL beakers containing 25 mL of the appropriate test solution, covered with clear acrylic. To

determine whether a 5-d embryonic exposure, similar to that of the fathead minnow FET test, was sufficient to induce alterations in neurological function and development, half of the individuals from each group were transferred into clean water, while the remaining half were maintained in their respective exposure solutions (n= 12/group) (Fig. 11). Individual larvae were tracked throughout the exposure, so that direct comparisons of individual performance in different assays could be made. Starting at 6 dpf, larvae were fed 1.06 mg newly hatched (< 24h) *Artemia* nauplii/larvae twice daily. Solution replacements (~80%) were completed daily. The mean (± standard deviation) pH, conductivity (µS/cm), hardness (mg/L) and alkalinity (mg/L) of the dilution water were 8 ± 0.1, 323.7 ± 73.6, 110.5 ± 3.4, and 101.6 ± 8.7, respectively. The mean (± SD) pH, conductivity (µS/cm), hardness (mg/L) and alkalinity (mg/L) of the highest exposure concentration (120 µg CPF/L) were 8.1 ± 0.1, 326.6 ± 52.1, 115.3 ± 27.3, and 103.7 ± 7.9, respectively. Embryos and larvae were checked daily for standard markers of mortality (i.e., coagulation, lack of tail detachment, lack of somite development, and absence of heartbeat) until the conclusion of the test at 12 dpf. In addition, assays related to neurological function and development were evaluated throughout the duration of the exposure as shown in Fig. 11 and described below.

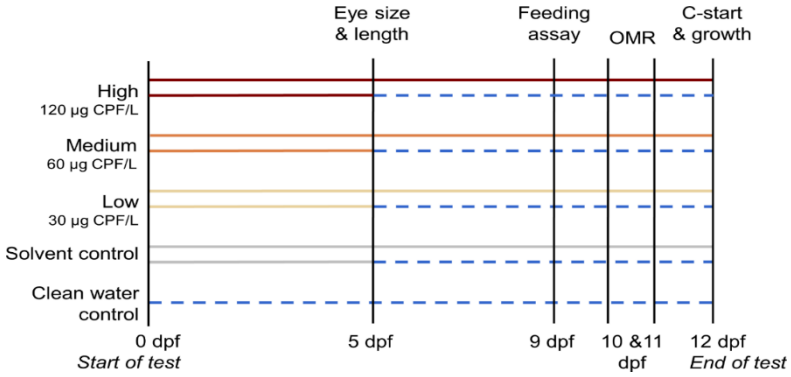

Figure 11. Experimental timeline indicating the times at which each endpoint was evaluated in control and chlorpyrifos (CPF) exposed embryos/larvae, as well as the exposure regime. A solid line indicates chemical exposure and the dash line indicates clean water. dpf = days post fertilization, OMR = optomotor response assay.

Eye size and 5 dpf Length Measurements

Photographs were taken at 5 dpf using a Leica DMi1 inverted microscope (25X) with a warmed stage for measurement of eye size and length. All surviving eleutheroembryos were anesthetized using buffered MS-222 (0.1 g MS-222 /L) and positioned in 3% methylcellulose. Eye size was measured as eye size index, with eye area being divided by the length of the eleutheroembryos at 5 dpf. Eye size was corrected for body length, as previous research has demonstrated that the two metrics are correlated (Krzykwa et al. 2018).

Length was measured as snout-vent length. Measurements were made from photographs using ImageJ (Rasband 1997).

The Fathead Minnow Larval Feeding Assay

Feeding assays were conducted before the morning feeding at 8 dpf (Fig. 11). One larval fish was added per well of a 6-well plate with 12 mL of dechlorinated municipal water and 10 prey items (< 24 h *Artemia* nauplii) per well, for a total of 6 fish/group/replicate. Well plates containing larvae were placed in an incubator for 1 h before larvae were photographed for size and transferred back to their original exposure chambers. The number of remaining nauplii were quantified on a light table and the exact time each larva was added to the well and removed was recorded to account for variations in timing. Larval length was measured from photographs using ImageJ.

The Fathead Head Minnow Larval Optomotor Response Assay

The OMR methods used in the present study are based off a methods development paper co-published with this one (Krzykwa and Jeffries, *in review*). Briefly, OMR was measured on days 10 and 11 (Fig. 11) using two OMR chambers, illuminated from the bottom, kept inside of an incubation chamber (Panasonic, MIR-254) at ~27 °C. Individual larvae were rinsed in clean water then transferred to a glass vessel containing 10 mL warmed dechlorinated municipal water in the OMR chamber. Larvae spent ~14 min in the OMR chamber before being removed and transferred back to their original exposure container. For the first 9 min of recording there were no direction changes, with the drum turning in the same direction the whole time for measurement of total following time--how long the fish spent swimming in the same direction as the alternating black and white stripes. After the first 9 min, the rotational direction of the drum was altered every minute for 5 min for the measurement of response latency--how long it took the fish to adjust to the change in direction. To reduce any time-of-day effects, individual larvae were tested from treatments in a sequential pattern (control, SC, low, medium, high, control, SC, low, etc.). To reduce any effect of feeding, on mornings when OMR was measured fish were given 1 h to feed before the initiation of measurements and measurements were limited to as many videos as could be recorded within 6 h of the morning feeding

(McGee et al. 2009). Videos were analyzed blind as 10 fps TIFF stacks in ImageJ, starting 4 min into the recording to account for a 4 min acclimation time. The time spent following the stripes, the total time spent swimming, and response latency were all quantified.

The Fathead Minnow Larval C-Start Assay

The C-start methods utilized in the present study were based on methods published by McGee et al. (2009) and Painter et al. (2009). Briefly, 12 dpf larval fathead minnows were rinsed in clean water and then individually transferred to a 5-cm glass petri dish containing 20 mL dechlorinated municipal water at ~27 °C and placed on top of a 1 mm grid in the C-start staging arena. A vibrational trigger system using a haptic motor mounted to the bottom of the platform was used to induce the C-start response. Initiation of the vibrational stimulus was indicated by the simultaneous appearance of a laser pointer in a corner of the filming arena. The escape response was filmed at 1000 fps using a FASTEC IL4-100 high-speed digital camera with FastMotion Controller software (v. 1.8.25) positioned ~ 24 cm above the filming arena. Individual larvae were tested from treatments in a sequential pattern (control, SC, low, medium, high, control, SC, low, etc.). Like with OMR, on mornings when C-start was measured fish were given 1 h to feed before the initiation of measurements and measurements were limited to as many videos as could be recorded within 6 h. In instances when larvae did not respond to the first vibrational stimulus, they were given up to 3 opportunities to respond with a 30 s interval between vibrations to confirm the lack of response to the vibrational stimulus. Videos were analyzed as TIFF stacks using ImageJ. The escape latency, velocity, angle, and total escape response were quantified, as well as the percentage of fish that responded to the vibrational stimulus.

Growth

At 12 dpf, after the C-start assay, fish were euthanized in buffered MS-222 (0.3 g MS-222/L), and wet-weight and length were measured to evaluate growth.

Statistical Analysis

Data from the 3 individual exposures were combined after confirmation that there were no significant differences between control fish from the three replicates (ANOVA or Pearson, $p > 0.12$). There were significant differences between replicates for growth related endpoints (i.e., length and mass, ANOVA, $p < 0.01$), which was expected, as it is known that there can be a genetic component to growth rate (Therkildsen et al. 2019) and the parentage of the different cohorts used in each exposure were likely different. To test for significant differences between exposure groups, an ANOVA was used followed by Tukey post-hoc multiple comparisons test. In cases of unequal variance as determined by a Levene's test, a Wilcoxon test followed by a Steel-Dwass post-hoc all-pairs nonparametric test was used. For categorical data (i.e., response/no response to vibrational stimulus in the C-start assay), a Pearson's chi-squared test was used to test for significant differences and post-hoc analysis was performed via pairwise comparisons of groups with a Holm-Bonferroni adjustment. The results of the behavioral assays were normalized to the performance of the solvent control group. Statistical analysis was performed using JMP 14.0.

RESULTS AND DISCUSSION

Eye Size

There were no significant differences in relative eye size between any of the CPF groups and the SC group (Fig. 12A, ANOVA, $p = 0.45$). The lack of alteration in relative eye size in response to CPF was unexpected, as previous work has reported alterations in eye size in response to neurotoxicant exposure (Bilotta et al. 2002; Wold et al. 2017; Krzykwa et al. 2019), including other AChE inhibitors (Cook et al. 2005; Altenhofen et al. 2019). It is of note that neither of the studies investigating AChE inhibitors that reported significant decreases in eye size corrected for the size of the animal, despite significant decreases in body size. Eye size—as measured by eye area—is strongly correlated with bodyfish size (Krzykwa et al. 2018); therefore, the alterations in eye size reported by Cook et al. (2005) and Altenhofen et al. (2019) may be an artifact of decreases in the overall size of the organisms. For example, in the present study there were significant

decreases in eye area when the measurements were not corrected for fish length (*data not shown*, mean ± SE varied from 0.074 ± 0.001 to 0.079 ± 0.001 mm², ANOVA, $p < 0.01$); however, there were also significant decreases in overall body size, as indicated by decreases in snout-vent length. When eye area was corrected for body size there were no longer significant differences between exposure groups, indicating that the decreases in eye area were a result of decreases in overall body size.

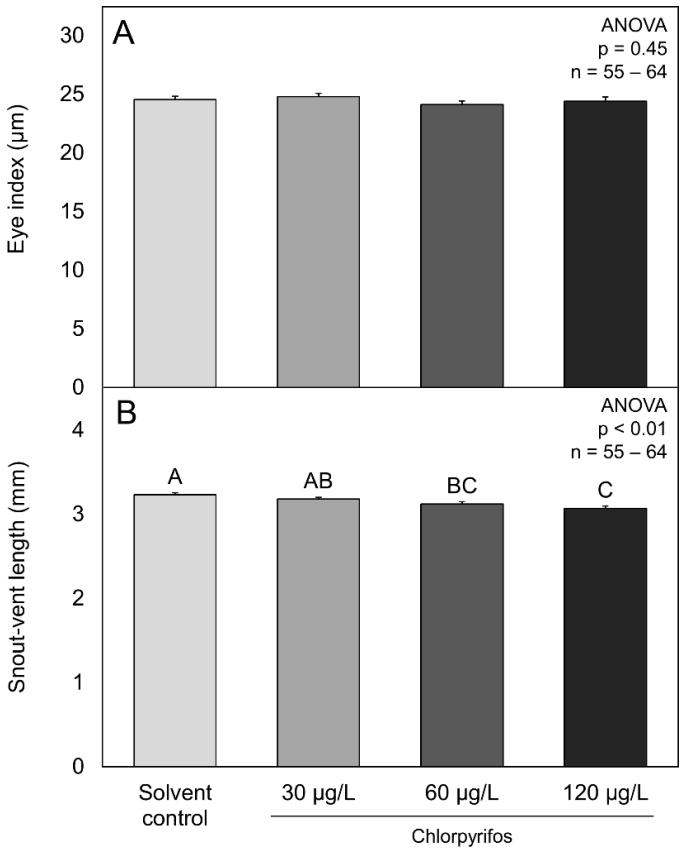

Figure 12. Average eye index (**A**) and snout-vent length (**B**) of embryos exposed to chlorpyrifos. Error bars represent standard error. Different letters above bars indicate significant differences between groups.

Although eye size was not sensitive to CPF exposure, it was an easy endpoint to measure, with the analysis and photographing taking little time compared to the behavioral assays. Given its ease of measurement, and the results of previous studies indicating its responsiveness to a variety of compounds with varying modes of action including heavy metals (Kim et al. 2013; Wold et al. 2017; Krzykwa et al. 2019), references toxicants (Krzykwa et al. 2018), and polycyclic aromatic hydrocarbons (Sørhus et al. 2017) in addition to AChE inhibitors (Cook et al. 2005; Altenhofen et al. 2019), it may still be worth considering for inclusion in future studies. Reductions in eye size have been associated with decreased development of neural/brain tissue (Cheng et al. 2013; Wold et al. 2017), and behavioral alterations later in development (Kim et al. 2013; Wold et al. 2017). However, the link to alterations in neurological function is still tenuous with further research needed to uncover the relationship between eye size, neurological development/function, and behavior.

Growth

There were significant alterations in growth at 5 dpf resulting from 5-d embryonic CPF exposure (Fig. 12B, ANOVA, $p < 0.01$), with the 60 µg CPF/L group being significantly shorter than the SC group, and the 120 µg CPF/L group being significantly shorter than both the SC and the 30 µg CPF/L groups. Significant alterations in eleutheroembryo body size have also occurred after exposure to other AChE inhibitors (Cook et al. 2005; Altenhofen et al. 2019) as well as other neurotoxicants (Floyd et al. 2008; Krzykwa et al. 2019).

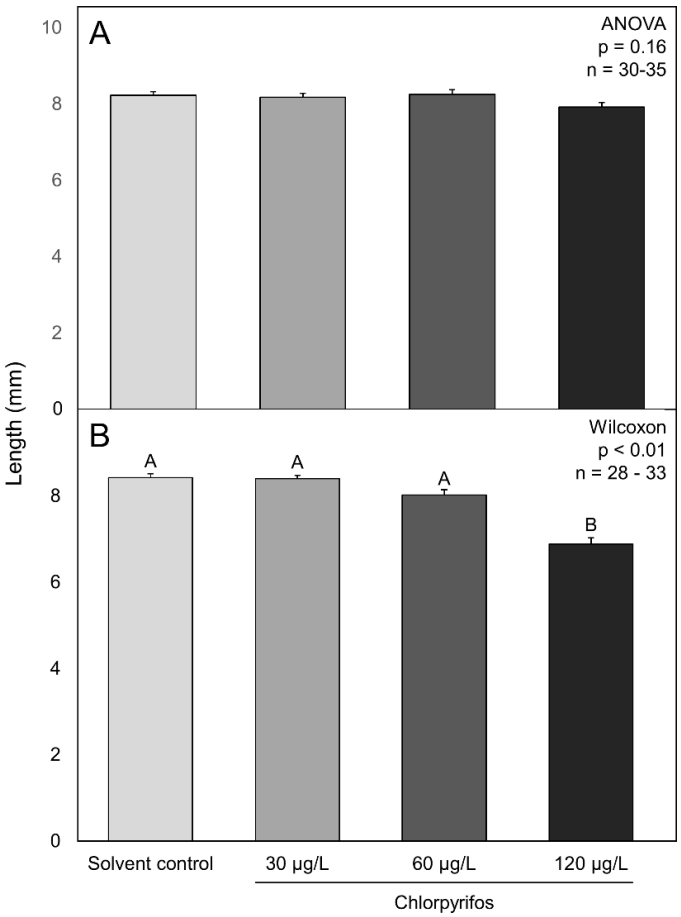

Figure 13. Average total length of larvae at 12 days post fertilization exposed to chlorpyrifos during embryonic development (**A**) and of larvae exposed to chlorpyrifos during embryonic and larval development (**B**). Error bars represent standard error. Different letters above bars indicate significant differences between groups.

Growth was also measured at the end of the exposure period (12 dpf). Alterations in body length at 5 dpf persisted for fish that were continuously exposed to CPF through 12 dpf (Fig. 13B, Wilcoxon, $p < 0.01$), as fish in the highest exposure group (120 µg CPF/L) were significantly shorter than fish from all other groups. However, for fish that were only exposed to CPF as embryos and moved to clean water at 5 dpf, concentration-dependent differences in body-length were not detected (Fig. 13A, ANOVA, $p = 0.16$), suggesting that the embryos were able to quickly overcome the early-life stage reduction in size when the exposure was limited to the embryonic stage. Although growth is not a behavioral endpoint, it is an easily measurable, traditional toxicity endpoint that was responsive to a continuous embryo larval CPF exposure. Additionally, it has ecological relevance as decreases in growth are linked to decreases in reproductive output and recruitment (Houde 1989; Groh et al. 2015).

Feeding Assay

There were no significant alterations in the feeding behavior of larvae subject to an embryonic-only CPF exposure (Fig. 14A, ANOVA, $p = 0.28$). The lack of alteration after the 5-d embryonic exposure suggests that the exposure to CPF during embryonic development does not induce lasting alterations in physiological parameters underlying feeding behavior (e.g., locomotion ability, visual acuity, etc.). Fathead minnow larvae exposed to the pyrethroid esfenvalerate demonstrated a similar trend (Floyd et al. 2008). When prey capture was measured immediately after exposure, a decrease in prey consumption was noted; however, this decrease was not detected when fish were given a depuration period of ≥ 8 d (Floyd et al. 2008).

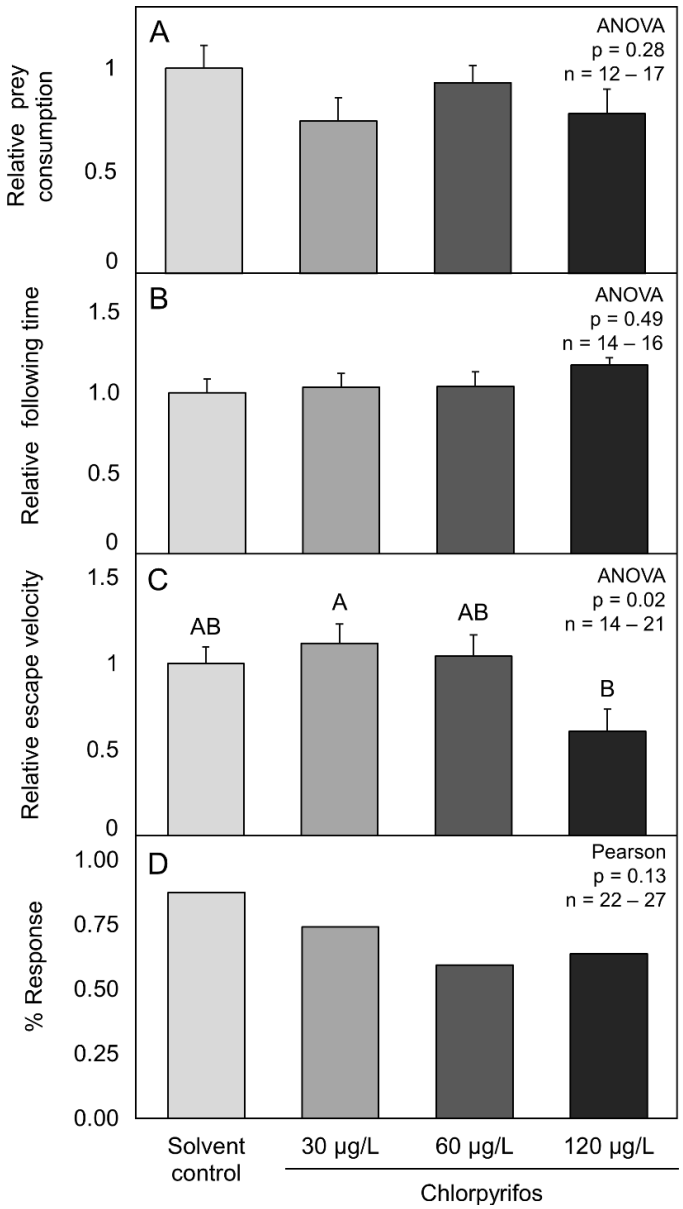

Figure 14. Average relative prey consumption (**A**), relative optomotor response following time (**B**), relative C-start escape velocity (**C**), and percent response to the vibration stimulus in the C-start assay (**D**) for 12-dpf larvae exposed to chlorpyrifos during embryonic development. Error bars represent standard error. Different letters above bars indicate significant differences between groups.

There were significant alterations in the feeding behavior of larvae continuously exposed to CPF through 12 dpf (Fig. 15A, ANOVA, $p < 0.01$), with larvae from the 120 µg CPF/L group consuming significantly fewer prey items than larvae from the 30 µg CPF/L group. Exposure to acid mine drainage (Castro et al. 2004; Abdel-moneim et al. 2015), pyrethroids (Floyd et al. 2008; Moreira et al. 2010), and methylmercury (Samson 2001) have also resulted in alterations in feeding, indicating that alterations in feeding behavior can result from varying types

of toxicant exposure and that the assay is sensitive to toxicant-induced alterations in physiological parameters underlying feeding behavior. In addition, decreases in feeding can lead to decreased growth, which as previously stated, is associated with decreases in reproductive output and larval recruitment (Houde 1989; Groh et al. 2015).

This assay was practical for a single individual to run, taking approximately 3 h (including 1 h for the fish to feed) for one technician to run the assay for 54 fish (9 groups, n = 6/group). In addition, this assay had the lowest associated cost of the three behavioral assays investigated. All that was required to run the assay was the prey that the larvae were already being fed, containers in which to keep the larvae while they were feeding, and a light table for the quantification of artemia.

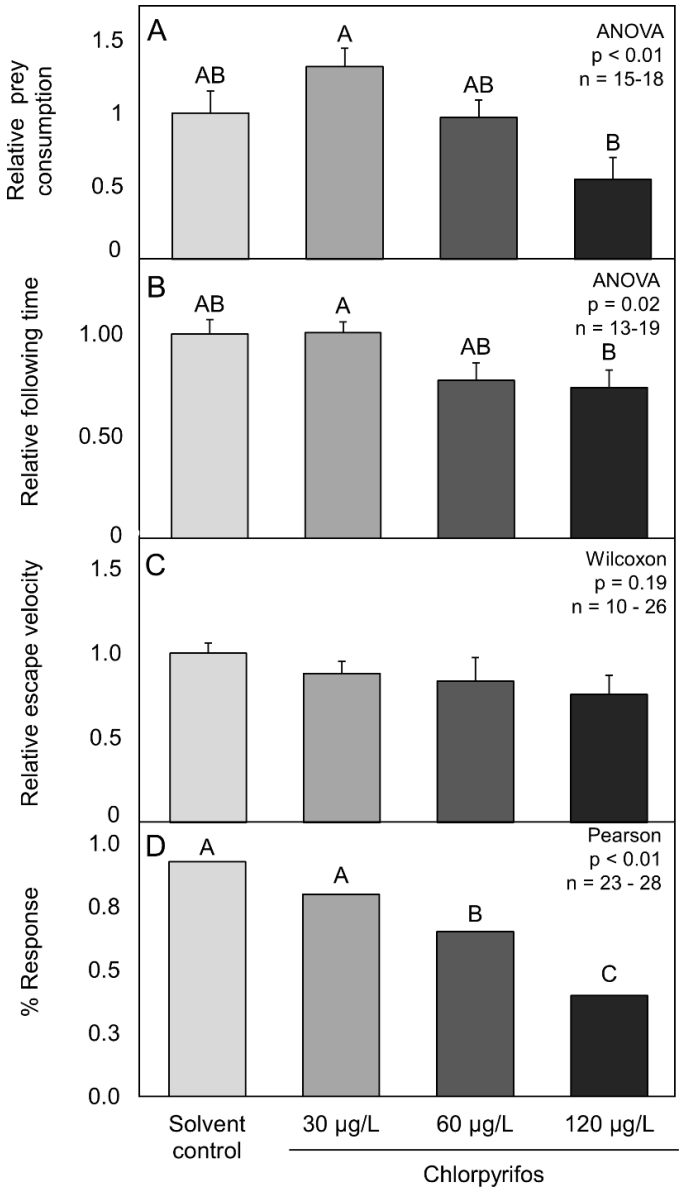

Figure 15. Average relative prey consumption (**A**), relative optomotor response following time (**B**), relative C-start escape velocity (**C**), and percent response to the vibration stimulus in the C-start assay (**D**) for 12-dpf larvae exposed to chlorpyrifos during embryonic and larval development. Error bars represent standard error. Different letters above bars indicate significant differences between groups.

Optomotor Response Assay

For fish that were exposed to CPF for 5 d during embryogenesis, there were no significant differences in relative following time (Fig. 14B, ANOVA, p = 0.43), relative response latency (*data not shown*, mean ± SE varied from 0.81 ± 0.77 to 1.53 ± 0.35, Wilcoxon, p = 0.72), or in the relative time spent not swimming (*data not shown*, mean ± SE varied from 0.72

± 0.25 to 1.67 ± 0.52, ANOVA, p = 0.53) in the OMR assay. These results indicate that the 5-d embryonic exposure did not have lasting impacts on the visual function of the exposed minnows.

For fish that were continuously exposed to CPF throughout embryonic and larval development, there were no significant differences in relative response latency (*data not shown*, mean ± SE varied from 0.65 ± 0.16 to 1 ± 0.27*,* Wilcoxon, p = 0.72). However, significant alterations in relative following time (Fig. 15B, ANOVA, p = 0.02) were noted, with fish exposed to the highest concentration of CPF (120 μg CPF/L) spending less time swimming in the same direction as the stripes than those exposed to the lowest concentration of CPF (30 μg CPF/L). This change can be attributed to CPF-induced alterations in visual function rather than alterations in baseline swimming activity given that there were no significant differences in the relative amount of time the fish spent not swimming while in the chamber (*data not shown*, mean ± SE varied from 1 ± 0.36 to 2.31 ± 0.58, ANOVA, p = 0.10). The results of previous studies also support the conclusion that the alterations in OMR noted in the present study are indicative of changes in visual function resulting from CPF exposure. For example, chronic (30 d) exposure to CPF resulted in alterations to the photoreceptor layer of the eye in fingerling barramundi (*Lates calcarifer*) at concentrations lower than those used in the present study (≤ 0.56 ug/L; Marigoudar et al. 2018). In addition, when AChE activity in the eyes of adult Japanese medaka (*Oryzias latipes*) was quantified, a significant reduction was noted in exposed fish relative to controls, suggesting that CPF exposure may directly impact ocular tissues and subsequently visual function (24 μg CPF/L; Qiu et al. 2017).

Alterations in OMR are not specific to AChE inhibitors. Exposure to a variety of toxicants including ethanol (Bilotta et al. 2002), heavy metals (LeFauve and Connaughton 2017), pesticides (Peyster and Long 1993; Floyd et al. 2008), and even parasite infections (Shirakashi and Goater 2002) have resulted in altered OMR, demonstrating that the OMR assay is sensitive to a variety of types of neurological disfunction. Additionally, vision is an ecologically important endpoint, as alterations are likely to impact prey capture (Gahtan 2005) and predator response (Fuiman et al. 2006; Preuss 2006).

Although OMR was sensitive to CPF-induced alterations, and may have ecological relevance, it was the least practical of the behavioral assays investigated in the present study. Sample sizes were limited to 18 fish/OMR chamber/day due to the time constraints associated with constricting the assay to within 6 h after the morning feeding. In the present study, this limitation was addressed by recording observations over a 2-day period (at 10 and 11 dpf), because there were no differences in average fish performance between these days (Krzykwa and Jeffries, *in review*), and by using two OMR chambers simultaneously. The analysis of the recorded videos was also time-consuming with each video taking ~ 20 min to analyze. In the present study, a total of 144 fish were evaluated; therefore, upwards of 48 hours were spent analyzing OMR videos. The OMR assay also required building specialized OMR chambers and the purchasing of a Go-Pro camera for each chamber. This, combined with the time requirements associated with the OMR assay, should be considered when deciding whether to incorporate the OMR assay into a study.

C-start Assay

There was a significant alteration in the escape velocity of larvae exposed to CPF during embryonic development, with larvae exposed to the lowest concentration of CPF (30 μg CPF/L) having a significantly faster escape velocity than those exposed to 120 μg CPF/L (Fig. 14C, ANOVA, $p = 0.02$). However, there were no alterations in relative C-start latency (*data not shown*, mean ± SE varied from 1 ± 0.16 to 2.49 ± 0.65, Wilcoxon, $p = 0.15$), relative escape angle (*data not shown*, mean ± SE varied from 0.98 ± 0.02 to 1.02 ± 0.01, ANOVA, $p = 0.28$), or the number of fish that responded to the vibrational stimulus after embryonic exposure to CPF (Fig. 14D, Pearson, $p = 0.13$). These results indicate that there may be some lasting impact of early-life stage CPF exposure on the physiological processes that govern the vibrational startle response, specifically those related to escape velocity.

Continuous 12-d exposure to CPF did not result in significant alterations in relative escape velocity (Fig. 15C, Wilcoxon, $p = 0.19$), relative C-start latency (*data not shown*, mean ± SE varied from 1 ± 0.001 to 1 ± 0.02, Wilcoxon, $p = 0.25$), or relative escape angle (*data not shown*, mean ± SE varied from 0.76 ± 0.11 to 1.00 ± 0.06, ANOVA, $p = 0.18$). However, there was a significant difference in the number of fish that

responded to the vibrational stimulus (Fig. 15D, Pearson, $p < 0.01$). There was a concentration-response trend, with fish from the 60 μg CPF/L group responding significantly less frequently than fish from the SC and 30 μg CPF/L groups, and with fish from the 120 μg CPF/L group responding less frequently than fish from the SC, 30 or 60 μg CPF/L groups. Exposure to CPF at concentrations as low as 3 and 24 μg CPF/L resulted in alterations in startle/predator response in adult California killifish (*Fundulus parvipinnis*; Renick et al. 2016) and adult Japanese medaka, respectively (Qiu et al. 2017). However, for the medaka, the effects was ameliorated after a 7-d depuration period aside from an increase in response latency (Qiu et al. 2017).

Alterations in C-start/vibrational startle assays have been reported after exposure to methylmercury (Alvarez et al. 2006; Weber 2006), pesticides (Carlson et al. 1998), and pharmaceuticals (McGee et al. 2009; Painter et al. 2009; Simmons et al. 2017), demonstrating that C-start is sensitive to compounds with a variety of modes of action.

The C-start assay was quick to run, with up to 90 fish being used in the assay in a single day. This was the maximum number of surviving fish in a replicate, so it is possible that more fish could have been measured. Analysis of the recorded videos was also quicker than in the case of OMR, with each video taking ~ 3 min to analyze. Based on this estimate C-start videos took ~ 28.5 h to analyze all 226 recorded videos, ~ 20 h less than it took to analyze the 144 OMR videos. Because the video recording and analysis took less time, it was far easier to achieve a higher sample size in the C-start assay than in the OMR assay, and thus more statistical power. However, the initial costs of the C-start assay were most expensive of the three behavioral assays investigated. This is due to the need for a high speed camera, as cameras capable of filming at 1000 fps cost upwards of $3000 USD (https://www.krontech.ca/). In addition, technical knowledge was needed to build the haptic motor vibrational stimulus set up. Although the C-start assay was the most sensitive and practical in terms of time it also had the highest associated costs, an aspect to be considered when deciding what behavioral assays should be included in future studies.

CONCLUSIONS

The 5-d embryonic exposure did not affect the performance of fish in the feeding or OMR assays, but did affect their performance in the C-start assay (Table 1). The lack of adverse impacts after embryo-only exposure could be a result of the exposure concentration used in the present study. During range finding studies, larvae were found to be more sensitive to CPF exposure than embryos and when LC_{50} values for embryo acute toxicity tests have been compared to other fish acute toxicity test types there has been a noted lack of toxicity of neurotoxicants to embryos (Lammer et al. 2009; Knöbel et al. 2012; Klüver et al. 2015; Glaberman et al. 2017; Krzykwa et al. 2019). Therefore, the lack of behavioral alterations resulting from embryonic exposure may be a result of the exposure concentration being too low to elicit long-lasting impacts on neurological function rather than an indicator that embryonic exposure to CPF does not have impacts on neurological function/development. Alternatively, the lack of behavioral alterations may have been a result of depuration, as previous studies have indicated that CPF- and esfenvalerate-induced changes in C-start and feeding behavior were no longer apparent after depuration periods of 7 and 6 d, respectively(Floyd et al. 2008; Qiu et al. 2017).

Table 1. Summary table of the sensitivity of the selected assays to chlorpyrifos exposure; − indicates there was no significant difference from control in response to exposure, + indicates there was a significant difference from the control in response to exposure.

	Chlorpyrifos exposure regime	
Assay	5-d Embryo	12-d Embryo-larval
5-d Eye size	−	
5-d Snout-vent length	+	
12-d Length	−	+
Feeding	−	+
Optomotor response:		
Time Following	−	+
Time spent swimming	−	−
Response latency	−	−
C-start:		
Latency	−	−
Escape angle	−	−
Escape velocity	+	−
Percent response	−	+

There were significant alterations in the performance of fathead minnow larvae in all three behavioral assays (feeding, OMR, and C-start) in response to a 12-d embryo-larval exposure to CPF (Table 1). Although there were significant alterations in OMR resulting from CPF exposure, the amount of time it takes to conduct the assay was found to limit sample sizes and reduce the practicality of the assay. The C-start assay

was the most sensitive of the three behavioral assays investigated in the present study as both the 60 and 120 μg CPF/L groups were found to be significantly different from the 30 μg CPF/L exposure group (Fig. 15D). Additionally, the C-start assay was the only behavioral assay that detected any impacts resulting from the 5-d embryonic exposure (Table 1). Feeding and C-start were the most practical of the selected assays, because they took less time and allowed for larger samples sizes. These assays are also tied to ecologically-relevant behaviors that have been associate with population-level impacts making them suitable for incorporation into environmental risk assessments and/or population models (Ågerstrand et al. 2020). Further work to standardize behavioral testing methods, and to link alterations to ecologically relevant behaviors, will help promote the use of these assays when investigating the potential environmental impacts of neurotoxic compounds.

Chapter 4: A Comparison of Two Methods for Estimating Critical Swimming Speed (U_{CRIT}) in Larval Fathead Minnows: The Laminar Flow Assay and The Spinning Task Assay

INTRODUCTION

Critical swimming performance (U_{CRIT}; Brett 1964) has been directly tied to cardiac output of fish and is thought to be a good predictor of swimming capability (Claireaux et al. 2005). The most common method of estimating U_{CRIT} is through the use of a swim flume in which the fish is confined to a portion of the flume and exposed to a constant laminar current (Brett 1964). The speed of the water flow is incrementally increased until the fish is fatigued and can no longer maintain its position against the current. The amount of time required to push the fish to fatigue is then used for the calculation of U_{CRIT}:

$$U_{CRIT} = U + \left[U_i \left(\frac{T}{T_i}\right)\right]$$

where U is the highest water velocity at which the fish was able to maintain its position against the current (cm/s), U_i is the increase in velocity after each time interval (cm/s), T is the time that the fish spent at its highest velocity (U) before fatigue (s) and T_i is the prescribed interval time between each incremental increase in velocity (s) (Brett 1964).

Though methods for performing laminar flow assays are well documented, there are feasibility issues associated this assay, such as the cost and relatively large footprint of commercial recirculating flumes and the time required to assess multiple fish (Killen et al. 2017). A potential alternative to the laminar flow assay is the spinning task assay. This assay is a modification of an existing respirometry method (Nilsson et al. 2007; Munday et al. 2009) and was originally developed to assess the motor coordination and swimming behavior of zebrafish (Blazina et al. 2013); however, it has also been proposed as an alternative method for estimating U_{CRIT} (Usui et al. 2018). In this assay, a fish is placed in a 1 L beaker with a magnetic stir bar. The beaker is then placed on a magnetic stir plate and the speed of the magnetic stirrer is incrementally increased, generating a current for the fish to swim against. Similar to the laminar flow assay, the current is increased

until the fish is fatigued and can no longer maintain its position against the current. The speed of the water, as well as how long the fish spent swimming at that speed, is then used for the calculation of U_{CRIT}. The spinning task assay could provide a more feasible alternative to the laminar flow assay as the supplies needed for the spinning task assay are less costly (both stir plates and stir bars are common laboratory equipment), and the assay requires less space than that needed for a large swim flume. The reduced cost and size of the apparatus may allow for multiple testing setups to be run simultaneously and therefore allow for increased sample size.

Although laminar flow and spinning task assays have been compared for the purposes of determining metabolic rate (Rummer et al. 2016), to our knowledge there have been no direct comparisons between these assays when utilized for the purpose of estimating U_{CRIT}. Therefore, our goal was to determine whether the spinning task assay is a suitable alternative to the traditional laminar flow assay for calculating U_{CRIT}. To achieve this goal, U_{CRIT} values estimated from each assay were compared for fathead minnow larvae at 3 time points (14, 19, and 24 days post fertilization, dpf).

METHODS

Maintenance of Brood Stock

All procedures involving fathead minnows were conducted in accordance with Texas Christian University (TCU) Institutional Animal Care and Use Committee approved methods (Protocol 18-12). Fathead minnow larvae utilized in the present study were produced by the TCU fathead minnow colony which was originally obtained from Hydrosphere. To procure larvae, 10 breeding colonies, each consisting of 1 male and 8 females, were housed in 30 L glass aquaria. Fish were maintained under optimal breeding conditions (26°C, 16 h light: 8 h dark photoperiod). Aquaria were subject to daily 1/3 water changes, and fish were fed commercially available flake food (Tetramin) to satiation twice daily. Breeding structures (10-cm-long section of 7.6-cm diameter polyvinyl chloride schedule 40 pipe halved lengthwise) were examined at least daily for the presence of eggs. Breeding structures with adhered eggs were placed in aerated beakers and kept in an

incubation chamber (Panasonic, MIR-254) at ~27 °C until hatch, at which point larvae were transferred to 1-L beakers containing dechlorinated municipal water.

Laminar Flow Assay

The swim flume used for the laminar flow assay was designed and built according to descriptions by Stobutzki and Bellwood (1994, 1997) and Faria et al. (2009). Briefly, the six-lane flume was made from clear acrylic according to the specifications shown in Fig. 16. Each parallel lane (18 cm long x 3 cm x 11 cm deep) was enclosed by mesh on either end to prevent fish from leaving their lane and flow straighteners (a bundle of plastic straws cut to a length of 4 cm) were positioned directly upstream of each lane to reduce turbulence and promote laminar flow. To maintain constant flow, water was pumped through the flume via a recirculating system powered by a 2600 gph pump at 14 dpf and a 4400 gph pump at 19 and 24 dpf. A larger pump was utilized at 19 and 24 dpf due to increased swimming capabilities of the fish. Flow rate was regulated by a ball valve, which was calibrated based upon the position of the valve handle relative to a protractor mounted behind it. Speeds were calibrated by determining how long it took for water flowing from the valve to fill a 5-L container divided by the total cross-sectional area of the flume and the number of lanes (Faria et al. 2009). At each setting, the flow was measured 3 times and the resultant average flow rate was used to generate a calibration curve demonstrating the relationship between the angle of the valve handle and water velocity. The flow speeds used in the experiment varied from ~2.5 - 24.5 cm/s at 14 dpf and from ~3 - 63 cm/s at 19

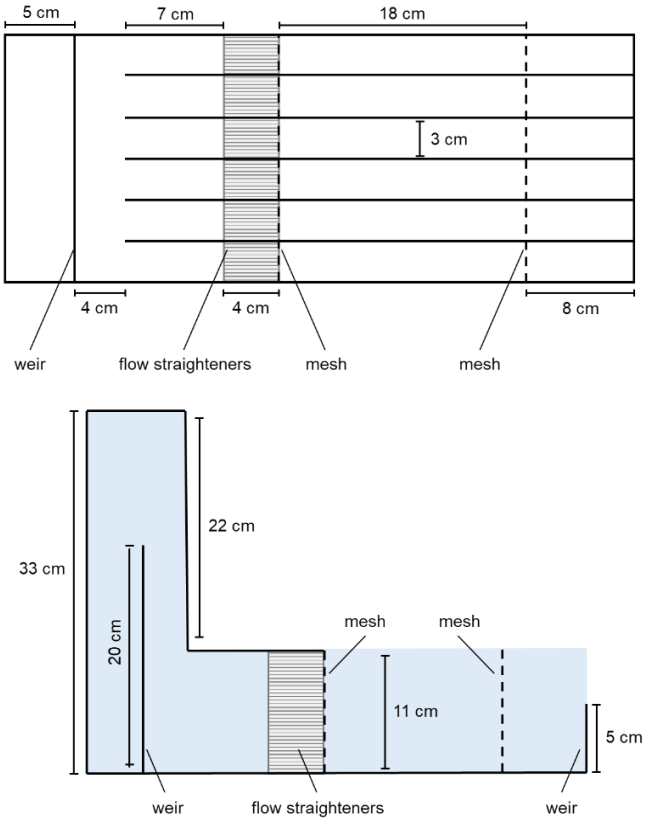

Figure 16. Top-down and side view of laminar swim flume design with dimensions indicated.

and 24 dpf. The water current in the flume was increased at 2-min intervals (T_i) by ~2 cm/s (U_i) on day 14 and by ~6.5 cm/s (U_i) on days 19 and 24. Fish were given a 5-min acclimation period at the lowest speed for that day to allow the fish to acclimate to the flume prior to increasing flow rates. Failure was defined to occur when a fish was unable to pull themselves away from the mesh at the end of lane, even after prodding via a plastic transfer pipette.

Spinning Task Assay

The spinning task assay was carried out using the methods described by Usui et al. (2018). Briefly, 750 mL of water was added to a 1-L French press coffee maker (Ikea) with the mesh guard submerged so that ~ 400 mL of water was displaced above the mesh. A magnetic stir bar was added to the bottom compartment of each coffee maker prior to depression of the mesh guard and each French press was placed on a single-plate magnetic stir plate that had been calibrated for speed to ensure uniformity across multiple apparatuses. Water temperature in each French press was checked prior to the initiation of all trials and water was replaced between each trial to ensure water temperatures of ~26 °C. A total of 5 individual French press swim chambers and stir plates were used in the present study. Water velocity at each speed setting on the stir plate was determined by monitoring a free-floating 1 x 1 cm piece of tape on the surface of the water and counting the number of rotations during a 30 s interval. To allow for direct comparisons between the results of the spinning task and laminar flow assays, the median circumference of each French press was estimated and used to convert water velocities from rpm to cm/s. Water speeds varied from ~1.6-22.6 cm/s. Fish were allowed 5 minutes at 1.6 cm/s to acclimate to the swim chamber. After the initial acclimation period, the water speed was increased by ~2 cm/s every 2 minutes until failure. Failure was defined as the time at which the fish was unable to maintain its position against the water current for ≥ 30 s.

Timing Comparison

Newly hatched (< 24 h) eleutheroembryos were transferred to 1-L beakers containing 650 mL of clean water for a total of 25 fish per beaker. Beakers were covered with clear acrylic to reduce evaporation and maintained at ~27 °C in a Panasonic MIR-254 incubator with gentle aeration. Water changes of 80% were performed daily and fish were fed newly-hatched live *Artemia* nauplii twice daily. On each assessment day (14, 19, and 24 dpf), one group of 20 fish was selected and divided into 2 groups of 10 with one group designated for the laminar flow assay and one for the spinning task assay (Fig. 17). One hour after the morning feeding 5 fish from the laminar flow group were subjected to the laminar flow assay, while 5 fish from the spinning task group were subjected to the spinning task assay. This was repeated until all 10 fish from each group had been assessed. After the groups completed their respective assays, the fish were allowed to rest for 3 h. After this rest period, fish were fed again to account for the potential influence of feeding on swim performance, and an hour later the group that had previously completed the laminar swim assay was subjected to the spinning task assay and vice versa. Individuals were tracked to allow for direct comparison of the two assay types. After the conclusion of the second assay, the mass and length of each fish was measured, as size is known to impact U_{CRIT}. Different groups of fish were used on each assessment day to limit the potential impacts of

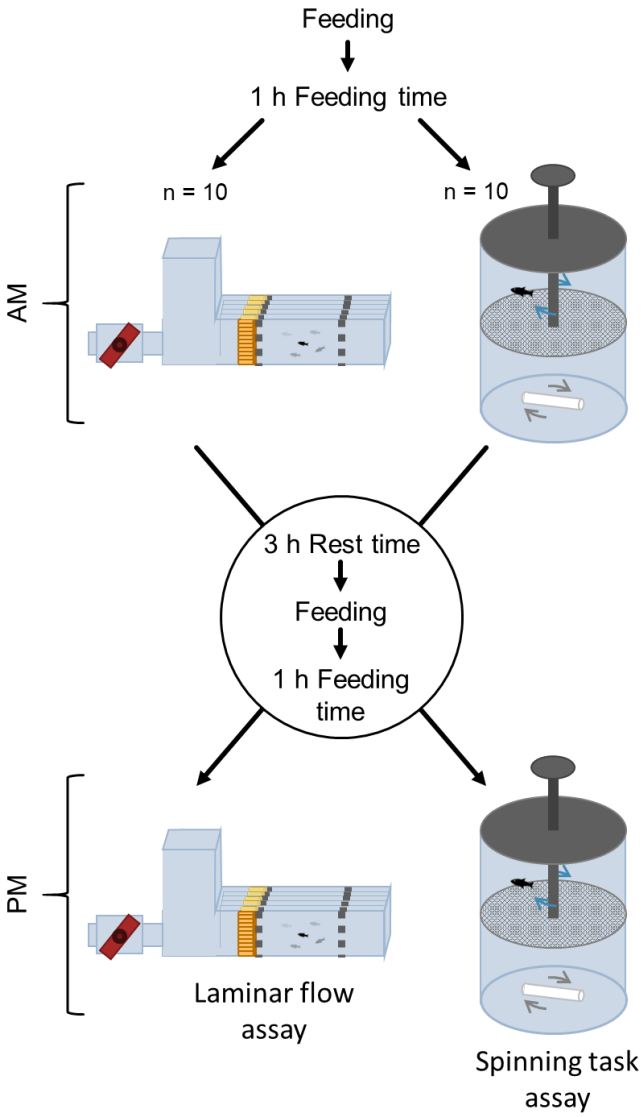

Figure 17. Schematic showing the experimental design for the timing comparison.

exercise training (Palstra et al. 2010; Usui et al. 2018). In cases where U_{CRIT} could not be calculated due to a lack of failure (i.e., the swimming capabilities of the fish exceeded the maximum water velocity within a given assay), the fish were excluded from the analysis at that time point.

Statistical Analysis

Paired t-tests were usedperformed to compare the performances of fish in each assay at each time point. Comparisons between time points were performed via one-way analysis of variance (ANOVA), followed by a Tukey post-hoc comparison to identify significant differences between groups, after confirmation of equal variance via a Levene's test for equality of variance. In cases of unequal variance, a Wilcoxon test followed by a Steel-Dwass post hoc all-pairs nonparametric test was utilized. All statistical analysis was performed using the statistical software package JMP 14.0.

RESULTS AND DISCUSSION

When the performance of fish in each assay was compared, there were no significant differences between the two assay types at 14 dpf (Fig. 18, Matched-pairs t-test, $p = 0.91$). However, significant differences between the two assays were detected at 19 and 24 dpf (Fig. 18, Matched-pairs t-test, $p < 0.01$). The lack of comparable results at 19 and 24 dpf was likely due to differences in how fish performed in the two assays with increasing age and size. When U_{CRIT} was estimated via the laminar flow assay, there were significant differences between days (Fig. 18, Wilcoxon, $p < 0.01$), with fish having significantly higher U_{CRIT} at 19 and 24 dpf than at 14 dpf. Although there was also a significant difference across days for U_{CRIT} values

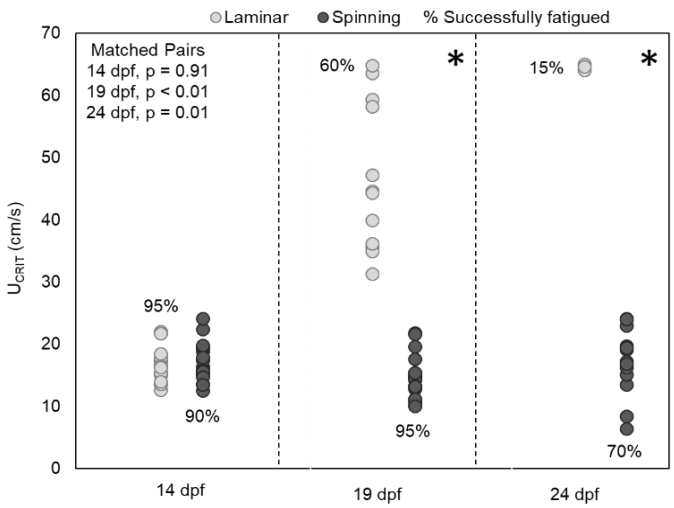

Figure 18. Critical swimming speeds (U_{CRIT}) estimated via the laminar flow and spinning task assays at 14, 19 and 24 days post fertilization (dpf). * indicates significant increase in laminar flow assay U_{CRIT} values at that time point when compared to 14 dpf. Percentages indicate the percentage of fish for which U_{CRIT} values could be calculated for in each assay.

estimated via the spinning task assay (Fig. 18, ANOVA, $p = 0.04$), post-hoc analysis was unable to determine which days were different from one another. The increase in performance of fish in the laminar flow assay was likely due to growth of the fish, because there was a significant correlation between length and U_{CRIT} as measured by the laminar flow assay (Fig. 19, $p < 0.01$, $R^2 = 0.64$), but not for U_{CRIT} as measured by the spinning task assay (Fig. 19, $p = 0.73$, $R^2 = 0.00$).

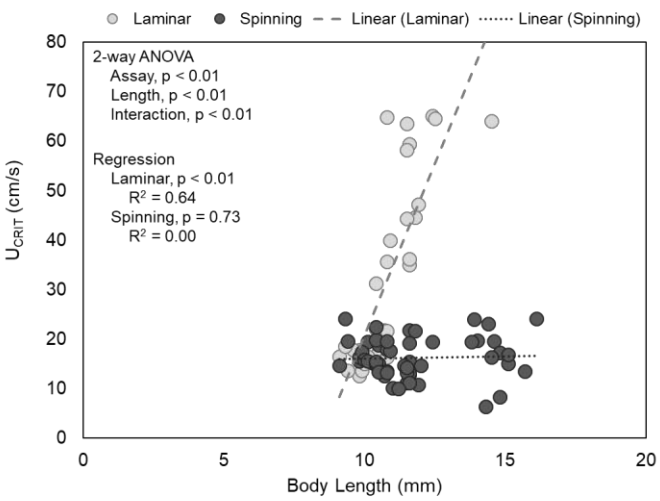

Figure 19. Estimated critical swimming speeds (U_{CRIT}) from the laminar flow and spinning task assays.

The significant differences in U_{CRIT} values estimated from the two different assays at 19 and 24 dpf suggests that the spinning task assay is not a suitable substitute for the laminar flow assay. The lack of a significant and positive correlation between length and U_{CRIT} values estimated via the spinning task assay was unexpected, as previous research, performed with laminar flow style flumes, has repeatedly demonstrated that the U_{CRIT} values of larval fish increase as age and size increase (Faria et al. 2009; Silva et al. 2015). In addition, when the spinning task assay was used to estimate the endurance of adult zebrafish, there were significant differences in how long the fish spent swimming at the maximum speed they could maintain between two different size classes of fish (Blazina et al. 2013); however, analysis was not done to fully characterize the relationship between length and the time that each fish swam at maximal speed. This lack of relationship between U_{CRIT} values generated by the spinning task assay and fish length may suggest that the spinning task assay is not estimating U_{CRIT} in a manner consistent with that of the laminar flow assay. This is further supported by the results of Rummer et al. (2016), which showed differences in estimated maximal metabolic rates (MMR) when two respirometry methods (laminar current vs. circular current) were compared. The authors hypothesized that lower MMR measured via circular respirometry methods were a result of muscular exhaustion, but not true fatigue, due to an imbalance in muscle use resulting from constant rotational motion

In addition, it is likely that the speed of the current in spinning task assay chambers is not as consistent as it is in the laminar flow chambers due to varying speeds across the area of the vortex (i.e., velocity at the outermost edge of the chamber may not be equivalent to that at the center of the chamber) (Nilsson et al. 2007; Blazina et al. 2013; Rummer et al. 2016).

Although these results indicate that the laminar flow assay was a more reliable method for estimating U_{CRIT} in larval fish, especially in cases where comparisons may be made to other studies, there are practicality issues associated with the laminar flow assay. One limitation that was encountered in the present study was achieving fast enough water flow to successfully fatigue fish. By the time larval fathead minnows had reached 24 dpf, the laminar flume used in the present study could only successfully exhaust 15% of fish utilized in the assay (Fig. 18). Additionally, the same constraints mentioned previously (i.e., size of the flume, building a flume, etc.) were hinderances encountered during the present study. For example, during the methods development of the laminar flow assay the size of the pump was increased 4 times, and two different swim flumes were built in order to successfully exhaust fish, and even then, only 60% of the 19 dpf fish were fatigued. Despite the practicality issues associated with the laminar flow assay, the lack of correlation between U_{CRIT} values calculated from the spinning task assay and the laminar flow assay, as well as the lack of correlation between body size and U_{CRIT} as estimated from the spinning task assay, suggests that the spinning task assay may not be a viable alternative to the laminar flow assay for the evaluation of swim performance.

Chapter 5: Comparing the Sensitivity of Two Methods for Estimating Critical Swimming Speed (U_{CRIT}) in Larval Fathead Minnows: The Laminar Flow Assay and The Spinning Task Assay

INTRODUCTION

The cardiovascular impacts of environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs), heavy metals, flame retardants and dioxins are well documented (Revis et al. 1981; Antkiewicz et al. 2005; Hicken et al. 2011; Du et al. 2015; Isales et al. 2015; Incardona and Scholz 2016). The cardio-toxic effects of these compounds have been characterized through a variety of methods including, but not limited to, the presence of edema, gene expression changes, and histology (Antkiewicz et al. 2005; Incardona et al. 2006; Adeyemo et al. 2015). Although these metrics are adequate for elucidating what physiological alterations may have taken place, they are not direct measures of cardiac function and performance. Thus, studies seeking to evaluate the impacts of toxicants more broadly employ measures of critical swimming performance, or U_{CRIT} (Brett 1964), which measures the cardiovascular functioning of fish by evaluating the fish's endurance.

As described in Chapter 4, this project sought to compare two different methods of estimating U_{CRIT}, the spinning task assay and the laminar flow assay. Previous work (Chapter 4) indicated that the two methods were comparable when evaluating the swim performance of 14 day post fertilization (dpf) larval fathead minnows, suggesting that either method would be suitable for estimating the U_{CRIT} of larval fathead minnows at that age. The goal of the present study was to compare the sensitivity of the two methods for detecting chemically induced alterations in cardiovascular function. To achieve this goal, we compared the U_{CRIT} values generated from each assay for 14 dpf fathead minnow larvae that were exposed to the known cardiotoxicant, phenanthrene (PHN).

METHODS

Maintenance of brood stock

All procedures involving fathead minnows were conducted in accordance with Texas Christian University (TCU) Institutional Animal Care and Use Committee approved methods (Protocol 19/07). Fathead minnow embryos utilized in the present study were produced by the TCU fathead minnow colony which was originally obtained from Hydrosphere. To procure embryos, 10 breeding colonies, each consisting of 1 male and 8 females, were housed in 30 L glass aquaria. Fish were maintained under optimal breeding conditions (26°C, 16hr light: 8hr dark photoperiod). Aquaria were subject to daily 1/3 water changes, and fish were fed commercially available flake food (Tetramin) to satiation twice daily. Breeding structures (10-cm-long section of 7.6-cm diameter polyvinyl chloride schedule 40 pipe halved lengthwise) were examined at least daily for the presence of eggs. Eggs were gently removed from the structures using a metal spatula, transferred into petri dishes containing the appropriate test solution, and assessed for viability using a Leica DMi1 inverted microscope with a heated stage.

Chemicals

Phenanthrene (PHN, 98% purity) was purchased from Sigma Aldrich. Stock solutions were made in methanol and stored in the dark at 4°C. Test solutions were made directly from stock solutions using dechlorinated municipal water, with solvent concentrations of 0.01% (OECD 2013). Due to differences in embryo and larva sensitivity to PHN embryos were exposed to nominal concentrations of 0 (solvent control), 231, 263 μg PHN/L, while larvae were exposed to nominal concentrations of 0 (solvent control), 178, and 318 μg PHN/L starting at 5 days post fertilization (dpf). The highest concentrations embryo/larvae were exposed to were the calculated LC25 values for that life stage, and the low concentrations were the calculated LC10 values for that life stage based off range finding studies. A clean water control was run alongside all PHN exposures to ensure that there were no effects resulting from the solvent exposure.

Phenanthrene exposure procedures

Three independent exposures were performed for PHN. Starting at < 2 hours post fertilization (hpf), fathead minnow embryos were transferred into 100 mm x 50 mm glass crystallizing dishes containing 250 mL of test solution. Dishes were covered with clear acrylic to reduce evaporation and maintained at ~27°C in a Panasonic MIR-254 incubator with gentle aeration. Test solutions were refreshed daily, with 80% of the solution being replaced. Embryos/larvae were monitored daily for mortality. The mean (± standard deviation) pH, conductivity (µS/cm), hardness (mg/L) and alkalinity (mg/L) of the dilution water were 7.9 ± 0.3, 314.4 ± 16.2, 107.2 ± 6.6, and 108.0 ± 5.7, respectively. The mean (± SD) pH, conductivity (µS/cm), hardness (mg/L) and alkalinity (mg/L) of the highest exposure concentrations were 8.0 ± 0.1, 302.6 ± 11.4, 108.0 ± 0.0, 107.2 ± 1.8, respectively. Starting at 6 dpf fish were fed 0.06 g *Artemia* nauplii/fish twice daily. Swim performance was assessed on day 14 using the same methods as those used on assessment days for the timing comparison (Chapter 4; Fig. 17).

Statistical Analysis

Comparison of the two assay types was performed via matched pairs t-test. Comparisons between exposure groups were performed via one-way analysis of variance (ANOVA), followed by a Tukey post-hoc comparison to identify significant differences between groups, after confirmation of equal variance via a Levene's test for equality of variance. In cases of unequal variance, a Wilcoxon test followed by a Steel-Dwass post hoc all-pairs nonparametric test was utilized. Statistical analysis was performed using JMP 14.0.

RESULTS AND DISCUSSION

There was no significant difference in U_{CRIT} values generated by each assay type (Fig. 20, Matched-pairs t-test, p = 0.86). There were also no significant differences in U_{CRIT} values between exposure groups, as generated from the laminar flow assay (Fig. 21, Wilcoxon, p = 0.36) after a 14-d PHN exposure. This same held true when U_{CRIT} values were calculated from the spinning task assay (Fig. 22, Wilcoxon, p = 0.34). The lack of alterations in swim performance was unexpected, as fish exposed to PAH mixtures during development have been shown to experience lasting changes to swimming performance (Hicken et al. 2011; Mager et al. 2014; Incardona et al. 2015), and PHN in particular has been demonstrated to induce cardiovascular abnormalities such as pericardial edema and altered heart rate (Incardona et al. 2004). It is possible that using a complex PAH mixture, such as crude oil, rather than PHN alone would have resulted in the expected reductions in U_{CRIT}. It is also possible that the lack of alterations in U_{CRIT} could be a factor of the age of the fathead minnows. Although previous work in marine fish has measured U_{CRIT} in early life stage organisms (Stobutzki and Bellwood 1997; Faria et al. 2009; Silva et al. 2015), the previous studies that saw reductions in U_{CRIT} after embryonic exposure to PAH mixtures allowed the fish to develop for longer, 25 d – 10 mon, before evaluating their swimming performance (Hicken et al. 2011; Mager et al. 2014; Incardona et al. 2015).

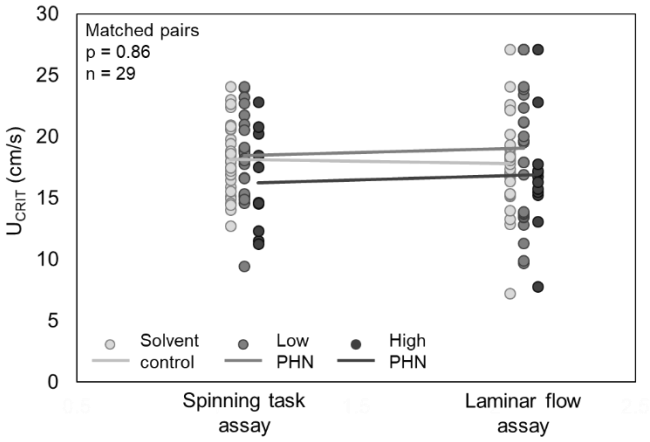

Figure 20. Critical swimming speed (U_{CRIT}) of larval fish exposed to phenanthrene (PHN) estimated via the spinning task assay and the laminar flow assay.

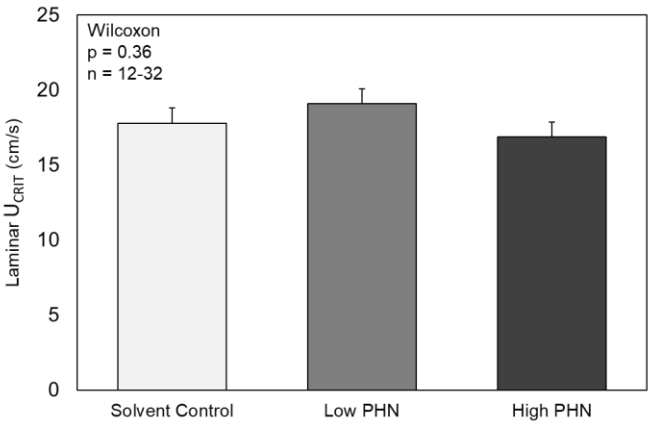

Figure 21. Average critical swimming speed (U_{CRIT}) of fathead minnow larvae exposed to phenanthrene (PHN) estimated via the laminar flow assay. Error bars indicate standard error.

Although there were no significant differences in the U_{CRIT} values of fish exposed to PHN during development, there were significant differences in the survival of fish exposed to PHN (Fig. 23, ANOVA, $p < 0.01$), with fish in the high-dose PHN group having significantly reduced survival. Anecdotally, it was the fish that had obvious edema that did not survive to 14 dpf, the day at which swim performance was measured. Therefore, the fish with visually obvious cardiovascular abnormalities were not assessed for their swim performance, as they did not survive. As such, it is possible that fish that survived the PHN exposures, and for whom swim performance data exists, did not experience the adverse cardiovascular impacts that their deceased counterparts did. Although the lack of alterations in cardiovascular performance among surviving larvae was not expected, the high mortality rates observed in response to PHN concentrations that induced edema are consistent with the findings of previous studies. Exposures to PAHs at concentrations that induced significant edema in fish embryos also impacted larval survival (Colavecchia et al. 2006), even in a study in which embryos were moved into clean water after hatch (Incardona et al. 2004). This high mortality rate among individuals in the high PHN exposure group resulted in modification of the original hypothesis that pericardial edema is indicative of altered cardiovascular performance. The modified hypothesis was that pericardial edema is predictive of reduced survival. This hypothesis was tested in the experiments outlined in Chapter 6.

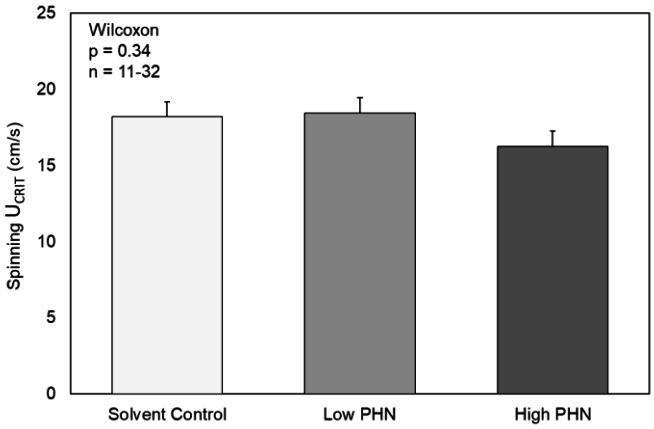

Figure 22. Average critical swimming speed (U_{CRIT}) of fathead minnow larvae exposed to phenanthrene (PHN) estimated via the spinning task assay. Error bars indicate standard error.

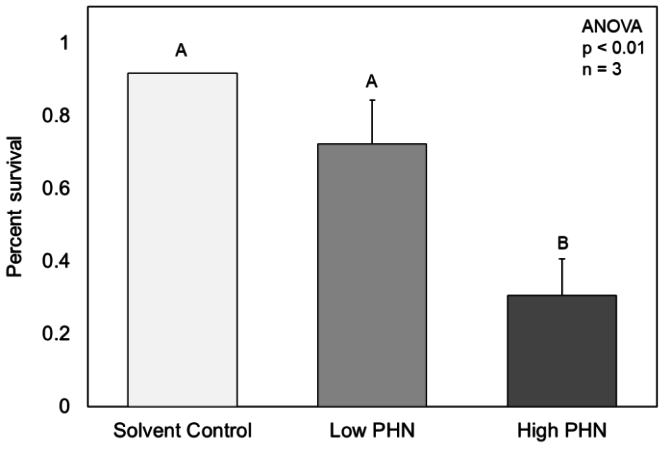

Figure 23. Average percent survival of fathead minnow larvae exposed to phenanthrene (PHN). Error bars indicate standard error. Different letters above bars indicate significant differences between groups.

Chapter 6: Investigating the Predictive Power of Three Potential Sublethal Endpoints for the Fish Embryo Toxicity Test: Snout-Vent Length, Eye Size and Pericardial Edema

INTRODUCTION

Fish have long been used as model organisms in aquatic toxicology, and there are a number of standardized test methods using juvenile/adult fish, such as the fish acute toxicity test (OECD 1992) and the fish early-life stage toxicity test (US EPA 1996). However, recent legislative demands, such as REACH in the EU (EU 2006) and the Frank R. Lautenberg act in the USA (US Congress 2016), mandate that alternatives to vertebrate animal testing be utilized whenever possible. This has led to increased interest in using fish embryos in place of more developed organisms (Lammer et al. 2009; Embry et al. 2010; Knöbel et al. 2012; Strähle et al. 2012; Belanger et al. 2013) as embryos are thought experience less distress during toxicant exposures than older fish (EFSA 2005; Strähle et al. 2012). This interest in developing aquatic toxicity testing methods that use fish embryos as a model organism has led to the development of the fish embryo acute toxicity (FET) test (OECD 2013).

Though FET test procedures have been carried out with several species (Braunbeck 2005), a FET test featuring zebrafish (*Danio rerio*) has been adopted as the standard method for assessing whole-effluent acute toxicity in Germany (DIN 2003). It has also been adopted as a standardized OECD test method for evaluating chemical toxicity (OECD 2013); though the data derived from the zebrafish FET test cannot be used alone to satisfy the regulatory requirements of REACH and thus, must be combined with other data in a weight-of-evidence approach (Sobanska et al. 2018). Further adoption and enhanced use of the FET test as a standard method for assessing toxicity has been limited due, in part, to two primary factors: 1) unlike other fish-based test types, the FET test lacks endpoints capable of estimating sublethal adverse effects (US EPA 2002b; OECD 2013); and 2) the FET test has been found to be less sensitive than other test types for some chemicals, particularly those classified as neurotoxicants (Klüver et al. 2015; Sobanska et al. 2018). The development of sublethal endpoints for the FET test has been proposed to overcome these limitations and to broaden the appeal of the FET test (Voelker et al. 2007; Weil et al. 2009; Jeffries et al. 2015; Roush et al.

2018; Stengel et al. 2018; Krzykwa et al. 2019). A variety of potential sublethal endpoints have been proposed, including the expression of genes related to development (Voelker et al. 2007; Adeyemo et al. 2015), embryonic behaviors (Selderslaghs et al. 2013), and the presence of developmental abnormalities (Selderslaghs et al. 2012; Jeffries et al. 2015; Krzykwa et al. 2018; Roush et al. 2018; Krzykwa et al. 2019). Of these proposed sublethal endpoints, developmental abnormalities may offer the most promise for inclusion as standard FET test endpoints. In general, developmental abnormalities can be easily visualized during routine mortality assessments conducted as part of the FET test making them feasible with regard to time, cost, and equipment required. Furthermore, developmental abnormalities are robust in that they are observed in response to chemicals with diverse modes of action, suggesting that their utility is not limited to specific classes of chemicals (Weichert et al. 2017; Wold et al. 2017; Krzykwa et al. 2019). Three developmental abnormalities that have been demonstrated to have potential for inclusion as sublethal FET test endpoints are eye size, pericardial edema, and snout-vent length (Krzykwa et al. 2018; Krzykwa et al. 2019). Alterations in these metrics have been observed in response to a variety of toxicants with different modes of action, including heavy metals (Cheng et al. 2000; Kim et al. 2013; Chen et al. 2015; Wold et al. 2017), pharmaceuticals (Carlsson and Norrgren 2004; Akande et al. 2010), dioxins (Henry et al. 1997; Belair et al. 2001; Hill 2004; Antkiewicz et al. 2005), pesticides (Cook et al. 2005) and polycyclic aromatic hydrocarbons (PAHs; Colavecchia et al. 2006; Ana dos Anjos et al. 2011; He et al. 2012; Sørhus et al. 2017).

Despite the wide-spread responsiveness of eye size and pericardial edema to contaminants, to our knowledge, no research has been done to elucidate the ability of eye size and pericardial edema to serve as early predictors of longer-term adverse outcomes. Consequently, the predictive power and utility of eye size and pericardial edema as sublethal FET test endpoints is limited. It is reasonable to hypothesize that reductions in eye size may serve as an early warning sign of longer-term adverse effects given evidence from previous studies showing that contaminant-induced reductions in the eye size of zebrafish embryos are associated with alterations in the development of neural tissues (Cheng et al. 2000; Wold et al. 2017), embryonic behavior (Kim et al. 2013; Wold et al. 2017), and visual acuity (Bilotta et al. 2002). Likewise, the development of pericardial edema may serve as a predictor of longer-term cardiovascular dysfunction as

previous studies have shown that fish exposed to concentrations of crude oil that induced pericardial edema as embryos also had reduced cardiovascular function as juveniles/adults (Hicken et al. 2011; Mager et al. 2014; Incardona et al. 2015).

The goal of this project was to advance the use of alternative methods in toxicity testing by investigating whether these developmental abnormalities are suitable sublethal endpoints for the FET test. To achieve this goal, three sublethal developmental abnormalities--snout-vent length, eye size, and pericardial area--were measured at the termination of fathead minnow (*Pimephales promelas*) FET tests. Exposed embryos were then transferred to clean water and the ability of these endpoints to predict alterations in feeding behavior, predator response, critical swimming speed, and/or mortality were investigated over a 9-d period after FET test termination. Three specific objectives were met: 1) to investigate whether altered snout-vent length is predictive of longer-term changes in growth and delayed mortality, 2) to determine whether alterations in eye size are predictive of reduced feeding behavior, altered predator response, and delayed mortality. and 3) to determine whether alterations in pericardial area are predictive of altered cardiovascular performance (as measured by critical swimming speed) and delayed mortality. In addition, the utility of these metrics as additional endpoints in the FET test was evaluated by estimating LC50 values with and without these metrics being included as lethal endpoints when appropriate. Though standardized methods are available for zebrafish, fathead minnows were utilized given that they are a preferred species in much of North America and to expand upon previous efforts to develop a fathead minnow FET test (Braunbeck et al. 2005; Ankley and Villeneuve 2006; Jeffries et al. 2014).

METHODS

Maintenance of Brood Stock

All procedures involving fathead minnows were conducted per methods approved by the Texas Christian University (TCU) Institutional Animal Care and Use Committee (Protocol 1907). Fathead minnow embryos and larvae used in the present study were produced by the TCU fathead minnow colony, which was originally obtained from Hydrosphere. To obtain embryos and larvae, 10 breeding colonies, each consisting

of 1 male and 8 females, were housed in 30 L glass aquaria. These ratios were selected based on the results of Roush et al. (2018). Fish were kept under optimal breeding conditions (26 °C, 16 h light: 8 h dark photoperiod). Aquaria were subject to daily 1/3 water changes and fish were fed commercially available flake food (Tetramin) to satiation twice daily. Breeding structures (10-cm-long section of 7.6-cm diameter polyvinyl chloride schedule 40 pipe halved lengthwise) were examined at least once daily for the presence of eggs. Eggs were removed from the structures using a metal spatula, transferred into petri dishes containing the appropriate test solution, and assessed for viability using a Leica DMi1 inverted microscope with a heated stage.

Chemicals

Cadmium chloride (Sigma-aldrich, 99.99% trace metals basis) was used to make a Cd stock in NanoPure water to achieve a concentration of 100 mg Cd/L and stored in a glass container wrapped in foil at 4°C. Test solutions were made daily via serial dilution using dechlorinated municipal water. Nominal concentrations were 0, 10, 20, 40, and 80 µg Cd/L. Water samples (125 mL) were taken from the highest concentration renewal solutions on days 0, 2, and 4 and from the dilution water on day 2. Each sample was spiked with 1 mL nitric acid and then stored in a light-impermeable, acid-washed Nalgene bottle at 4°C. Sample concentrations were measured via ICP-OES on an Ametek SPECTROBLUE ICP-OES with Ametek Smart Analyzer Vision software version 6.01.

A DCA (Alfa Aesar , 98% purity) stock solution was made in NanoPure water to achieve a concentration of 100 mg DCA/L. This stock solution was stored for ≤ 60 d in a glass container wrapped in foil at 4°C. Test solutions, which were made daily via serial dilution using dechlorinated municipal water, had nominal concentrations of 0, 87.5, 175, 350, and 700 µg DCA/L. Water samples (50 mL) were taken from the highest-concentration renewal solutions on days 0, 2, and 4 and from the dilution water on day 2. Samples were stored in amber glass vials at 4°C for < 24 h prior to extraction via solid phase on HyperSep C18 (500 mg/6 mL) cartridges as described by Field et al. (1997). Cartridges were prepared with 8 mL acetone, followed by 8 mL of methanol and 10 mL NanoPure water. Cartridges were dried under a vacuum for 10 min

and then eluted with 1.5 mL methanol/acetonitrile (50:50). Standards were processed in the same fashion as the samples. Extracts were quantified using a NanoDrop microvolume spectrophotometer (ThermoFisher) at 247 nm absorbance. Concentrations of the highest exposure solution were found to be 113% of nominal, with no DCA detected in dilution water.

Perfluorooctanesulfonic acid potassium salt (Matrix Scientific, ≥ 98% purity) was diluted in NanoPure water to create a 500 mg/L stock solution of PFOS. The stock solution was stored in a polypropylene container wrapped in foil at 4°C for ≤ 40 days. Test solutions were made daily via serial dilution using dechlorinated municipal water. Nominal concentrations were 0, 625, 1,250, 2,500, and 5,000 μg PFOS/L. Water samples (14 mL) were collected from the highest concentration renewal solutions on days 0, 2, and 4 and from the dilution water on day 2. Samples were filtered via cellulose acetate vacuum filters with a 0.22 um pore size (Corning) before storage in 15 mL polypropylene centrifuge tubes at - 20°C. Samples were then sent to Todd Anderson at Texas Tech University for analysis. Concentrations of the highest exposure solutions were found to be 127% of nominal, with no PFOS detected in dilution water.

Experimental Exposure Procedure

Embryo exposures were conducted using modified OECD protocols and previously published fathead minnow FET test methods (Braunbeck et al. 2005; OECD 2013; Jeffries et al. 2014). Fathead minnow embryos at ≤ 32 cell stage were randomly sorted into 24-well plates, with one embryo and 2.5 mL of the appropriate test solution per well. Plates were covered with manufacturer-supplied lids and kept in an incubation chamber (Panasonic, MIR-254) at ~27 °C under a photoperiod 16 h light: 8 h dark. At 5 dpf, individual embryos were transferred into gently aerated 30 mL beakers containing 25 mL of clean dechlorinated municipal water and covered with clear acrylic. Individual larvae were tracked throughout the exposure so that the performance of larvae in the selected behavioral assays could be correlated to alterations in eye size or pericardial area. Starting at 6 dpf, larvae were fed 1.06 mg newly hatched (< 24h) *Artemia* nauplii/larvae twice daily, this amount was increased to 2.12 mg nauplii/larvae starting at 12 dpf. All time points were pushed back by a day for the DCA exposure as a result of delayed hatch in all exposure groups.

Although fathead minnows typically hatch at 72 – 96 hpf (Devlin et al. 1996), the embryos in the DCA exposure did not hatch until 96 - 120 hpf. Thus, the exposure was continued for another 24 h so that embryos would be exposed for ~ 24 h post hatch in a manner similar to the Cd and PFOS exposures. Water changes (~80%) were completed daily. Water quality parameters are given in Table 2. Embryos and larvae were checked daily for standard markers of mortality (i.e., coagulation, lack of tail detachment, lack of somite development, and absence of heartbeat) until the conclusion of the test at 14 dpf. In addition, several behaviors associated with neurological and cardiovascular development were evaluated throughout the duration of the exposure as shown in Fig. 24 and described below.

Table 2. Water quality parameters (mean ± standard deviation) measured in dilution water and in the renewal solution for the highest test concentration for tests with cadmium (Cd), 3,4-dichloroaniline (DCA), or perfluorooctanesulfonic acid (PFOS).

Compound		pH	Conductivity (µS/cm)	Hardness (mg/L)	Alkalinity (mg/L)
Cd	Dilution	7.9 ± 0.2	355.3 ± 19.0	109.0 ± 6.0	112.5 ± 5.3
	Renewal	7.9 ± 0.1	332.5 ± 1.0	117.3 ± 12.2	112.3 ± 5.9
DCA	Dilution	7.9 ± 0.1	316.5 ± 19.7	105.0 ± 2.0	102.0 ± 2.3
	Renewal	8.1 ± 0.1	303.8 ± 4.6	103.0 ± 2.0	98.0 ± 2.3
PFOS	Dilution	8.3 ± 0.1	348.3 ± 15.7	126.0 ± 7.7	110.0 ± 5.2
	Renewal	8.4 ± 0.1	336.3 ± 10.0	127.0 ± 14.4	112.0 ± 7.3

Assessment of Length, Eye Size, and Pericardial Area at FET Test Termination

At 5 dpf, all surviving eleutheroembryos were anesthetized using buffered MS-222 (0.1 g MS-222/L), positioned in 3% methylcellulose and photographed using a Leica DMi1 inverted microscope (25X) with a warmed stage for measurement of snout-vent length, eye size and pericardial area using ImageJ (Rasband 1997). Length was measured as snout-vent length. Because previous research has revealed a significant correlation between eye size and body length (Krzykwa et al. 2018), eye size was measured as eye size index, which was calculated by dividing eye area by the length of the eleutheroembryos. Pericardial area was measured as total area.

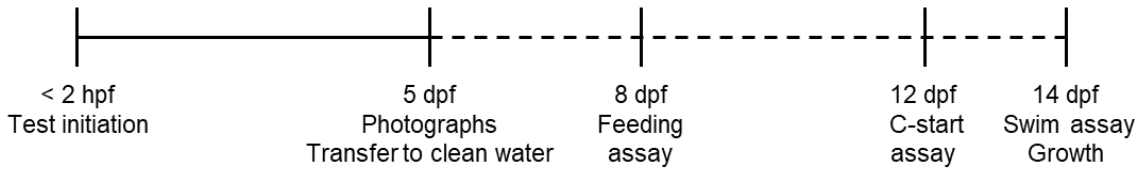

Figure 24. Experimental timeline indicating behavioral assays. Dashed line indicates when fish were moved from exposure solutions into clean dechlorinated municipal water. hpf = hours post-fertilization, dpf = days post fertilization.

The Fathead Minnow Larval Feeding Assay

Feeding assays were conducted prior to the regularly scheduled morning feeding at 8 dpf for Cd and PFOS and at 9 dpf for DCA (Fig. 24). One larval fish was added to each well of a 6-well plate with 12 mL of dechlorinated municipal water and 10 prey items (< 24 h *Artemia* nauplii), for a total of 6 fish/group. Well plates containing larvae were placed in an incubator and larvae were given 1 h to consume the nauplii before larvae were photographed for the determination of length and transferred back to their original exposure chambers. The number of remaining nauplii were quantified on a light table.

The Fathead Minnow Larval C-Start Assay

The C-start methods utilized in the present study were the same as those described in Chapter 3 and are based on methods published by McGee et al. (2009) and Painter et al. (2009). Twelve-thirteen dpf larval fathead minnows were individually transferred to a 5-cm diameter glass petri dish containing 20 mL of warmed (~27°C) dechlorinated municipal water and placed atop a 1 mm grid in the C-start staging arena. Initiation of the vibrational stimulus via a haptic motor mounted to the bottom of the platform was indicated by the simultaneous appearance of a laser pointer in a corner of the filming arena. Individual larvae were evaluated from treatments in a sequential pattern (control, low, medium, high, control, low, etc.). On mornings when C-start was measured, fish were given 1 h to feed before the initiation of the C-start assays and measurements were limited to as many videos as could be recorded within a 6 h time frame. In instances when larvae did not respond to the first vibrational stimulus, they were given up to 3 opportunities to respond with a 30 s interval between vibrations to confirm the lack of response to the vibrational stimulus. Videos were analyzed as TIFF stacks using ImageJ. The percentage of fish responding to the stimulus, escape latency, angle, and total escape response were quantified.

Swimming Performance Assay

Critical swimming speed (U_{CRIT}) was measured at 14-15 dpf using the laminar flow assay as described in Chapters 4 and 5. The flow speeds used in the experiment varied from ~2.5-24.5 cm/s. Fish were given a 5-min acclimation period at ~2.5 cm/s prior to increasing flow rates. Failure, which was deemed to occur

when a fish was unable to pull away from the mesh at the end of lane even after prodding via a plastic transfer pipette, was recorded for each fish and U_{CRIT} was calculated as follows:

$$U_{CRIT} = U + \left[U_i \left(\frac{T}{T_i}\right)\right]$$

where U is the highest velocity the fish was able to maintain (cm/s), U*i* is the velocity increment (cm/s), T is the time elapsed at fatigue velocity (s), and T*i* is the prescribed interval time (s) (Brett 1964). The water current in the flume was increased at 2-min intervals (T*i*) by ~2 cm/s(U*i*).

Growth

Immediately following the swim performance assay, 14-15 dpf fish were euthanized in buffered MS-222 (0.3 g MS-222/L) and wet-weight and length were measured to evaluate growth.

Statistical Analysis

For each test chemical, significant differences between exposure groups were tested for using analysis of variance (ANOVA) followed by Tukey post-hoc multiple comparisons tests. In cases of unequal variance as determined by a Levene's test, a Wilcoxon test followed by Steel-Dwass post-hoc all-pairs nonparametric test was used. For categorical data (i.e., response/no response to vibrational stimulus in the C-start assay and mortality data), a Pearson's chi-squared test was used to test for significant differences and post-hoc analysis was performed via pairwise comparisons of groups with a Holm-Bonferroni adjustment. For mortality data, the pairwise comparison was performed via a Fisher's Exact test, as some exposure groups had values of zero in the contingency table. Within each replicate, measurements of eye size and pericardial area, as well as the results of the behavioral assays, were normalized to the control group. Estimations of mortality resulting from the proposed sublethal endpoints were calculated via inverse prediction. Statistical analysis was performed using JMP 14.0.

RESULTS AND DISCUSSION

Snout-Vent Length as a Predictor of Longer-Term Changes in Growth and Mortality

PFOS did not have a significant impact on 5-d snout-vent length (Fig. 25, Wilcoxon, $p = 0.06$), unlike Cd and DCA, both of which had significant effects (Fig. 25, ANOVA, $p < 0.01$). Fish exposed to 700 μg DCA/L were on average significantly shorter than fish from the control group and other DCA exposure groups. Fish exposed to 40 and 80 μg Cd/L were significantly shorter than fish from other Cd exposure groups and the control group. Both Cd and DCA have previously been shown to reduce the snout-vent length of fathead minnow eleutheroembryos (Krzykwa et al. 2018; Krzykwa et al. 2019). The lack of alterations in body length after PFOS exposure was unexpected, as chronic (150 d) exposure to 250 μg PFOS/L significantly impacted growth in zebrafish (Wang et al. 2011) and embryonic exposure to 3 and 5 mg PFOS/L resulted in significantly reduced length in zebrafish eleutheroembryos (Shi et al. 2008).

There were no significant differences in length measured at 14 dpf after embryonic exposure to Cd or PFOS (Fig. 25, Wilcoxon or ANOVA, $p \geq 0.05$). In contrast, significant alterations in 14-d length were noted in response to DCA (Fig. 25, ANOVA, $p < 0.01$); though, post-hoc analysis was unable to determine which groups differed from one another. When the relationship between 5-d snout-vent length and 14-d length was investigated in fish exposed to PFOS, no significant relationship emerged (Fig. 25, $p = 0.25$). However, significant, but very weak, positive relationships between the two metrics were detected for fish that were exposed to either DCA (Fig. 25, $R^2 = 0.08$, $p < 0.01$) or Cd (Fig. 25, $R^2 = 0.29$, $p < 0.01$). This lack of a strong relationship between the two metrics indicates that alterations in snout-vent length measured at the conclusion of the FET test are not necessarily predictive of longer-term impacts to growth. However, the Cd and DCA exposure groups that experienced significant decreases in mean 5-d snout-vent length relative

to the controls each exhibited ≥ 80 % mortality at 14 d (Fig. 26), indicating that significant reductions in 5-d growth may be predictive of decreased survival.

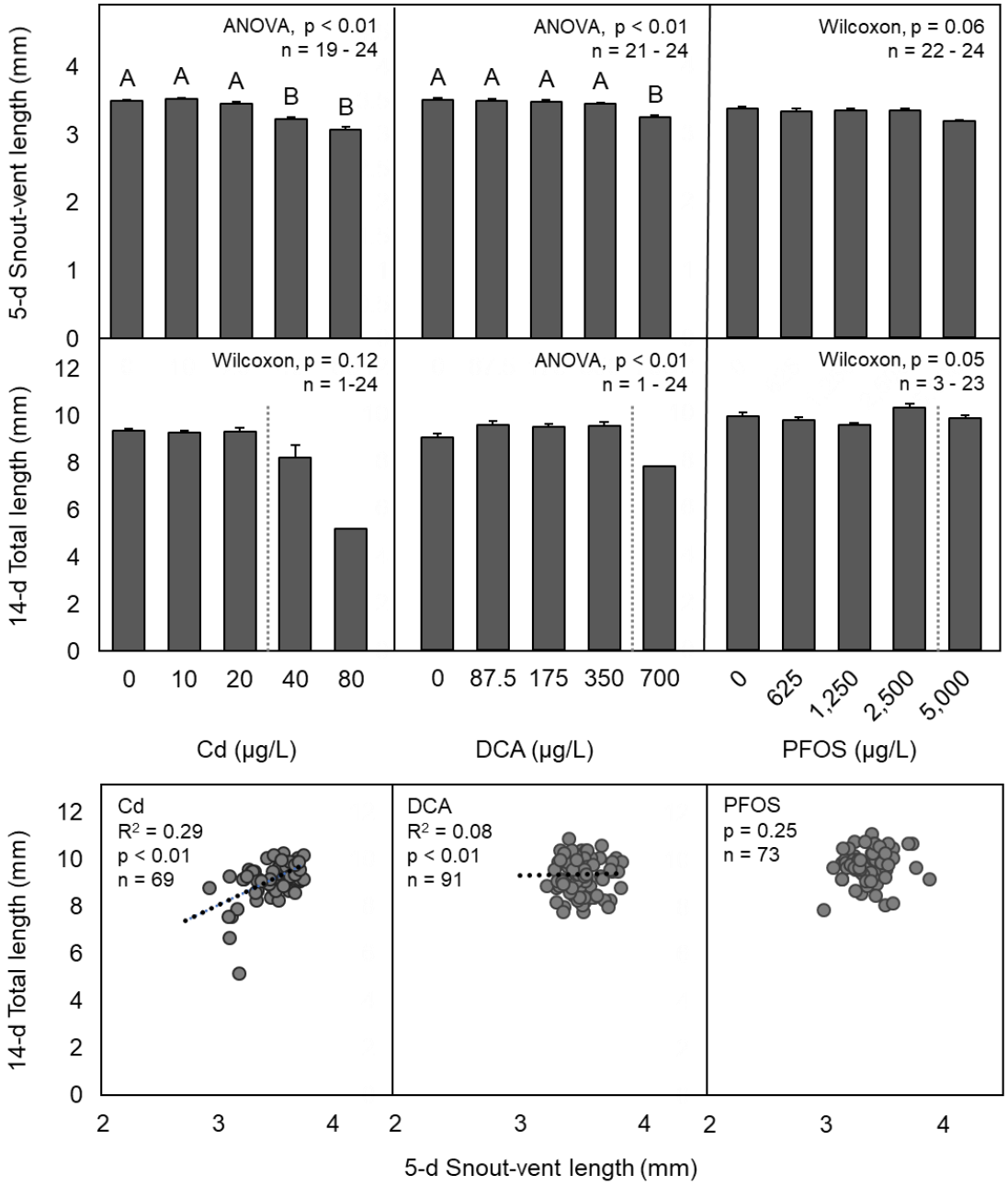

Figure 25. Average snout-vent length at 5 days post fertilization (dpf) (top panels), total length at 14 dpf (middle panels), and the regression between the two endpoints (bottom panels) for fathead minnow embryos/larvae exposed to cadmium (Cd), 3,4-dichloroaniline (DCA), or perfluorooctanesulfonic acid (PFOS). Error bars indicate standard error. Different letters above bars indicate significant differences between groups. Exposure groups to the right of the grey dashed line had ≥ 80% mortality at 14 dpf.

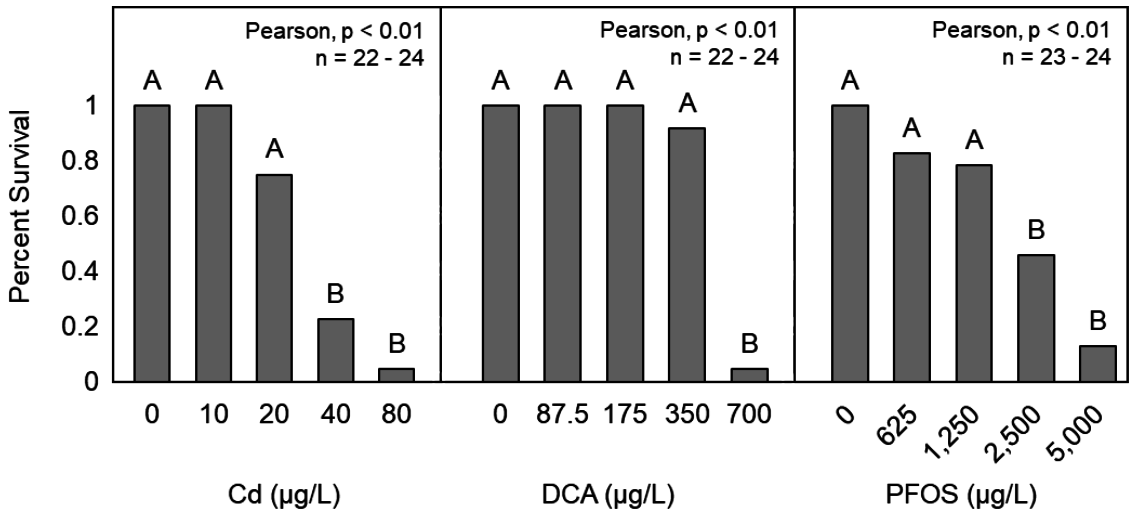

Figure 26. Percent survival, at 14 d, of fathead minnow larvae exposed to cadmium (Cd), 3,4-dichloroaniline (DCA), or perfluorooctanesulfonic acid (PFOS) as embryos. Different letters above bars indicate significant differences between groups.

Although 5-d snout-vent length did not appear to be a good predictor of long-term reductions in growth, it may offer promise as an additional FET test endpoint given its apparent ability to predict mortality. A comparison of 5-d snout-vent length of fish from all 3 exposures to the longer-term survival of those fish at 14 dpf revealed that fish with snout-vent lengths ≤ 2.75 mm had a 95% probability of mortality and fish with a snout-vent length of ≤ 2.99 mm had an 80% probability of mortality (Fig. 27). When snout-vent length was used as a marker of mortality for estimating LC50 values for each chemical in the present study, there were not enough individuals with snout-vent lengths < 2.99 mm to calculate an LC50 for DCA. However, the calculated LC50 values (lower 95 % confidence interval, upper 95 % confidence interval) for Cd and PFOS were 107.99 µg Cd/L (66.13, 622.29) and 10.5 mg PFOS/L (5.7, 33,600), respectively. This Cd LC50 value is similar to that reported by larval exposure tests (113.3 ± 24.9 µg Cd/L) and ~80 fold lower than that previously estimated

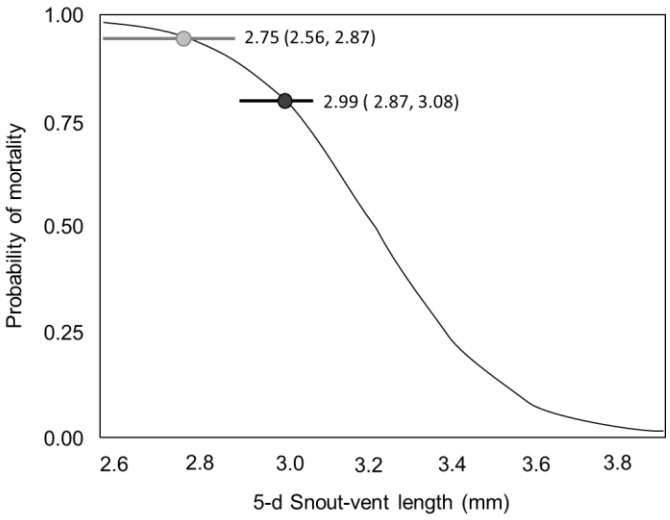

Figure 27. Predicted mortality (lower 95% confidence interval, upper 95% confidence interval) by snout-vent length at 5 dpf for fathead minnows. Light grey indicates 95% likelihood of morality, dark grey indicates 80% likelihood of mortality

by the fathead minnow FET test (8285.2 ± 342.5 µg Cd/L; Krzykwa et al. 2019). Similarly, the PFOS LC50 value is much lower than previously reported LC50 values of 70.17 and 34.2 mg PFOS/L in zebrafish embryos (Ding et al. 2011; Stengel et al. 2017). These results suggest that for some compounds, such as Cd and PFOS, the inclusion of snout-vent length as a marker of mortality for the FET test could increase test sensitivity.

Eye Size as a Predictor of Behavioral Alterations and Mortality

Of the chemicals utilized in the present study, PFOS was the only one that did not have a significant impact on relative eye size index (Fig. 28, ANOVA, $p = 0.56$). Significant differences in relative eye size index resulted from Cd exposure (Fig. 28, Wilcoxon, $p < 0.01$), as fish exposed to 80 µg Cd/L had significantly smaller eyes than control and 10 µg Cd/L-exposed fish. Fish exposed to 40 µg Cd/L had significantly smaller eyes those in the control, 10, and 20 µg Cd/L groups. The decrease in eye size as a result of Cd exposure was expected as previous work has reported decreases in eye size resulting from Cd exposure (Cheng et al. 2000; Wold et al. 2017; Krzykwa et al. 2019) and other heavy metals (Kim et al. 2013). Fish exposed to DCA also had significant alterations in relative eye size index (Fig. 28, ANOVA, $p < 0.01$), with fish exposed to 350 and 700 µg DCA/L having significantly increased relative eye size index compared to controls. This increase in eye size was unexpected in light of previous findings indicating reductions in eye size in DCA-exposed fathead minnow embryos (Krzykwa et al. 2018). It is possible that the discrepancy between the current and previous studies is related to differences in the concentrations of DCA utilized, as the concentration range used in the previous study (1 - 4 mg DCA/L) exceeds that of the present study (87.5-700 µg DCA/L). Further, it is possible that the increases in eye size index were an artifact of the observed decreases in length; however, this possibility can be eliminated given the results of a post-hoc analysis showing that eye area itself was significantly increased in DCA-exposed fish (*data not shown*, means ± standard error ranged from 0.089 ± 0.002 to 0.096 ± 0.002 mm^2, ANOVA, $p = 0.01$). It remains unclear how such increases in eye size arise or

how they should be interpreted, as reports of reduced eye size in response to chemical exposure are far more common (Cook et al. 2005; Li et al. 2012; Kim et al. 2013; Zhang et al. 2015; Houbrechts et al. 2016; Sørhus et al. 2017; Wold et al. 2017).

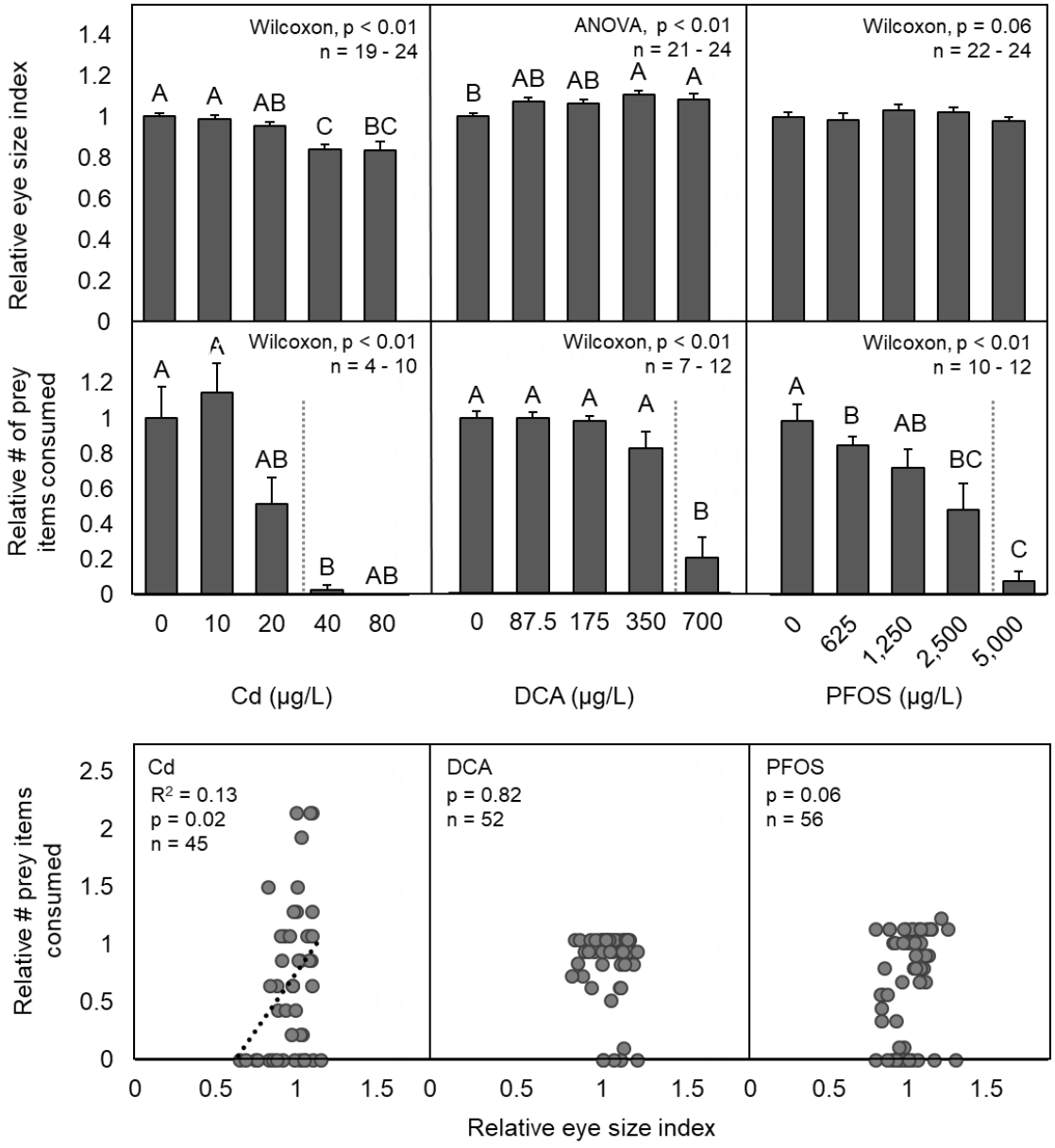

Figure 28. Average relative eye size index at 5 days post fertilization (dpf) (top panels), relative number of prey items consumed in the feeding assay (middle panels), and the regression between the two endpoints (bottom panels) for fathead minnow embryos/larvae exposed to cadmium (Cd), 3,4-dichloroaniline (DCA), or perfluorooctanesulfonic acid (PFOS). Error bars indicate standard error. Different letters above bars indicate significant differences between groups. Exposure groups to the right of the grey dashed line had ≥ 80% mortality at 14 dpf.

The only significant alteration in C-start response in the present study was a significant reduction in the number of the fish that responded to the vibrational stimulus after exposure to DCA (Table 3, Pearson chi-square, $p = 0.03$), with fish exposed to 175 μg DCA/L responding less frequently than fish exposed to 87.5 μg DCA/L. Aside from this, no significant differences in relative response latency, total escape response, or escape angle after exposure to Cd, DCA, or PFOS (Table 3, ANOVA, $p \geq 0.20$ in all cases) were detected. Nor were alterations in the number of fish that responded to the vibrational stimulus after Cd or PFOS exposure detected (Table 3, Pearson chi-squared, $p \geq 0.07$). The general lack of alteration in C-start behaviors was unexpected as previous evidence suggests that Cd, PFOS, and DCA can all alter neurodevelopment. For example, Cd has been shown to impact behaviors such as place preference (Wold et al. 2017) and hyperactivity (Ruiter et al. 2016), while PFOS has been shown to negatively impact the average number of neuromast cells present along the lateral line of zebrafish embryos (Stengel et al. 2018) and to alter C-start responses of juvenile qingbo (*Spinibarbus sinensis*) (Xia et al. 2015). Embryonic exposure to DCA (500 μg

Table 3. Average (± standard error) C-start response for fathead minnow embryos/larvae exposed to cadmium (Cd), 3,4-dichloroaniline (DCA), or perfluorooctanesulfonic acid (PFOS) and the regression between said C-start response and 5-d relative eye size index. Error bars indicate standard error. Different letters next to values indicate significant differences between groups ($p = 0.03$). * indicates that the exposure group had ≥ 80% mortality at 14 dpf.

		C-start (relative)			C-start response (%)
		Response latency	Total escape response	Escape angle	
Cd (μg/L)	0	1.00 ± 0.16	1.00 ± 0.32	1.00 ± 0.05	79
	10	0.68 ± 0.12	1.07 ± 0.28	1.02 ± 0.05	83
	20	1.11 ± 0.20	1.01 ± 0.27	1.14 ± 0.04	95
	40*	0.53 ± 0.29	0.31 ± 0.11	0.93 ± 0.15	100
	80*	0.37 ± 0.05	0.60 ± 0.49	1.10 ± 0.11	50
	Regression	p = 0.70	p = 0.80	p = 0.60	
DCA (μg/L)	0	1.00 ± 0.17	1.00 ± 0.34	1.00 ± 0.03	71 ^{AB}
	87.5	0.88 ± 0.19	1.69 ± 0.41	0.99 ± 0.03	79 ^{A}
	175	1.00 ± 0.34	1.19 ± 0.39	0.96 ± 0.04	42 ^{B}
	350	0.75 ± 0.25	1.29 ± 0.36	0.96 ± 0.04	52 ^{AB}
	Regression	p = 0.98	p = 0.75	p = 0.57	
PFOS (μg/L)	0	1.00 ± 0.33	1.00 ± 0.21	1.00 ± 0.02	75
	625	0.51 ± 0.24	1.07 ± 0.22	0.97 ± 0.04	79
	1,250	0.68 ± 0.29	1.01 ± 0.24	0.97 ± 0.03	79
	2,500	0.98 ± 0.41	0.91 ± 0.26	0.94 ± 0.04	100
	5,000*	0.30 ± 0.07	0.84 ± 0.41	0.91 ± 0.01	67
	Regression	p = 0.27	p = 0.56	p = 0.25	

DCA/L) resulted in decreased locomotor activity in zebrafish larvae (Scheil et al. 2009). The lack of alterations in C-start responses after embryonic Cd and PFOS exposure in the present study may indicate that any impacts on neuromast cells after a 5-d embryonic exposure were not sufficient to impact behavior. In fact, previous studies have shown that sublethal exposure to Cd (5 μg Cd/L) had no impact on C-start performance in juvenile sea bass (*Dicentrarchus labrax*; Faucher et al. 2006) nor on tap-startle responses in zebrafish larvae embryonically-exposed to Cd (concentrations ≤ 3.59 mg Cd/L; Ruiter et al. 2016). However, it is also possible that fish did experience alterations in neuromast cells, but that fish experiencing such alterations did not survive until 12 dpf when C-start response was assessed. Alternatively, it is also possible that the effects of Cd and PFOS were diminished after a 7-d depuration period (the time frame between the cessation of exposures and measurement of C-start behaviors). Unsurprisingly, consideringBecause of the lack of significant alterations in C-start behavior, there was no significant correlations between relative eye size index and any of the metrics by which C-start response was assessed (Table 3, $p \geq 0.25$). This would indicate that while there were reductions in eye size in response to Cd exposure, said alterations were not predictive of altered neurological function as assessed via the C-start assay.

Unlike the C-start assay, there were significant decreases in feeding behavior as a result of exposure to all 3 compounds utilized in the present study (Fig. 28, Wilcoxon, $p < 0.01$ in all cases). Fish from the 40 and 80 μg Cd/L groups consumed significantly fewer prey items than fish from the 10 μg Cd/L group, and fish from the 40 μg Cd/L consumed significantly fewer prey items than fish from the control group. Fish exposed to 700 μg DCA/L consumed significantly fewer prey items than fish from all other groups. The fish in the PFOS exposure demonstrated a concentration-response trend with fish exposed to 625, 2,500, or 5,000 μg PFOS/L consuming significantly fewer *Artemia* nauplii than control fish, and fish exposed to 5,000 μg PFOS/L consuming fewer prey items than fish exposed to 625 or 1,250 μg PFOS/L. The alterations in feeding behavior in response to all 3 compounds investigated suggests that the feeding behavior assay is sensitive to compounds with varying modes of action. This is likely because feeding behavior involves the integration of multiple physiological systems to identify and capture prey (Floyd et al. 2008; Portugues and Engert 2009). It is worth noting that the exposure groups that had significant reductions in feeding behavior

compared to the controls in the Cd and DCA experiments also had ≥ 80% mortality at 14 dpf (Fig. 26), indicating that significant reductions in feeding behavior may be a predictor of reduced larval survival.

Although the feeding assay was more sensitive to chemical exposure than the C-start assay, there was no significant relationship between relative eye size index and performance in the feeding assay for fish exposed to either DCA or PFOS (Fig. 28, $p \geq 0.06$). This is interesting, as there were significant alterations in feeding behavior in response to exposure to both chemicals; however, neither induced reductions in relative eye size index. For Cd-exposed fish, a significant, though weak, positive relationship between relative eye size index and feeding behavior was detected (Fig. 28, $R^2 = 0.13$, $p = 0.02$). The lack of a strong relationship between eye size and feeding behavior, despite the noted alterations in feeding behavior in response to all 3 test chemicals, indicates that alterations in eye size are not predictive of altered feeding behavior. Again, this is likely due to the fact that feeding behavior requires the integration of multiple physiological systems to both identify and capture prey (Floyd et al. 2008; Portugues and Engert 2009).

In addition to eye size not being predictive of alterations in behavior as hypothesized, it also does not seem to be suitable for predicting mortality. A relative eye size index of ≤ 0.41 is predicted to result in an 80% chance of mortality, while a relative eye size index of ≤ 0.01 is predicted to result in a 95% chance of mortality (Fig. 29). The minimum relative eye size index recorded in the present study (0.50 in response to 80 μg Cd/L) was larger than the relative eye size index predicted to cause 80% mortality. As such, it was not possible to use relative eye size index to estimate LC50 values for Cd, DCA, or PFOS. Without a metanalysis of other studies that measured eye size index to determine if a relative eye size index of ≤ 0.41 is

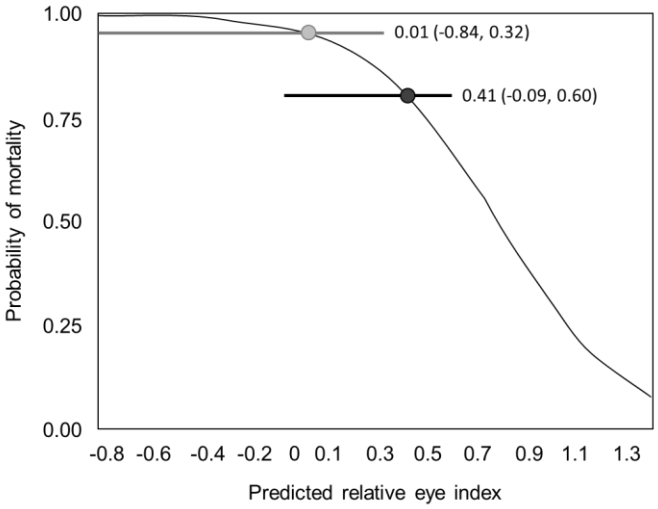

Figure 29. Predicted mortality (lower 95% confidence interval, upper 95% confidence interval) by relative eye size index at 5 dpf for fathead minnows. Light grey indicates 95% likelihood of morality, dark grey indicates 80% likelihood of mortality.

physiologically possible, it is unclear if relative eye size index could be suitable for use as a marker of mortality for the FET test.

Pericardial Edema as a Predictor of Alterations in Swim Performance and Mortality

There were significant differences in relative pericardial area in response to exposure to all 3 compounds investigated in the present study (Fig. 30, Wilcoxon, $p < 0.01$). Fish exposed to ≥ 20 μg Cd/L had significant increases in pericardial area compared to fish in the control and 10 μg Cd/L groups, while fish from the 700 μg DCA/L group had significantly larger relative pericardial areas than those from all other groups. Exposure to PFOS resulted in a concentration-response trend with fish in the 2,500 μg PFOS/L group exhibiting significantly larger pericardial areas than fish in the control, 625, and 1,250 μg PFOS/L groups, and fish exposed to 5,000 μg PFOS/L group had significantly larger pericardial areas than fish from all other groups. The observed increases in pericardial area were anticipated as exposures to Cd, DCA, and PFOS have previously been demonstrated to result in increases in pericardial area (Zheng et al. 2012; Krzykwa et al. 2018; Krzykwa et al. 2019). Of the endpoints evaluated as potential FET test endpoints in the present study, only pericardial area was found to be significantly altered in response to all 3 compounds, indicating its responsiveness to compounds with varying modes of action.

There were no significant differences in U_{CRIT} (Fig. 30, ANOVA, $p \geq 0.63$) after exposure to any of the compounds investigated in the present study, nor were there significant correlations between relative pericardial area and U_{CRIT} (Fig. 30, $p \geq 0.27$). This lack of correlation between pericardial area and U_{CRIT}, or even significant alterations in U_{CRIT} in response to chemical exposure, was unexpected. Previous research investigating the cardiovascular toxicity of PAHs reported significant alterations in U_{CRIT} in juvenile and adult fish exposed during embryonic development to PAH concentrations that induced pericardial edema (Hicken et al. 2011; Mager et al. 2014). It seems likely that in the present study fish with significant increases in relative

pericardial area did not survive until 14 dpf, when U_{CRIT} was measured, as concentrations that induced significant increases in pericardial area also resulted in ≥ 80% mortality (Fig. 26).

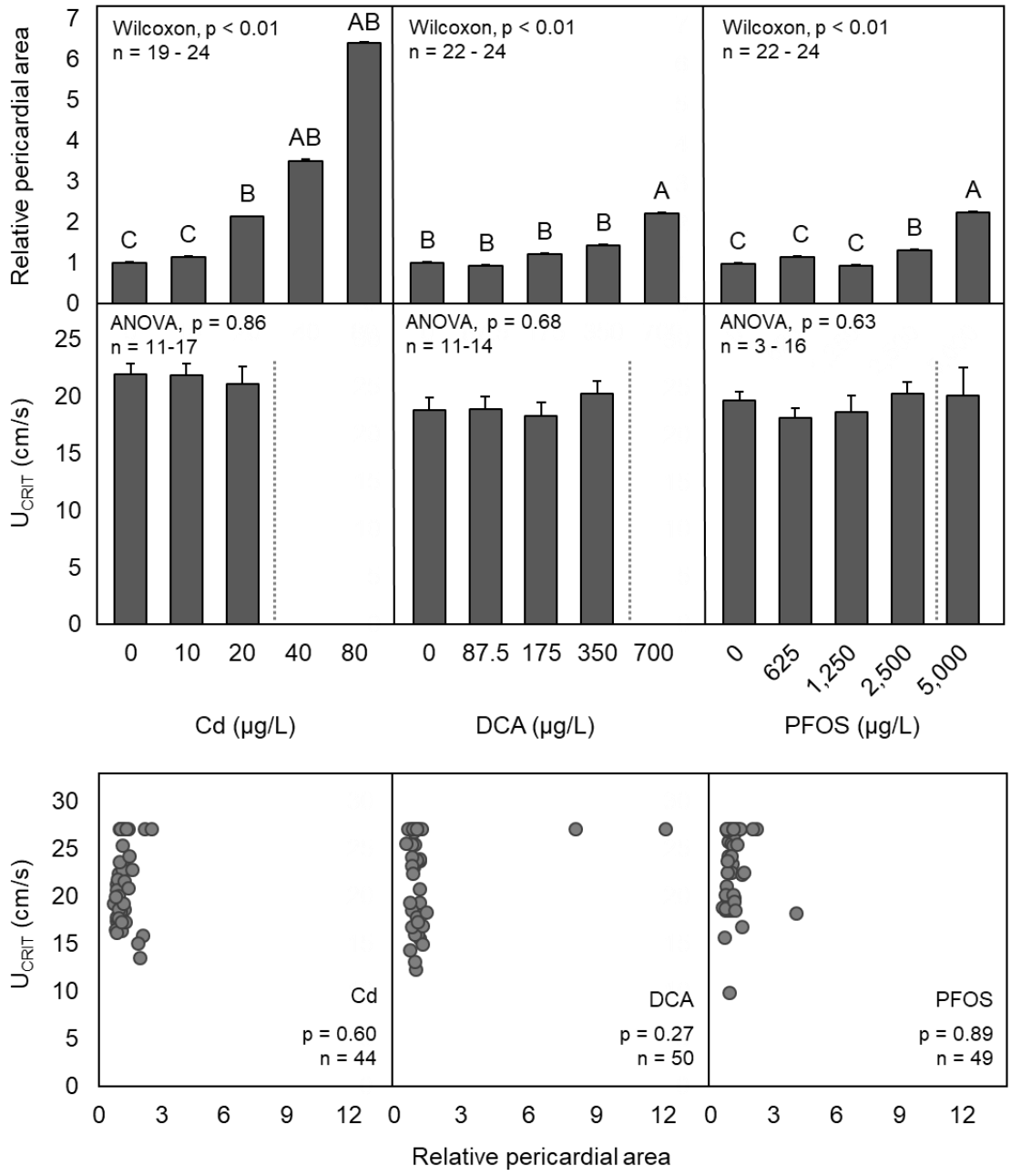

Figure 30. Average relative pericardial area at 5 days post fertilization (dpf) (top panels), critical swimming speed (U_{CRIT}, middle panels), and the regression between the two endpoints (bottom panels) for fathead minnow embryos/larvae exposed to cadmium (Cd), 3,4-dichloroaniline (DCA), or perfluorooctanesulfonic acid (PFOS). Error bars indicate standard error. Different letters above bars indicate significant differences between groups. Exposure groups to the right of the grey dashed line had ≥ 80% mortality at 14 dpf.

Although alterations in pericardial area were not correlated with alterations in cardiovascular performance as estimated by U_{CRIT}, pericardial area may be a suitable endpoint for predicting mortality. Fish with relative pericardial areas ≥ 3.54 were estimated to have an 80% probability of mortality, while fish with relative pericardial areas ≥ 4.89 have a 95% probability of mortality (Fig. 31). When relative pericardial area was treated as a marker of mortality, there were not enough fish in the DCA exposure with relative pericardial areas ≥ 3.54 to calculate an LC50 value; however, LC50 values could be generated for both Cd and PFOS. For Cd, the calculated LC50 value (lower 95% confidence interval, upper 95% confidence interval) was 56.86 μg Cd/L (43.37, 87.71). This value is roughly half of that calculated from larval exposure tests (113.3 ± 24.9 μg Cd/L) and ~ 160 fold lower than those previously estimated by the fathead minnow FET test (8285.2 ± 342.5 μg Cd/L; Krzykwa et al. 2019). In addition, it is roughly half the estimated LC50 generated for Cd when using reduced snout-vent length as a marker of mortality. For PFOS the calculated LC50 value (lower 95% confidence interval, upper 95% confidence interval) was 14.23 mg PFOS/L (6.15, 8.14 x 10^{19}). This value is higher than the value estimated by using snout-vent length as a marker of mortality, but still lower than previously reported LC50 values of 70.17 and 34.2 mg PFOS/L in zebrafish embryos (Ding et al. 2011; Stengel et al. 2017). These results indicate that the inclusion of increased pericardial area as a marker of mortality would increase the sensitivity of the FET test, especially for compounds such as Cd where the FET test has historically been less sensitive than other test types using larval fish as a model organism (Krzykwa et al. 2019).

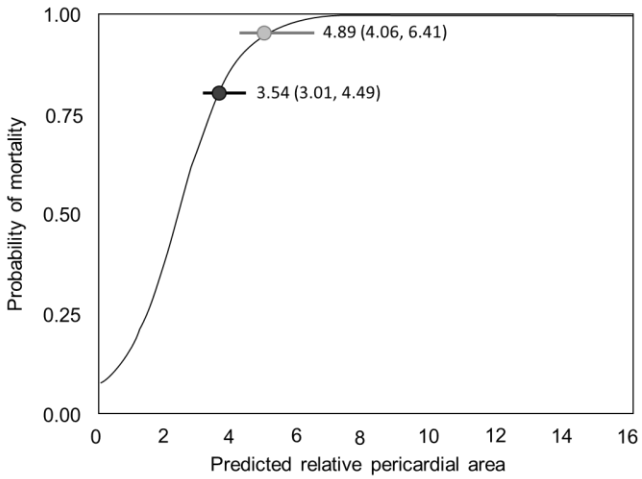

Figure 31. Predicted mortality (lower 95% confidence interval, upper 95% confidence interval) by relative pericardial area at 5 dpf for fathead minnows. Light grey indicates 95% likelihood of morality, dark grey indicates 80% likelihood of mortality.

CONCLUSIONS

The development and inclusion of alternative endpoints for the FET test has the potential to increase its sensitivity. In addition, the inclusion of such endpoints may harmonize LC50 values generated via the FET test with those from other test types for chemicals where embryos have been deemed less sensitive than older fish based upon traditional markers of mortality (Krzykwa et al. 2019). Although reductions in eye size were not predictive of altered behavior, and relative eye size index seems unlikely to increase the sensitivity of the FET test, both snout-vent length and pericardial area may prove to increase the sensitivity of the FET test despite not having the predictive power anticipated. When both metrics were used to predict mortality the estimated LC50 values were less than those found in the literature (Table 4). Although this relationship between altered snout-vent length and mortality and between increased pericardial area and mortality seems promising, further research is needed to determine whether these two metrics are predictive of increased mortality for other compounds with other modes of action. In addition, further work elucidating the threshold at which these two metrics are predictive of mortality would increase the likelihood of them being embraced as potential endpoints for the FET test.

Table 4. Summary table indicating the sensitivity of the selected endpoints to cadmium (Cd), 3,4-dichloroaniline (DCA), and perfluorooctanesulfonic acid (PFOS); + indicates that LC50 values generated using that endpoint as a marker of mortality were less than those in the literature, -- indicates that no LC50 value could be generated.

	Snout-vent length	Relative eye size	Relative pericardial area
Cd	+	--	+
DCA	--	--	--
PFOS	+	--	+

Chapter 7: Project Summary and Conclusions

The overarching goal of this project was to investigate whether alterations in proposed sublethal endpoints for the fish embryo toxicity (FET) test (i.e., reduced snout-vent length, reduced eye size index, and increased pericardial area) were predictive of long-term adverse outcomes as assessed through behavioral and cardiovascular alterations. To achieve this goal the three objectives were met. The major finding associated with each objective, are listed below.

Objective 1: Develop and validate methods for assessing neurological function and behavior in fathead minnow larvae. This objective had 2 associated sub-objectives:

a) Develop standardized methods for evaluating optomotor response (OMR) in larval fathead minnows (Chapter 2)
    - OMR assays with larval fathead minnows should utilize fish at a minimum age of 9 days post fertilization (dpf) as this was the earliest time point that larval fathead minnows displayed OMR.
    - Fish subjected to the OMR assay should be given an acclimation period of at least 4 min, as the OMR of fish progressively increased during the first 4 min of the assay then plateaued until termination of the assay.
    - The volume of water in the OMR chamber does not appear to impact OMR performance, as no differences in performance were noted when fish were held in OMR chambers containing 10 or 20 mL of water.
    - Response latency, the amount of time it took fish to identify a directional change, does not appear to be a viable endpoint for the OMR assay as the high variation in this endpoint requires unrealistic sample sizes
b) Compare 3 different behavioral assays (i.e., feeding, C-start, and OMR assays) to identify which behavioral assays were sensitive to chemically induced alterations in performance (Chapter 3)

- A 5-d embryonic exposure to chlorpyrifos (CPF) did not impact feeding or OMR performance, but fish exposed as embryos to 120 μg CPF/L did exhibit a significant decrease in escape velocity in the C-start assay.
- A 12-d embryo-larval exposure to CPF resulted in significant alterations in all 3 behavioral assays. Both feeding and OMR were similar in sensitivity, with significant alterations only being observed in fish exposed to 120 μg CPF/L; however, there was significantly reduced C-start response in fish exposed to 60 and 120 μg CPF/L. As such, it appears that the C-start assay was the most sensitive of the three assays evaluated.
- When the practicality of the three different behavioral assays were compared, the C-start and feeding assays were more practical than the OMR assay due to the amount of time needed to analyze OMR video recordings.

Objective 2: Identify and validate methods for assessing cardiovascular function in early life stage fathead minnows. This objective also had 2 associated sub-objectives:

a) Compare two methods (the spinning task assay and the laminar flow assay) for assessing cardiovascular function via critical swimming speed (U_{CRIT}) in larval fathead minnows (Chapter 4)
    - There were limitations to the swim flume design utilized for the laminar flow assay in this project, particularly for fish ≥ 19 dpf. Specifically, 19 and 24 dpf fish did not exhibit swim failure in this assay; therefore, U_{CRIT} estimates could not be made. In contrast, most fish subjected to the laminar flow assay at 14 dpf or to the spinning task assay at 14, 19 or 24 dpf were successfully fatigued making it possible to estimate U_{CRIT}.
    - The spinning task assay and laminar flow assay resulted in significantly different estimates of U_{CRIT} at 19 and 24 dpf. The U_{CRIT} estimates from the laminar flow assay increased as fish matured, as expected, whereas those from the spinning task assay did not. This indicates that the two assay types evaluate different types of swimming ability.

b) Compare the ability of these two methods to detect chemically induced alterations in cardiovascular performance of larval fathead minnows resulting from exposure to phenanthrene (PHN) (Chapter 5)

- Neither the spinning task assay nor the laminar flow assay detected significant reductions in U_{CRIT} resulting from exposure to PHN. There were, however, significant alterations in the survival of fish in the high PHN exposure group.
- The results of this experiment indicated a need to change the hypothesis that pericardial edema would be predictive of decreased cardiovascular performance to pericardial edema being predictive of reduced survival as there was high mortality observed in fish that had pericardial edema.

Objective 3: Determine if chemically induced alterations in snout-vent length, eye size and/or pericardial area at the conclusion of the FET test were predictive of longer-term adverse effects, indicated by increased mortality or altered performance in the validated neurological and cardiovascular assays. (Chapter 6)

- Snout-vent length was not a strong predictor of reduced longer-term growth; however, it was predictive of mortality. Thus, its inclusion as a FET test endpoint could still improve the sensitivity of the FET test.
- Eye size was not predictive of alterations in predator response, nor was it strongly predictive of altered feeding behavior. Additionally, the reduction in eye size that would be predictive of decreased survival was not observed in the present study. As such, the inclusion of eye size as an endpoint in the FET test would likely not increase the predictive power of the test.
- Increased pericardial area was not predictive of alterations in cardiovascular function; however, it was predictive of mortality. Thus, its inclusion as a FET test endpoint could still improve the predictive power of the FET test.
- When snout-vent length and relative pericardial area were used as predictors of mortality, the resulting estimated LC50 values were lower than those previously

generated by fish embryo tests for both cadmium and perfluorooctanesulfonic acid. In the case of cadmium, a toxicant that is known to have reduced embryotoxicity, the resultant LC50 values were more like those generated by acute toxicity tests using larval fish which have historically been more sensitive to cadmium exposure than embryos. As such, the inclusion of snout-vent length and pericardial area as predictors of increased mortality increased the sensitivity of the FET test.

The results of this project expanded the utility of the fathead minnow as a model organism in toxicology through the development/modification of multiple assays associated with both neurological function and cardiovascular function for use with the fathead minnow. Although the fathead minnow has long been the US EPA standard model organism, many behavioral assays have been developed for use with zebrafish, such as the OMR assay and the spinning task assay. This dissertation helped develop methods that would allow for such assessments to be done using the fathead minnow. One roadblock encountered in this project was a lack of information concerning methodology, and why specific methodological choices were made, for the behavioral assays selected. As such, the methods development work done as a part of this project was included in publishing efforts so that other researchers would have access to that information. In addition, as a part of the methods development portion of this project the selected assays were compared to one another to evaluate which method(s) would be the best investment of time, effort, and funds. This information will be helpful to other researchers who may also be interested in assessing either neurological or cardiovascular function in larval fish.

This dissertation also indicates that developmental abnormalities such as reduced snout-vent length and/or increased pericardial area may be predictive of reduced survival. For two of the three compounds investigated inclusion of these metrics as markers of mortality resulted in estimated LC50 values lower than those previously generated using embryos. For the third compound, 3,4-dichloroanline (DCA), the inability to estimate LC50 values using snout-vent length or relative pericardial area was likely a result of the dosing utilized. Re-analysis of previous DCA FET test data revealed an estimated LC50 value of 2.49 mg DCA/L

when pericardial area was included as a marker of mortality and an estimated LC50 value of 1.70 mg DCA/L when snout-vent length was included as a marker of mortality (Krzykwa et al. 2018). These values are lower than the LC50 value generated by the FET test without the inclusion of these metrics (8.8 mg/L; Krzykwa et al. 2018), though they are still higher than values in the literature from larval tests (0.35 mg DCA/L; (Jeffries et al. 2014). This would suggest that while inclusion of these endpoints would increase the sensitivity of the FET test, more work may be needed to further harmonize results from FET tests with those from other test types for compounds that have been demonstrated as having reduced embryotoxicity.

Although the results of this project elucidate the utility of developmental abnormalities as potential endpoints for the FET test, further research is needed before such endpoints are likely to be adopted. One question that has come up frequently is whether the relationship between snout-vent length/pericardial area and mortality will hold true for compounds with differing modes of action. Investigating such a relationship would require a larger scale effort of screening a wide variety of compounds. Such work would also lead to a more precise estimate of when these metrics are predictive of reduced survival. In addition, it is necessary to investigate whether the inclusion of these metrics would increase the sensitivity of the FET test for compounds for which it has previously been shown to be less sensitive than other test types, such as neurotoxicants. This could be done via meta-analysis of already available datasets; however, many researchers report edema as either presence/absence or via a severity score. This is likely because the measurement of pericardial area is more time consuming than these other methods of identifying pericardial edema. Additional work investigating whether these metrics of pericardial edema are as predictive of reduced mortality as measurements of relative pericardial area would be helpful for both being able to do meta-analyses of existing datasets as well as for increasing the likelihood that researchers will begin to include pericardial edema as a marker of mortality. Although there is still work to be done, this project reveals that there is reason to continue developing alternative endpoints for the FET test, both to increase its utility/sensitivity and to further its adoption by regulatory agencies.

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VITA

Professional Preparation

Ph.D. Biology, Texas Christian University, Expected graduation May 2020
Field of Study: Biology
M.S., Biology, Texas Christian University, Graduated May 2017
Field of Study: Biology
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Selected Publications

Krzykwa JC and Jeffries MKS. 2020. Comparison of behavioral assays for assessing toxicant-induced alterations in neurological function in larval fathead minnows. Chemosphere. *Accepted, pending revision.*

Krzykwa JC and Jeffries MKS. 2020. Development of a larval fathead minnow optomotor response assay for assessing visual function. MethodsX. *Accepted, pending revision.*

Krzykwa JC, Saeid A, Jeffries MKS. 2019. Identifying sublethal endpoints for evaluating neurotoxic compounds utilizing the fish embryo toxicity test. Ecotoxicol Environ Saf. 170:521–529. doi:10/gfrgd6.

Krzykwa JC, Olivas A, Jeffries MKS. 2018. Development of cardiovascular and neurodevelopmental metrics as sublethal endpoints for the fish embryo toxicity test. Environ Toxicol Chem. 37(10):2530–2541. doi:10/gdqbfn.

Awards

2020 Ida M. Green Fellowship from the Office of Graduate Studies, Texas Christian University
2019 TCU Student Travel Award
2018 TCU Science and Engineering Research Center Grant
2017 The SETAC / Procter & Gamble Fellowship for Doctoral Research in Environmental Science
2016 Third Place Master's Student Platform Presentation, SETAC North America Annual Meeting
2016 Second Place Graduate Student Platform Presentation, Lone Star Society of Toxicology Meeting
2016 Student Travel Award, SETAC
2016 Second Place Poster Presentation, South-Central Regional SETAC Annual Meeting
2016 Adkins Fellowship, Texas Christian University

Teaching Experience

Graduate Instructor, BIOL 10003 Contemporary Issues in Biology (Fall 2019) – Texas Christian University
Guest Lecturer/Facilitator, BIOL 40473 Vertebrate Endocrinology Laboratory (Spring 2019) – Texas Christian University
Guest Lecturer/Facilitator, BIOL 60131 Introduction to Scientific Research and Writing (Fall 2018 & 2019) – Texas Christian University
Guest Lecturer/Facilitator, BIOL 40403 Mammalian Physiology (2018) - Texas Christian University
Teaching Assistant, BIOL 40403 Mammalian Physiology (Fall 2017 & 2018) - Texas Christian University,
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ABSTRACT

DEVELOPMENT OF CARDIOVASCULAR AND NEURODEVELOPMENTAL METRICS AS SUBLETHAL ENDPOINTS FOR THE FISH EMBRYO TOXICITY TEST

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The fish embryo toxicity (FET) test has been proposed as a more humane alternative to other aquatic toxicology test methods. However, its adoption by regulatory agencies has been limited due to its inability to predict sublethal adverse effects and its lack of sensitivity for some test compounds, such as neurotoxicants. A proposed solution to these limitations is the development of alternative endpoints for the FET test. The goal of this dissertation was to investigate the predictive power of three developmental abnormalities that have been proposed as sublethal endpoints for the FET test: snout-vent length, eye size, and pericardial edema, quantified as pericardial area. To evaluate the ability of these metrics to predict adverse effects, methods were developed for assays that assessed the neurological and cardiovascular function of larval fathead minnows (*Pimephales promelas*). The ability of the proposed sublethal endpoints to predict alterations in the developed assays was evaluated using three compounds: cadmium, 3,4-dichloroaniline, and perfluorooctanesulfonic acid. The results of this dissertation indicate that while the proposed endpoints are not predictive of altered neurological or cardiovascular function/development, snout-vent length and pericardial area may have potential to be markers of mortality. The inclusion of both endpoints led to LC50 estimates that were lower than those published in the literature for fish embryo acute toxicity tests for two out of the three compounds evaluated. This project both expanded the utility of the fathead minnow as a model organism by developing assays for the assessment of neurological and cardiovascular function in larval fathead minnows, and revealed that the incorporation of snout-vent length and pericardial edema as markers of mortality has the potential to increase the sensitivity of the FET test.