Video Article

Quantifying Fish Swimming Behavior in Response to Acute Exposure of Aqueous Copper Using Computer Assisted Video and Digital Image Analysis

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Abstract

Behavioral responses of aquatic organisms to environmental contaminants can be precursors of other effects such as survival, growth, or reproduction. However, these responses may be subtle, and measurement can be challenging. Using juvenile white sturgeon (*Acipenser transmontanus*) with copper exposures, this paper illustrates techniques used for quantifying behavioral responses using computer assisted video and digital image analysis. In previous studies severe impairments in swimming behavior were observed among early life stage white sturgeon during acute and chronic exposures to copper. Sturgeon behavior was rapidly impaired and to the extent that survival in the field would be jeopardized, as fish would be swept downstream, or readily captured by predators. The objectives of this investigation were to illustrate protocols to quantify swimming activity during a series of acute copper exposures to determine time to effect during early lifestage development, and to understand the significance of these responses relative to survival of these vulnerable early lifestage fish. With mortality being on a time continuum, determining when copper first affects swimming ability helps us to understand the implications for population level effects. The techniques used are readily adaptable to experimental designs with other organisms and stressors.

Video Link

The video component of this article can be found at http://www.jove.com/video/53477/

Introduction

Understanding how contaminant exposure can affect behavior is at times very challenging and subjective. Behavior is typically defined as a series of overt, observable, whole-body activities that operate through the central nervous system and enable an organism to survive, grow, and reproduce. Changes in behavior due to exposure to a toxicant are among the most sensitive indicators of environmental stress often between 10-100 times more sensitive when compared to survival[1]. The majority of these studies examined swimming activity, ventilation, and foraging behavior of fish[2,3,4]. Swimming activity is the most frequently used sublethal endpoint in determining a behavioral change in response to a contaminant in toxicity tests[5]. Swimming variables include the frequency and duration of movement, speed and distance travelled, the frequency and angle of turns, position in the water column and the pattern of swimming. Swimming activity is an effective measure of swimming behavior when assessing sensitivity to a toxicant based on the criteria proposed in Chapter 9 by Rand[6] in the book Fundamentals of Aquatic Toxicology.

This paper presents a toxicological study as an example of how the toxicity of copper to early life-stage white sturgeon (*Acipenser transmontanus*) at various stages of early development in water-only exposures relative to sturgeon swimming behavior was evaluated and illustrates methods for quantifying swimming behavior.

In previous studies, adverse behavioral responses were evident with early onset during the first few days of acute and chronic exposures to copper and became progressively more severe over exposure duration and concentration[7,8,9]. The magnitude and timing of the onset of these behavioral responses are likely sufficient to limit long-term survival and thus are of concern given the implications for recruitment failure[10]. In order to accurately interpret the significance of this sensitivity to metal exposure methods and procedures were developed for quantifying the time course and extent of behavioral impairments relative to copper concentrations.

Test protocols for behavioral function and development were established by analyzing overhead video samples of sturgeon in exposure chambers. The video samples provided qualitative assessment of development and function among copper treatments throughout the exposure period. Behavior and shelter-seeking activity were evaluated during exposures to aqueous copper concentrations to determine the time to effect and to characterize the temporal sequence of impairment that affects shelter seeking, lethargy, swimming coordination, equilibrium, and respiration. In addition, video recordings were made of sturgeon subsampled from each replicate for the purpose of quantitatively documenting spontaneous swimming activity. These endpoints included measures of duration or the time spent moving, swimming speed and distance travelled[5,11] using a commercially available digital image analysis software package. This software defines the outline of each image within a

field of view and from this, defines the centroid for each image. The software then can track the position of each centroid in a frame by frame succession to determine the paths of movement.

This study complied with all applicable sections of the Final Rules of the Animal Welfare Act regulations (9 CFR) and with all institutional guidelines for the humane treatment of the test organisms during culture and experimentation. Upon termination of the study, all fish were euthanized according to appropriate guidelines as approved by the Institutional Animal Care and Use Committee for the U.S. Geological Survey, Columbia Environmental Research Center.

Protocol

1. Setup Diluter for Larval Sturgeon Exposure to Aqueous Copper Concentrations

1. Set up exposures using a flow-through system such as a modified Mount and Brungs[12] diluter system following guidelines by the ASTM International[13,14,15].
2. Select 25 µg/L as the high copper concentration based on previous test results where effects were observed around 4-6 µg/L. Use reagent grade copper II sulfate pentahydrate (>98% purity) and mix up a test stock solution.
   NOTE: Example concentration series for the exposures are 25, 12.5, 6.25, 3.125, 1.0625, and 0 µg/L. Using 50% serial dilutions starting with 25 µg/L as the high concentration will target a range of concentrations observed to cause behavioral impairment.
3. Prepare the test stock solution in a volumetric flask 48 hours prior to the initiation of the exposure and set to deliver to spike the diluter mixing chamber using an automated syringe dispenser.
4. Use a spreadsheet template to determine the weight of chemical to be added to 1 L of deionized water that will result in 25 µg/L copper concentration, when diluter mixing chamber is spiked with 1 ml of the test stock solution.
   NOTE: **Figure 1** depicts an example of a spreadsheet template for chemical stock preparation for diluter toxicity tests.
5. Weigh out 195 mg of reagent grade copper II sulfate pentahydrate using an analytical balance and pour into a 1 L volumetric flask and mix with 1 L of deionized water for a stock concentration of 48.65 mg/L.
6. Place the intake tube from the automated syringe dispenser into the test stock solution and set the spike volume to 1 ml and turn the diluter on by flipping the power switch and let cycle for 48 hr allow it to equilibrate to the corresponding copper concentration prior to stocking sturgeon.
7. Attach an in-line 4-way flow splitter[16] to each delivery line to partition water flow to each of the four replicate exposure chambers in the diluter water bath. Gravity feed the water. Cycle the water through the diluter when the power to the diluter turns on and a solenoid valve is opened allowing water to flow to the dilution tanks. Set chiller unit to 15 °C and turn on water pump to circulate water in the diluter water bath.
   NOTE: This process is regulated by the use of an automated timer.
8. Set the diluter to cycle every 30 min using the automated timer and to deliver 250 ml of test water with each cycle, resulting in 12 volume additions per day to each replicate test chamber.
9. Select exposure test chambers based on the size of the sturgeon to maintain acceptable loading rate which is <10 g fish wet weight/L in any chamber at any given time. For example, to conduct exposures with 30 day-post-hatch (dph) white sturgeon (average weight in grams 0.17 g) use 12 x 21.5 cm^2 glass jars with a 4 cm hole in the side. Cover this side with a 30 micron mesh stainless steel screen to allow for flow through of the test water. The volume of test water in the exposure jars is 1 L.
10. Using a 50 ml plastic syringe take two replicate 50 ml water samples at each concentration for a total of 12 samples and dispense test water into 100 ml glass beakers and measure dissolved oxygen (DO), temperature, conductivity, pH, alkalinity, hardness, total ammonia, major cations, major anions and dissolved organic carbon using standard commercial equipment and follow the manufacturer's instructions.
    NOTE: samples should be taken at the initiation and end of exposure.
11. To collect sub-samples for chemical analysis, use a 25 ml plastic syringe, draw up approximately 24 ml of test water from the exposure chambers using a sipper straw attached to the syringe instead of a needle.
12. Remove the sipper straw from the syringe and place a polypropylene filter cartridge housing a 0.45 µm pore size, polyethersulfone membrane on the plastic syringe.
13. Push 4 ml of test water through the filter and dispose.
14. Dispense the remaining 20 ml of test water through the filter into an acid-cleaned polyethylene bottle and acidify to 1% volume/volume with high-purity, 16 M nitric acid for storage of up to 3 months.
    NOTE: Samples for chemical analysis should be taken at the initiation, middle and end of exposure to confirm copper concentrations.
15. Perform chemical analysis using inductively coupled plasma mass spectrometry following U.S. Environmental Protection Agency Method 6020a[17]
16. After taking all water samples and the diluter is cycling, stock 10 (random, haphazardly) sturgeon in each replicate test chamber. Collect the sturgeon from the culture tank where they are housed using a small mesh non-abrasive net. Place sturgeon in a small bucket with culture water. A total of 240 fish are needed to start the exposure.
17. Do not feed the fish during the exposure.
    NOTE: Please refer to **Figure 1** for a visual setup of the diluter layout.
18. Read the test every day for the duration of the exposure and record fish mortality and monitor swimming behavior.
    NOTE: Other endpoints to look for include lethargy, loss of equilibrium, changes in respiration, changes in pigmentation, position fish are in the water column, hiding activity and any other abnormalities that can be visually identified.
    NOTE: Read the test at the same time each day for consistency.
19. Measure and quantify swimming activity (time fish spent moving, velocity and the distance moved) using a commercially available digital tracking software program.

2. Observations and Mortality Counts During Exposure

1. Visually inspect each test chamber and note mortality and observations of abnormal behavior using a behavioral checklist (**Table 1**) on the datasheets approximately at the same time daily during the 96 hr exposure, preferably in the morning.

NOTE: Behaviors that are strikingly, uncharacteristically, subjectively, qualitatively different from the controls are considered abnormal. Optimally the observer is unaware of the treatments.
NOTE: The loss of equilibrium is defined as the inability of fish to maintain an upright position within the water column and immobility is defined as the inability of fish to move or swim unless prodded. Other abnormalities such as lethargy, hyperactivity, increases or decreases in respiration, color changes, tremors, spasms, bloated abdomens, position in the water column and any other unusual swimming patterns should also be recorded on the data sheet.
NOTE: Refer to Video 1 for examples of abnormal behavior.

2. Record and remove dead sturgeon daily.
3. Using a hand held Dissolved Oxygen (DO) meter with probe measure the dissolved oxygen *in situ* and record water temperature in two replicates of each exposure concentration and record on the data sheet.

3. Videotaping Swimming Activity

1. Capture video data samples using a hand-held video camera mounted to a tripod positioned directly overhead of the testing chamber to document behavioral abnormalities.
2. To quantify swimming activity, cut a piece of PVC pipe 13 cm in diameter and 13 cm tall to use as the test arena (**Figure 1**). Place the PVC pipe in the diluter within each corresponding copper concentration exposure tanks. Use the area within the PVC pipe as the test arena as this is large enough for sturgeon to swim about freely.
3. At the end of the 96 hr exposure, randomly subsample 5 surviving sturgeon from each copper concentration to measure for swimming activity and place them in to the test arena using a small mesh net.
NOTE: In the higher test concentrations where sturgeon mortality was prevalent, any remaining surviving sturgeon should be used to measure swimming activity and in some cases might be less than 5.
4. After placing the fish in to the test arena, allow the fish to acclimate for a period of 30 min.
NOTE: Successful, error free analysis of the video requires a high contrast image of the fish against a background with a minimum of structure that might obscure or hide the image of the fish. The image of the fish must be in good focus and must be free of surface glare or free of distortions due to moving water so the diluter system must be turned off.
5. After 30 min, turn the video camera ON and set to REC to record the swimming activity for a period of 2 min.
6. Euthanize the fish after taking the video recordings to determine behavior.
7. Place sturgeon in a concentrated solution of tricaine methanesulfonate (MS222) water for at least 10 min to allow for cessation of opercular movement.
NOTE: A concentration of at least 250 mg/L is recommended and might be much higher for certain species.
8. Place the euthanized sturgeon in a plastic zip lock bag and place in the freezer for disposal at a later time. Turn the video camera OFF and transfer all video files to a computer for post processing using digital tracking software.

4. Measures of Swimming Activity from Video Playback

1. Locate the data sheet and video data files for the experiment to be analyzed. Convert video files to a compatible format the digital analysis software can handle.
2. Upload all files to be processed into software. Open tracking software by clicking on the icon. Click on the "New default experiment" under the "Create a new experiment" option on main screen.
3. Enter name for experiment in "New Experiment" dialog box that appears on screen. Choose location experiment file is to be saved. Click "OK". Choose "Experiment Settings" option under "Setup".Choose "From video file" under "Video Source".
4. Select "1" for "Number of arenas". Select "3" for "Number of subjects per arena". Select "Center-point detection" under "Tracked Features". Select desired units.
5. Select "Trial list" option under "Setup". Click "Add Videos" at top of screen. Choose "Alphabetical order" under "Sort order" option on "Add Videos" dialog box that appears on screen. Click "Browse". Navigate to folder where video files are located.
6. Highlight all video files. Click "Open". Click "Add Variable" at top of screen. Enter "Concentration" into "Label" box. Enter "Copper concentration in micrograms/L" into "Description" box.
7. Choose "Numerical" from drop down list for "Type". Click on "Predefined Values" box. Choose "Define Individual values" option in the "Predefine Numerical Values" dialog box that appears.
8. Enter "0", "3", "6", "13", "25", and "50" in "Predefined Value" space. Click "Add >>" between each number addition. Uncheck "Allow other values" option. Click "OK".
9. Choose "Trial" from drop down menu in "Scope" box. Enter appropriate concentration for each trial in boxes provided. Click "Add Variable" at top of screen. Enter "Replicate" into "Label" box. Enter "Replicate number" into "Description" box. Choose "Numerical" from drop down list for "Type".
10. Click on "Predefined Values" box. Choose "Define Individual values" option in the "Predefine Numerical Values" dialog box. Enter "1", "2", "3" and "4" in "Predefined Value" space. Click "Add >>" between each number addition. Uncheck "Allow other values" option.Click "OK".
11. Choose "Trial" from drop down menu in "Scope" box. Enter appropriate replicate number for each trial in boxes provided. Choose "Arena Settings" under "Setup" tab at top of screen. Name first setting "Trial 1". Click "Grab Background Image" option from "Arena Settings (Trial 1)" dialog box.
12. Click "Browse" on the "Grab Background Image" dialog box. Locate video file for Trial 1 and click "Open". Click "Grab" option in the "Grab Background Image" dialog box after video appears. Click the white circle icon near top of screen under "Arena Settings".
13. Manipulate circle that appears so that entire swimming area is enclosed in the circle. Hatch marks appear where the arena area is defined. Click "Calibration Scale" icon near top of screen under "Arena Settings". Left click on one edge of arena. Hold and drag mouse over to opposite end of arena. Release left click.
14. Enter "10.5" in "Real world distance" box in the "Calibration Distance" dialogue box that appears. Click "OK". If necessary, adjust calibration line so that it spans the entire diameter of circular arena.

15. Click "Validate Arena Settings" option on "Arena Settings (Trial 1)" dialogue box. Address any issues if settings are not validated. Right click "Arena Settings" under "Setup" option from the "Experiment Explorer" toolbar on left of screen and choose "New" from menu.

16. Repeat steps 4.11-4.15 until arena settings have been created for each trial. Be sure to choose the appropriate video file for each trial. Choose "Detection Settings" under "Setup" option from the "Experiment Explorer" toolbar on left of screen.

17. Choose "Dynamic Subtraction" from dropdown menu under "Method" in the "Detection Settings: Detection Settings 1" dialogue box that appears.Choose different fill colors for each subject under "Subject Identification" in the "Detection Settings: Detection Settings 1" dialogue box.

18. Choose "Select Video" and locate video for Trial 1.Click "Open".Select "5.9941" from "Sample rate" box under "Video" in the "Detection Settings: Detection Settings 1" dialogue box. Click "Settings" for the "Reference image" option under "Detection" in the "Detection Settings: Detection Settings 1" dialogue box.

19. Click "Start Learning (C)" option in the "Reference Image" dialogue box.Wait for program to learn reference image. Once the image in "Reference Image" dialogue box appears without animals, click "Use dynamic reference image" under "Acquisition settings" within the dialogue box.

20. Click "Close". Choose "Darker" from the drop down menu for "Subject is" under "Detection" in the "Detection Settings: Detection Settings 1" dialogue box. Set smaller number to "33" and larger number to "153" for "Dark contrast" under "Detection" "Detection Settings: Detection Settings 1" dialogue box.

21. Click "Save Changes" at bottom right of "Detection Settings: Detection Settings 1" dialogue box. Click the play button on the "Playback Control" dialogue box and confirm that software is successfully tracking animals as opposed to shadows or debris. Adjust numbers for "Dark contrast" as necessary.

22. Once tracking is appropriate, click "Save Changes" at bottom right of "Detection Settings: Detection Settings 1" dialogue box. Choose "Acquisition" under "Setup" option from the "Experiment Explorer" toolbar on left of screen.

23. Click "Track next planned trial" in the "Acquisition Settings" dialogue box. Confirm the correct trial, video and arena setting is displayed under "Settings" in the "Acquisition Settings" dialogue box.

24. Check the "Detection determines speed" option in the "Acquisition Control" dialogue box. Click the button with the green circle enclosed in a white square to begin the acquisition process. Repeat steps 4.22-4.23 until all trials have been tracked.

25. Click "Data Profiles" under "Analysis" option from the "Experiment Explorer" toolbar on left of screen.Choose "Time" under "Nesting" option in the "Components" toolbar. Adjust "To" to "0:02:00" under the "Select track time interval" heading in the "Time" dialogue box. Click "OK".

26. Drag the "Nest" box between the "Start" box and the "Result 1" box in the "Data Profiles" area on the right side of the screen. Click "Analysis Profile" under the "Analysis" option from the "Experiment Explorer" toolbar on left of screen. Click "Velocity" under the "Distance and Time" heading in the "Dependent Variables" toolbar that appears.

27. Click "Add" on the "Velocity" dialogue box. Click "Distance moved" under the "Distance and Time" heading in the "Dependent Variables" toolbar. Click "Add" on the "Distance moved" dialogue box. Click "Movement" under the "Individual Behavior" heading in the "Dependent Variables" toolbar.

28. Adjust "Averaging interval" to "1" under the "Outlier filter" heading in the "Movement" dialogue box. Adjust "Start velocity" to "2.00" and "Stop velocity" to "1.75" under the "Threshold" heading in the "Movement" dialogue box.

29. Check both boxes for "Moving" and "Not moving" under the "Calculate statistics for" heading in the "Movement" dialogue box. Click "Add" at the bottom of the "Movement" dialogue box. Click "Analysis Output" under "Results" option in the "Experiment Explorer" toolbar on left of screen. Click "Calculate" at top of screen.

30. Once dependent variables are calculated, click "Export" at top of screen. Choose destination folder in "Export Analysis Output" dialogue box. Select "Excel" from "File type" dropdown menu in the "Export Analysis Output" dialogue box. Click "OK".

31. Click "Save Experiment" under the "File" tab at the top of the screen. Close the digital tracking software. Import the data to a spreadsheet file and analyze using a commercial statistical analysis software package.

Representative Results

Manually processing the visual observational data showed percent abnormalities increased with increasing copper concentration after just 72 hr of exposure initiated with 2 day post hatch (dph) sturgeon (**Figure 2**). The video samples documented the extreme impact of copper exposure on sturgeon swimming behavior (Video 2) and aided in defining the behavioral impairment as a result. In another example, sturgeon at 30 dph proved sensitive to copper exposure with a 96 hr median lethal effect concentration (LC50) of 40.3 µg/L based on mortality only. However, when sublethal behavioral endpoints of loss of equilibrium and immobilization are included along with mortality the sensitivity increases with an estimated 96 hr median effect concentration (EC50) ranging from 2.4 to 5.0 µg/L. Video documentation captured these sublethal effects and further validated human observations of behavioral abnormalities recorded during the exposure. The use of digital tracking software reduced the post processing time significantly when analyzing swimming activity. Fish swimming speed, the time spent moving, and the distance travelled all significantly decreased (**Figure 3**) with increasing copper concentration. Swimming paths were also reduced with increasing copper concentration (**Figure 4**).

CHEMICAL STOCK PREPARATION FOR DILUTER TOXICITY TESTS

USGS Columbia Environmental Research Center
Ecology Branch

Enter or check all values inside boxes
Compare highlighted cells to check calculations

Study Code:	10-20-07a,c	
Study Name:	Acute toxicity of copper, zinc, and cadmium to white sturgeon and rainbow trout	
Study Director:	R. Calfee	
Lead Technician:	Erinn Beahan and Holly Puglis	
Approx. Start Date:	1-Nov-10	

Test Description:	Consecutive early life stage acute toxicity tests with white sturgeon and rainbow trout starting with 18 dph fish	(e.g., test species, life stage, test duration)

Chemical Information:

1	Name	Copper (II) sulfate pentahydrate: $CuSO_4.5H_2O$	
2	Chemical Storage ID:	(new)	
3	Chemical Purity (%):	98.0	(from chemical label)
4	Molecular Weight:	249.68	(from chemical label)
5	Molecular or Atomic Weight of Active Ingredient*:	63.54	(e.g. metal atomic wt; may be same as #4)
6	Fraction Active Ingredient:	= 0.2494	** Must be less than or equal to 1.0 **

Diluter Information: Diluter #3 BioEast

7	Target High Concentration (mg/L):	0.025	
8	Dilution volume (mL):	1946	(usually diluter W1 cell volume)
9	Volume delivered per cycle (mL):	1.0	(planned Hamilton delivery volume)

Stock Solution Preparation:

10	Stock volume (L)	1.00	
11	Weight of chemical to add (g):	= 0.195	
12	Stock Concentration (mg/L):	= 48.65	

Add chemical to volumetric.
Add about one-half total volume of DI water (or solvent) and dissolve.
(For metals, add 0.5 mL concentrated ultrapure nitric acid per liter)
Bring to total volume with DI water (or solvent).
Label with name and weight of chemical, stock concentration, and date.

Hamilton Delivery Volumes:

13	Hamilton delivery volume, mL:	1.00	(enter value from #9 to check or enter new
14	Actual high concentration, mg/L (**CHECK vs. above**):	0.025	value to calculate new concentration)

Figure 1: Computer screenshot of a spreadsheet template used to determine test stock solution concentration. Chemical stock preparation for the diluter toxicity tests was determined using a spreadsheet template based on a target concentration. Please click here to view a larger version of this figure.
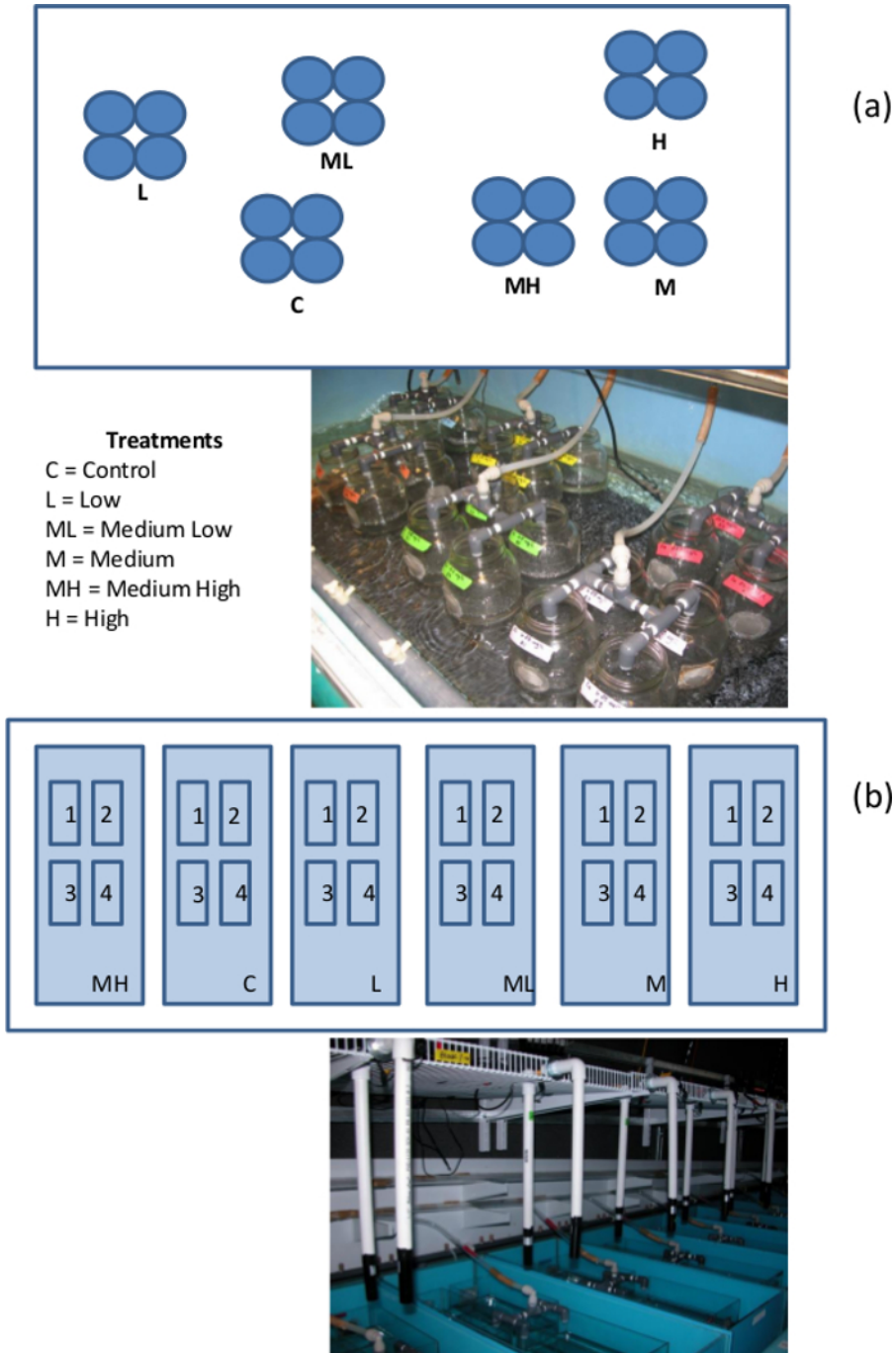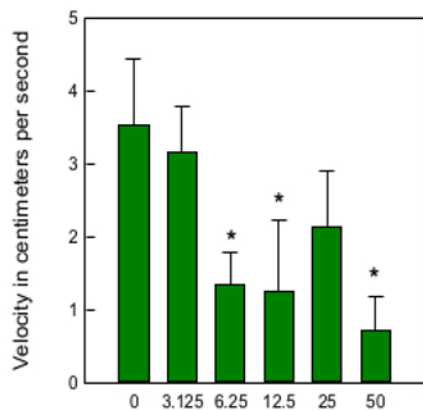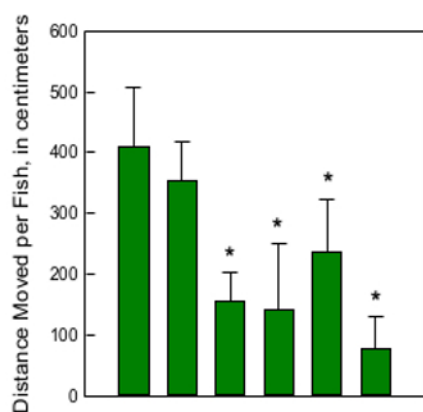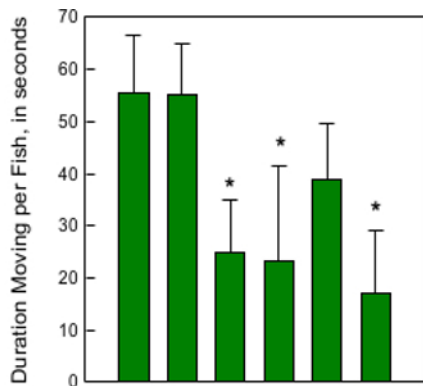
Figure 2: Diluter setup and experimental design. Various life-stages of white sturgeon were exposed to copper. The size of diluter used for the exposures was selected based on the size of the fish. (**a**) Early life-stage sturgeon were exposed using a small diluter setup and (**b**) older life-stage sturgeon were exposed using a large diluter setup.

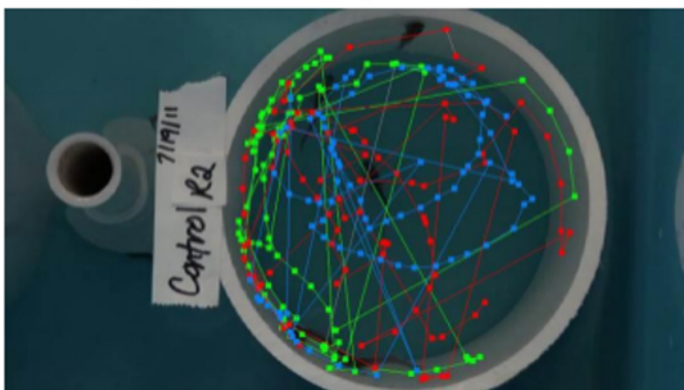Nominal copper concentration, in mmicrograms per liter

Figure 3: Swimming behavior results from an early life-stage white sturgeon (*Acipenser transmontanus*) 72 hr copper exposure (starting with 30 day post hatch [dph] fish). Swimming activity endpoints (**a**) duration of movement among 30 dph white sturgeon; (**b**) speed of swimming; and (**c**) distance moved declined with increasing copper concentration among white sturgeon exposed for 96 hr. Asterisk indicates significant difference from control, error bars represent standard deviation.
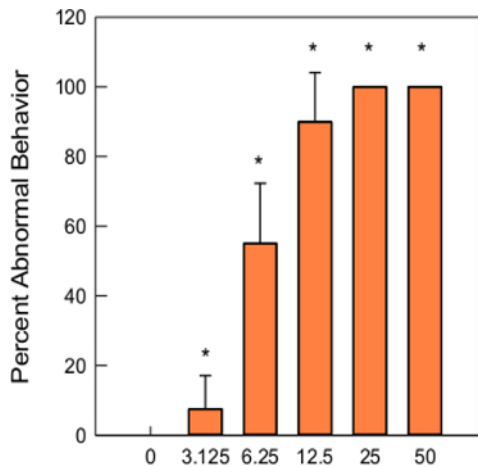
Copper concentration, in micrograms per liter

Figure 4: Results from an early life-stage white sturgeon (Acipenser transmontanus) 96 hr copper exposure (starting with 2 day post hatch [dph] fish). Surviving white sturgeon at 2 dph exhibiting loss of equilibrium and immobilization after 72 hr of a 96 hr exposure with increasing copper concentration. Asterisk indicates significant difference from control, error bars represent standard deviation.

A. Swimming paths of white sturgeon from control treatment

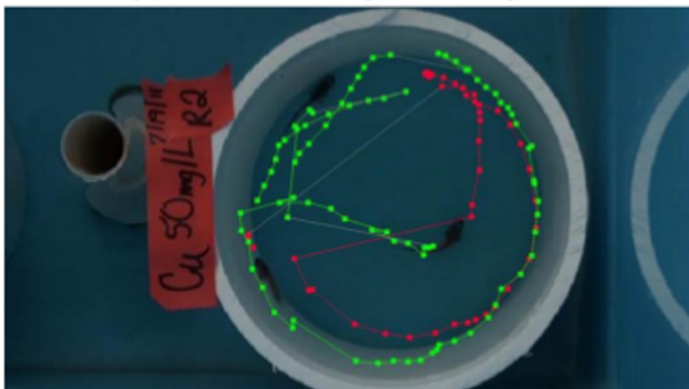B. Swimming paths of white sturgeon from high Cu treatment

Figure 5: Example swimming path results from an early life-stage white sturgeon (Acipenser transmontanus) 96 hr copper exposure (staring with 30 day post hatch [dph] fish). Swimming paths of sturgeon digitized using digital tracking software from (**a**) control replicate (n = 5 fish) and (**b**) from a high treatment (50 µg/L) replicate (n = 3 fish) after a 96 hr exposure. Note the number of swimming paths does not represent the number of fish present in the chamber because of some fish were inactive. Please click here to view a larger version of this figure.

Quantifiable Parameter	Observational Parameters
Velocity	Lethargy/Hyperactivity
Distance Moved	Loss of equilibrium
Duration of time spent in zone	Spasms/tremors/upside down
Zone transition-number of times organism moves between selected zones	Position in the water column
Heading to point-calculates the deviation of the animal's path towards a point of interest	Respiration (fast/slow)
Heading calculates the heading of the selected body point	Coloration
Turn angle-difference in heading between two samples	Hiding
Angular velocity-calculated by dividing the turn angle by the sample interval	
Meander-calculated by dividing the turn angle by the distance moved. Used to compare turning in animals moving at different speeds	
Time spent moving	
Mobility state-calculates the duration for which the complete area detected as animal is changing, even if the center point remains the same	
Rotation-one rotation is completed when the selected body point has a cumulative turn angle of 360°. Turns in the opposite direction of less than the threshold are ignored.	
Mobility continuous-calculates the percentage of mobility for the complete area of the detected animal, even if the center point remains the same.	
Distance between subjects-calculates distance between all actors and the selected receivers	
Proximity-calculates the duration for which the actor is or is not in proximity to the receiver	
Relative Movement	
Net weighted movement-The movement of the actor to (positive) and from (negative) the receiver, weighted by the distance between them	
Weighted movement from-movement of the actor from the receiver, weighted by the distance between them.	
Weighted movement to- movement of the actor to the receiver, weighted by the distance between them	
Trial control state- period between two events of the Trial Control elements, or within one element	
Trial control event-moment in which an event within an element you have defined in Trial Control occurs.	

Table 1: Behavioral endpoints quantified through digital tracking software. These endpoints can be used on individuals or groups and also serve as a list for visual observational checks of behavioral impairment during the exposure.

Video 1: Visual definition of abnormal behavior exhibited by affected white sturgeon. (Right click to download) A daily observational check list was used to document abnormalities. The loss of equilibrium and immobilization were the most prevalent abnormalities observed during the exposures. Modified from Calfee *et al.*[7]

Video 2: Visual documentation highlighting an example of white sturgeon swimming behavior. (Right click to download) White sturgeon swimming activity was greatly reduced with exposure to increasing copper concentration. The sturgeon depicted in this video are from a control, medium low, and a high treatment at the end of a 96 hr aqueous copper exposure. Although the white sturgeon were still alive it is evident the fish were greatly impaired in the treatments compared to the controls. Modified from Calfee *et al.*[7]

Discussion

Changes in behavior due to exposure to a contaminant are often used as an endpoint for sub-lethal toxicity, but can be difficult to measure. Generally, behavior responses are measured by visual observations and manual data analysis which requires a lot of time to process. However with advancing technology, methods for quantifying swimming activity have focused on using videography[18] and motion analysis or digital tracking software which reduces the processing and analysis time. During analysis of the video captured data, quantifying swimming variables manually would have been very time consuming so the use of video data recordings and fish tracking software provided a more effective and efficient way for analyzing sturgeon swimming behavior. Although the procedure highlighted swimming behavior of a fish, customizing for other organisms such as amphibians and aquatic invertebrates would require simple modifications. Depending on what behavioral endpoints are being addressed, experimental design and camera systems can be developed for use with just about any commercially available tracking software package.

The method is demonstrated using dissolved copper, but is applicable to other aqueous contaminants or characteristics such as temperature or oxygen content. The protocols developed and presented in this paper utilized a simple digital video camera as the recording device. The digital files are easily transferred to a computer and uploaded in to the motion analysis software. The methods are constantly being modified and refined to streamline the quantification process. It is imperative that the video quality be in high definition in order for the analysis software to identify each individual fish for tracking. Any background that does not contrast with the fish will cause problems when trying to process the data files. Another common problem with two dimensional video tracking is identifying individuals when swimming paths cross. This can be corrected manually by identifying each fish during the path crossing and linking up the path segments within the software. Alternatively, total activity can be

determined from each replicate chamber as a group average. Several individual chambers with each one containing one fish can be filmed in the same field of view to calculate movements of individual fish.

Currently we have upgraded to using a series of overhead surveillance cameras above the exposure chambers that are linked to a high definition digital video recording device (HD-DVR). However, the use of any camera system that can record high definition MPEG-4 videos will work. The HD-DVR can be set to record at a specified time and programmed for up to 7 days. This hands-off automated approach allows the capture of several videos at the same time to maintain consistency while minimizing external disturbances which could compromise fish behavior. The HD-DVR systems are connected to an internal network so transferring of files is relatively simple. While the automated camera system is a much improved technique for quantifying swimming behavior, it is still beneficial to conduct visual observations to serve as additional supporting information for documenting behavioral impairment during toxicity tests.

There is a long history of literature documenting altered fish behavior resulting from exposure to metals dating back to the early 1960s[19,20,21]. Copper has been shown to cause changes in activity levels such as hypoactivity in bluegill[22] (*Lepomis macrochirus rafinesque*) and changes in locomotor and feeding activity of brook trout[23] (*Salvelinusfontinalis*). At least some juvenile fish rely on their sense of smell to detect and avoid predators, and copper-induced chemosensory deprivation may impact behaviors related to detecting alarm chemicals[24,25,26]. The olfactory epithelium is damaged due to copper exposure thereby affecting sensory mechanisms which could result in disorientation, behavioral avoidance, reduced feeding and any other behaviors that are guided by olfaction[27]. These altered behaviors were consistent with what was observed during the exposures.

The swimming behavior of white sturgeon was greatly impacted during sublethal exposure to aqueous copper concentrations.. These results illustrate how behavior is affected at sublethal concentrations of copper and can be used as an indicator of toxic stress. The video based analysis proved effective in quantifying swimming behavior and also served as qualitative visual documentation of the severe impacts on sturgeon exposed to copper. The analysis software is also capable of quantifying other various behavioral endpoints. Please refer to **Table 1** for a list. The exposure system can be modified to address each endpoint in real-time mode and can be used to quantify differences in behavior associated with exposure to various contaminants of concern.

The use of behavioral endpoints in aquatic toxicological research is increasingly employed and should be considered when evaluating the effects of contaminants because adaptive behavioral function is crucial in the determination of environmental injury[9]. The impacts of environmental contaminants to fish behavior is often subjective and challenging particularly when dealing with sublethal endpoints in the absence of standard methods..

Swimming activity as quantified by using these methods can be rigorously monitored, is non-destructive with minimal stress to the organism and can be repeated. Swimming behavior is a valid and consistent index of sublethal toxicity that should be incorporated in test protocols to expand the sensitivity of standard toxicity tests[5].

Disclosures

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