A video-based movement analysis system to quantify behavioral stress responses of fish

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Abstract

Behavioral alterations can be measured as endpoints for sublethal toxicity, and serve as a tool for environmental risk assessment and analysis of toxicological impact. Numerous technical and biological factors have made sublethal effects on fish behavior difficult to quantify. In order to investigate stress- and contaminant-induced behavioral alterations, a video analysis system was designed by our laboratory. With this system up to 12 fish may be individually housed in 20 L exposure arenas and automatically videotaped at multiple and discrete intervals during an experimental period. Analog video data can then digitized, converted into x,y coordinates, and finally transformed into relevant behavioral endpoints using software designed for tracking fish movement combined with specific algorithms. These endpoints include velocity, total distance traveled, angular change, percent movement, space utilization, and fractal dimension (path complexity). Data from fish exposed to a reference toxicant, MS222, and simulation experiments, are presented to exemplify alterations in fish behavior associated with exposure, and accuracy and precision, respectively. The system provides flexibility to analyze any observed movement behavior, is remotely controlled, and can be transportable. These movement analyses can be used to identify characteristic behavioral responses to a variety of environmentally-relevant stressors, and assist in risk assessment and the development of more sensitive lowest observable effect level and no observable effect level for sentinel species.
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1. Introduction

Fish are ideal sentinels and test organisms for behavioral assays of various stress factors and toxic chemical exposure due to their ecological relevance in many natural systems (Little et al., 1990). In addition, fish may bioaccumulate various contaminants and/or play a role in food web biomagnification. Behavioral endpoints in fish, as well as other organisms, are valuable tools to discern and evaluate effects of environmental stress. These endpoints of exposure are important because they integrate endogenous and exogenous factors that can link biochemical and physiological processes, and can provide insights into individual- and community-level effects of environmental contamination

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(Brewer et al., 2001; Vogl et al., 1999). Quantifiable behavioral changes in organisms associated with stress and toxicant exposure provides novel information that cannot be gained from traditional toxicological methods, including short-term and sublethal exposure effects, and the potential for mortality (Henry and Atchinson, 1986; Bridges, 1997; Saglio and Trajasse, 1998; Little and Finger, 1990). Ecological relevance of such exposures may stem from altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Little and Finger, 1990; Zhou and Weiss, 1998). Changes in behavior may also alter juvenile recruitment, thereby disrupting population, community, and demographic dynamics over time (Bridges, 1997).

Basic knowledge of exposure related behavioral alterations relevant for ecotoxicological assays remain scarce, and systems that have the ability to link toxicology data with swimming and avoidance behaviors are needed (Vogl et al., 1999). In the past, conventional procedures for assessing behavioral swimming performance endpoints have been found to be insensitive. Lack of test standards, homogeneity of samples, variation in measured endpoints, and small sample sizes have hindered the progress of behavioral toxicology as a consensus-based discipline. Sound experimental design combined with proper statistical analyses are essential for developing approaches using fish movement for the bioindication of stressors (Vogl et al., 1999).

Despite historical shortcomings movement analysis and quantification of locomotion has progressed over the past 20 years mainly due to the continued improvement of computer processors and digital imaging methods. As a result, acceptability of behavioral tests for hazard evaluation has increased as technology has improved the quantification of behavioral observations (Kondaiah and Murty, 1994). A variety of methods have been used in order to quantify movement data, including hand scoring through direct observation (Hansen et al., 1999; Bjerselius et al., 2001; Teather et al., 2001; Wibe et al., 2001), telemetry (Wescott and Graham, 2000), and computer digitization into x,y coordinate data (Vogl et al., 1999; Beauvais et al., 2000; Suzuki et al., 2003). Several computer tracking hardware and software systems have been developed for the purpose of behavioral analysis (Miller et al., 1982; Godden and Graham 1983; Hoy et al., 1996; Dusenbery, 1985; Ye and Bell, 1991; Kato et al., 1996). More recently, the Motion Analysis NP110 system with expert vision software (Dodson et al., 1995; Brewer et al., 2001) and the BehavioQuant object tracing system (Vogl et al., 1999; Baganz et al., 1998; Steinberg et al., 1995), have been used for digitization of behavior into x,y coordinate data for subsequent analysis. In addition, commercial packages by Colombus, Leica, and Ethovision are

also available, but are expensive and may be cost prohibitive to research groups, particularly if custom endpoints need to be developed.

Once behavioral responses have been collected and converted into x,y coordinate data, they can be transformed by computer software into relevant endpoints. In addition to measurement sensitivity, a variety of factors are important in the choice of endpoints; they should be intuitive, reproducible, quantifiable and environmentally relevant (de Peyster and Long, 1993). Such endpoints may include: swimming activity and performance, feeding capacity, vulnerability as prey, rheotaxis, velocity, angular change, water column position, total distance traveled, acceleration, and surfacing (Little and Finger, 1990; Kato et al., 1996; Saglio and Trijasse, 1998; Weis and Weis, 1998; Zhou and Weiss, 1998; Beauvais et al., 2000; Brewer et al., 2001; Gipson, 2001). Other, more complex behaviors such as path tortuosity (fractal dimension), the ratio of net to gross movement, chemoreception, optomotor responses, respiration, aggression, and social interactions have also been investigated (Dicke and Burrough, 1988; Henry and Atchinson, 1986; de Peyster and Long, 1993; Wescott and Graham, 2000; Brewer et al., 2001; Gipson, 2001). Although an extensive amount of research has taken place and a variety of endpoints have been investigated, areas of uncertainty still exist in the analysis of behavioral modifications associated with toxin exposure.

The system described in this paper provides a mechanism to fill these gaps through the use of flow-through hardware and novel software. The analysis software was designed specifically to provide widely applicable endpoints, and adequate flexibility to compensate for variation in individual study aims and protocols. Our goal was to provide reproducible, reliable, relevant endpoints based on consistent behavioral changes that can link sublethal exposure of environmental contaminants (and other stressors) to behavioral modifications in fish.

Data from random walk and correlated random walk simulation experiments are presented to demonstrate the accuracy and precision of the tracking and analysis portions of the system. Data from fish exposed to a reference toxicant, tricane methanesulfonae (*m*-amino-benzoic acid ethyl ester methansulfonate, MS222), are presented to exemplify alterations in fish behavior associated with exposure. This compound is a licensed fish anaesthetic that is widely used in research and field settings. It is an isomer of benzocaine with the amine group being in the *meta* position on the benzene ring rather than the *para* position. The presence of the methane sulphonate grouping allows MS222 to be directly dissolved in water. MS222 is rapidly absorbed through the gills. Its mode of action is by preventing the generation and conduction of nerve impulses with direct

action on the central nervous system, cardiovascular system, neuromuscular junctions and ganglion synapses (Alpharma 2001; Brown 1993; Ross and Ross 1999). MS222 is a good model behavioral toxicant in our laboratory because of its effect on the CNS, ease of use, reproducibility of results, and the consistent ability to achieve a reliable dose response.

2. Methods

2.1. System design

Twelve 20-L exposure arenas were constructed from 14-in (35.6 cm) diameter polyvinyl chloride (PVC) pipes and end caps. Each arena had two 0.25-in (0.64 cm) threaded nipples that served as an input and drain. The drain line lead to an adjustable height standpipe, which was set to achieve a 5 L exposure volume. The input was bifurcated to accept both toxicant and dilution water flow lines. Toxicant and dilution water flow were electronically controlled with digital, multi-channel peristaltic pumps (Masterflex L/S, Cole-Parmer, Vernon Hills, IL) that were supplied by multiple, aerated 600-L carboys. The exposure arenas were illuminated with shadowless fluorescent lighting. Twelve color CCD cameras with manual iris and focus control were mounted above the respective arenas and were

connected to dedicated VCR decks with time-lapse and dry contact closure capability for recording. All 12 VCRs were connected to a multiplexer that supported real-time observation (Fig. 1). VCR recording and stop functions were synchronously activated remotely using X-10 technology.

Analog video data were then digitized in real time at 3 frames per second (Fig. 2), on a Macintosh platform (G4, 900 MHz, 850 Mb SDRAM). Digitized video data was analyzed using the VideoScript™ video and image processing system. The system analyzed each frame using a correlation-based search with sub pixel resolution (see for example, Maragos et al., 2000) for a
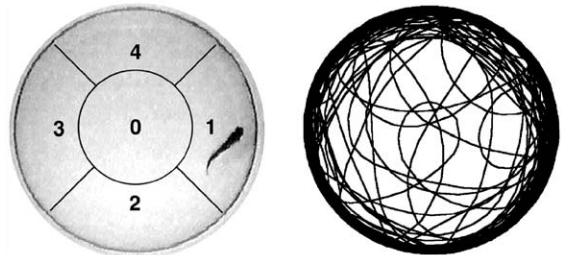

Fig. 2. Digitized image of a mummichog in an experimental arena overlaid with sector grid as used by tracking software to analyze space utilization (left), and a resultant 30-min path of an acclimated fish as generated by our tracking software (right).
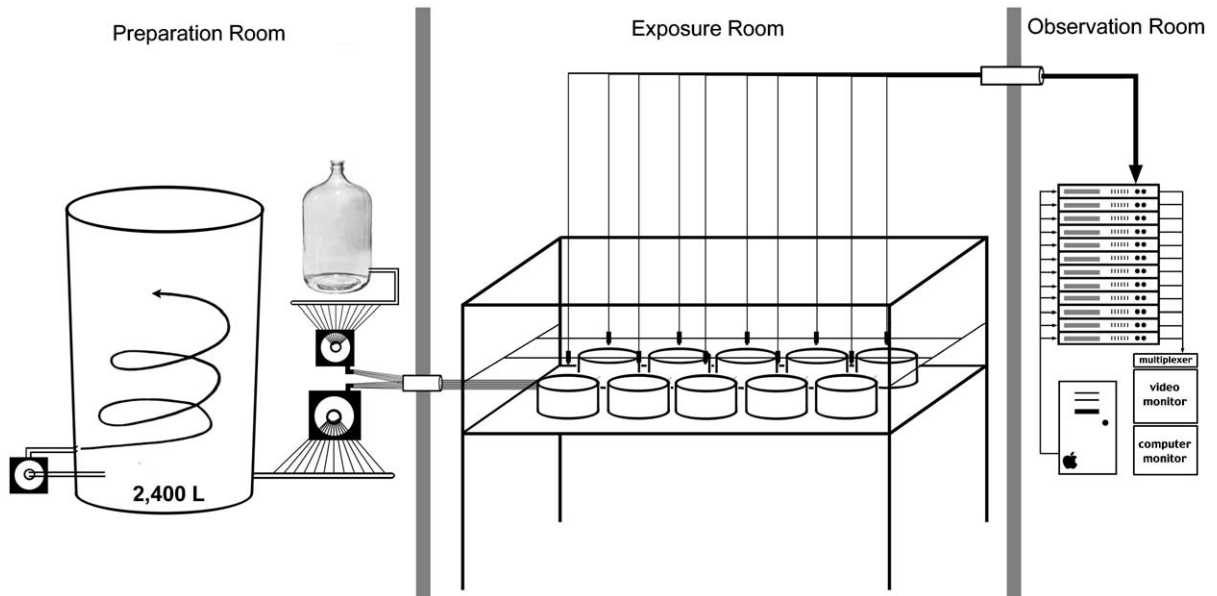

Fig. 1. Schematic diagram showing water preparation room, exposure room and remote computer/video control systems. Analog CCD cameras are mounted above respective exposure vessels. Fluorescent tubes (not shown) create shadowless illumination. Video signals are recorded on VCR decks that are controlled by X-10 computer software and hardware. A video multiplexer and display monitor permit simultaneous viewing of animals in real time during an experiment.

Table 1
Behavioral endpoints measured through time

Behavioral endpoint	Definition
Movement	Displacement ≥ 20 mm per second, or if the fish was classified as moving in the previous frame and the summed distance for both frames ≥ 40 mm (approximately $1/2$ body length)
Percent movement	The # of seconds the fish satisfies movement criteria divided by the total # seconds, multiplied by 100
Velocity	Average speed (cm/sec) while the fish is defined as having movement
Angular change	The difference ($0°$–$180°$) between the angular components of two consecutive 1 s movement vectors (degrees/sec) divided by the total # of consecutive 1 s movement events. Angular change was only calculated when two consecutive movement vectors met the movement criteria
Path tortuosity (fractal dimension)	Fractal dimension (D) is calculated using the hybrid divider method (Hayward et al., 1989) and is an indicator of path complexity. A series of path generalizations are created at various step sizes and the data are mathematically drawn on a Richardson plot (Log path length vs. Log step length). D is then calculated as 1 minus the slope of the Richardson plot. $D = 1$ if the path is a straight line and 2 if it completely fills the 2 dimensional plane
Space utilization	The # of frames fish spend in predefined regions (shown in Fig. 2, left image) of the exposure arena divided by the total # frames
Distance from center	Average distance from the center of the exposure vessel (cm) divided by the total # of frames satisfying the movement criteria. This is a measure of how close the fish swims to the wall of the arena
Relative burst frequency	The # of frames that velocity is >3 SDs above mean velocity

template fish head. The outputs were the x and y co-ordinates for the best-fit match to the fish head. Errors were reported if the best-fit correlation between the template and the video data fell below a threshold of 0.5. The x,y coordinate data were then analyzed using software designed at the Aquatic Pathobiology Laboratory to obtain the desired behavioral endpoints (Table 1).

Preliminary experiments were designed and conducted to determine appropriate acclimation time and video clip segment length (Kane et al., 2004). Based on these experiments, we determined that 24-h vessel acclimation time was sufficient to remove significant alterations in movement patterns over time for any of the endpoints measured ($p < 0.05$). We examined various video clip lengths required to capture sufficient fish motion for reproducible analysis. From these data, we determined that clip lengths of 30-min were appropriate.

2.2. Test species

The model species used for the sensitivity validation of our system was the mummichog, *Fundulus hetero-clitus*. This species of killifish is non-migratory (Fritz et al., 1975; Lotrich, 1975; Smith and Abel, 1994) and is commonly found in estuarine and brackish water along the Atlantic coast of the United States. Mummichog are euryhaline, eurythermal, relatively tolerant to environ-

mental stressors, easy to collect and maintain in the laboratory, and are an important prey species for many higher trophic level organisms. Due to their geographic range, tolerances, and ecological significance, they serve as a good laboratory model for ecotoxicology studies. Mummichog (70–90 mm total length) were collected from a reference site in Solomons, MD, treated for ectoparasites, and laboratory-acclimated for 4 weeks prior to experimentation. Fish were gradually acclimated to a 14:10 h (light:dark) photoperiod. Laboratory lighting to simulate dawn and dusk effects was controlled using X-10 computer hardware with an X-Tension (Sand Hill Engineering, Geneva, FL) software interface. Fish were fed three times per week (Trout Grower Standard $\frac{3}{32}$-in pellets, 38% protein, Ziegler Bros., Gardner, PA), and were fasted for 24 hours prior to vessel acclimation. Temperature, pH, and salinity of the experimental arenas were recorded and maintained at the same values as the holding tanks. Water quality (pH, temp, salinity, ammonia) was measured before and after exposures from multiple surrogate (not used for videography) exposure arenas.

2.3. Random walk and correlated random walk analyses

The accuracy and precision of our software to quantify movement of targets (i.e., fish) as coordinate data were evaluated using simulated random walk (RW)

and correlated random walk (CRW) datasets. Ten individual data sets were generated consisting of a series of 5400 x,y coordinate pairs for each analysis. At each time step (333 ms), both the RW and CRW targets had a 90% chance of moving between 1.35 and 1.50 cm from the previous position, and a 10% chance of remaining motionless. The actual distance a target traveled in the x and y directions at any given time step was randomly attributed from a uniform distribution.

Artificially generated RW and CRW data sets differed only in the maximum allowable angular change in either direction from one step to the next. No constraints were imposed on the RW data set, however the CRW data set had a maximum angular change of 45°. The actual angular change at each time step was based on a uniform distribution ranging from the maximum angular change in the clockwise direction (assigned a positive value) to the maximum angular change in the counter-clockwise direction (assigned a negative value) (e.g., CRW angular change per step = $U[-45°, 45°]$). The motion analysis software calculated the mean angular difference between consecutive 1-s displacement vectors. For this reason, the expected angular change values returned from the software for the RW and CRW were expected to be 90° and 30°, respectively, using the following equations:

$$\mu_{RWangch} = abs[U(-180°, 180°) - U(-180°, 180°)] = 90°,$$
(1)

$$\mu_{CRWangch} = abs[U(-45°, 45°) - U(-45°, 45°)] = 30°.$$
(2)

2.4. MS222 exposure

Twelve laboratory-acclimated fish were randomly selected and placed individually in respective exposure arenas 24 hours prior to recording observations. The arenas were maintained at a flow rate of 7 mL/min, i.e., approximately 2 exchanges/day, in order to assure adequate water quality over the 48-hour observation period. The cameras and automated VCRs recorded a 30-min baseline clip after the initial 24-hour acclimation period. Baseline data collection was initiated with a 5 mL bolus of dilution water pumped into the vessels to simulate the toxicant flow effect. After the 30-min baseline recording period, a 5 mL bolus of 60 g/L MS222 solution was pumped into the vessels to achieve a nominal exposure concentration of 60 mg/L. This exposure concentration was based on preliminary dose–response experiments in which empirical observations of altered behavior were collected; this exposure concentration was the highest concentration that failed to produce ataxia under static conditions. A flow rate of 10 mL/min for the first 5 min facilitated mixing of the

(dilution water and the) MS222 in the experimental vessels and was then turned off. This flow rate and time interval was determined from preliminary dye experiments (Kane et al., 2004). The first 30-min exposure video clip was recorded at the start of the injection of the MS222 bolus, followed by two consecutive 30-min observations. The second and third 30-min exposure periods were initiated with injection of dilution water (i.e., there was no maintenance dosing). The four 30-min video clips from each arena (1 baseline clip and 3 exposure clips), were subsequently digitized at 3 fps (this frame rate was determined appropriate based on preliminary experiments), 5400 frames/clip were tracked, and the coordinate data entered into the software program for analysis.

2.5. Statistical analyses

A completely randomized statistical design was used with behavior as the response and time as the categorical variable. Non-normal data were transformed to meet the assumptions of the ANOVA procedure. Data that could not be transformed to meet the assumptions were ranked prior to analysis. Given the nature of our data, i.e., non-independent time periods, repeated measures analysis provided the most power for the detection of differences. Repeated measures 1-way ANOVA (PROC MIXED, repeated, SAS, vs. 8.1, Cary, N.C.) was used to compare behavioral endpoints over time for the MS222 exposure. This analysis allowed for each animal to serve as its own baseline control. In addition, several covariance structures were investigated in an effort to discern a best fit structure for both sets of data. As a result of the fit and spacing between time periods, the Autoregressive (1) covariance structure (AR-1) was selected. A one-way ANOVA (PROC MIXED, SAS, vs. 8.1, Cary, N.C.) was used to compare the RW and CRW simulated datasets. A Tukey–Kramer post hoc mean comparison test was used to evaluate differences ($\alpha = 0.05$) between time periods in the event of a significant F statistic.

3. Results

3.1. System performance and random walk analyses

The system captured, digitized and tracked the movement patterns of mummichog over time with a high degree of consistency and accuracy based on empirical observations of the analog video data by 3 independent observers, and the ability to repeat tracking of multiple fish targets. Comparison of the resultant paths with visual observations from the videography data indicated that the analysis software correctly described the various aspects of fish motion, and that

graphical presentation of motion-related endpoints closely followed observed trends.

Random walk (RW) and correlated random walk (CRW) simulation models were tested using our motion analysis software to evaluate the accuracy and precision of the software to quantify movement. The software successfully described a more linear path for the CRW compared to the RW model, and the CRW model had significantly increased velocity ($p<0.001$) with corresponding significant decreases in angular change and fractal dimension compared to the RW ($p<0.001$). These results are intuitive since the CRW model had directional constraints on the angular deviation between each time step. The percent movement values calculated by our software for the ten RW and CRW simulated trials was 89.9% (expected value = 90.0%). The calculated RW and CRW angular change was 91.1° and 30.5°, respectively (expected values = 90° and 30°, respectively). Further, analyses of fractal dimension and velocity parameters from these model data sets reflected biologically relevant responses (i.e., CRW has lower fractal dimension and higher velocity, consistent

with less tortuous paths). These data support the ability of our analytical software to accurately depict and quantify movement patterns and describe biologically relevant, behavioral endpoints.

3.2. MS222 experiment

MS222 exposure resulted in several significant, quantifiable, behavioral alterations in mummichog. Significant increases in percent movement, velocity, distance from center, and relative burst frequency occurred in exposed fish between baseline and all subsequent time periods ($p<0.05$, Fig. 3). In addition, fractal dimension significantly decreased between baseline and subsequent time periods ($p<0.05$, Fig. 3c). Angular change was not significantly different between any of the time periods ($p>0.05$, Fig. 3f). Space utilization was also not significantly different between baseline and exposure timepoints (data not shown). Fish exposed to MS222 displayed an overall increase in movement endpoint behaviors combined with a less complex swimming path over the 1.5-hour exposure
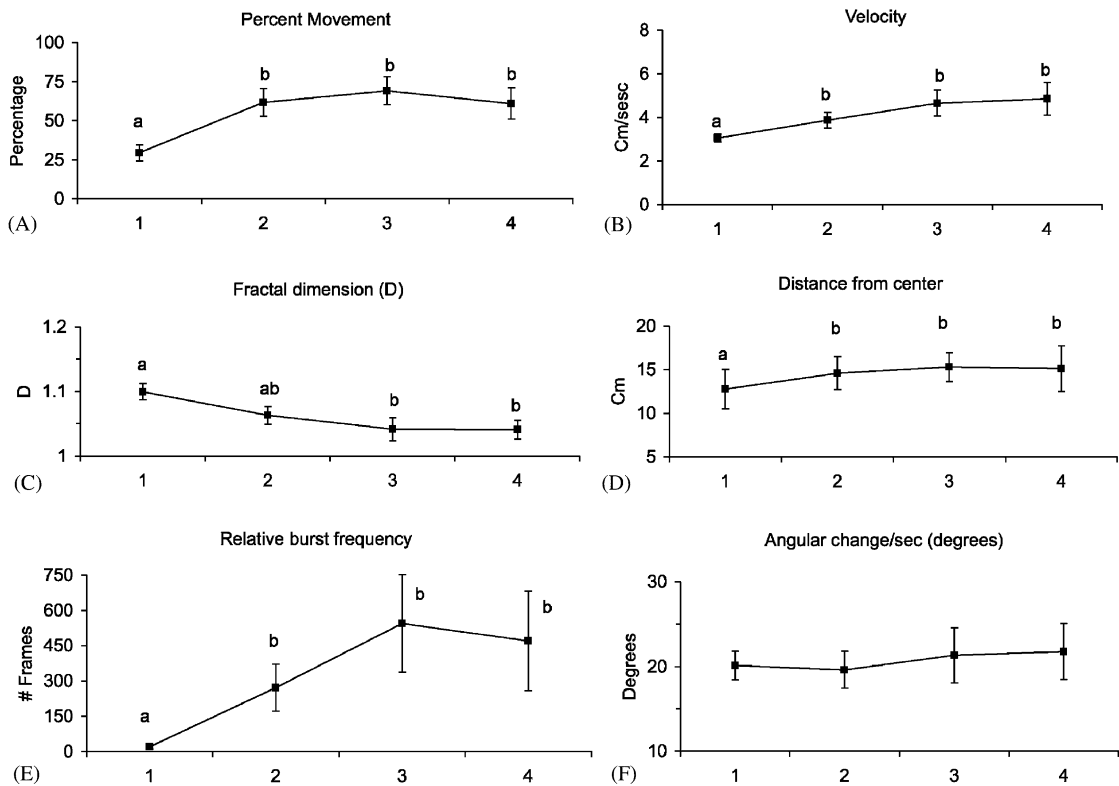

Fig. 3. Changes in (A) percent movement; (B) velocity; (C) fractal dimension; (D) distance from center; (E) relative burst frequency; and (F) angular change over time for the 60 mg/L MS222 exposure. The x-axis shows the four observation periods. The 1st observation in the 30-min baseline. The next three observations are the 1st, 2nd and 3rd 30-min exposure intervals. Groups with different letters are significantly different from each other at $p = 0.5$, Data are means $\pm$ SE.

period. Based on stable water quality parameters before and after the MS222 exposure, time dependent degradation of water quality can be eliminated as an elicitor of behavioral change.

4. Discussion

The objectives of this study were to develop a novel, video-based fish movement tracking and analysis system and to quantify changes in behavior resulting from sublethal exposure, using MS222 as a model stress agent. Results indicate that the described system has the capability and sensitivity to detect alterations in behaviors over time and is suitable to test the effects of contaminant and sublethal stress exposures on fish behavior. With additional modification, the system can be used to obtain quantifiable and automated recordings, in real time, of behavioral alterations resulting from environmental fluctuation. It should be noted, however, that real time data analysis in field situations may require longer observation times due to the "noise" of chronobiological effects.

Exposure to a sublethal concentration of MS222 resulted in significant differences in multiple locomotory variables between the 30-min baseline period compared with the three subsequent 30-min exposure periods. MS222 exposure was associated with significant increases in velocity, percent movement, relative burst frequency, and distance from center, with a significant decrease in fractal dimension. Empirical observations suggested that exposed fish were in a state of mild or partial equilibrium loss, which corresponds with stage 3 anesthesia on a scale of 0–6 as described by Bowser (2001) and a state of light narcosis as described by Brown (1993). Erratic swimming and an initial excitatory period have been associated with this state of anesthesia (Brown, 1993; Bowser, 2001). Increased activity, longer swimming paths, and navigation closer to the vessel wall all likely help fish to maintain equilibrium and their position in the water column under mild anesthesia. These results are consistent with the existing behavioral information on the effects of anesthesia in fish and support the use of quantitative behavioral changes to evaluate the efficacy of anesthetics and other drugs that can affect movement. At a sublethal, light anesthetic dose (60 mg/L) for mummichog, MS222 appears to have a stimulatory rather than sedative effect.

Since the analysis software was developed in-house, and can be altered or recoded to discern differences in various movement patterns, our system has improved flexibility in behavioral measurements over commercial behavioral quantification systems (e.g., Ethovision, Behaviouquant, and Expert Vision). Current system capabilities and development areas include quantifica-

tion of a wide range of behaviors, including daily swimming patterns, startle responses, avoidance behaviors and social interactions. In addition, the system can remotely dose and record up to 12 individual fish or 12 groups of at least 5 individuals simultaneously, for up to 1 hour, without the need to mark or tag the animal, thus reducing variance in behavior due to observer or handling disturbances. Finally, the system can be adapted for static and flow through exposures of environmentally relevant contaminants, and has the ability to be mobile for real-time field assessment.

This behavioral exposure, tracking, and analysis system can be applied to quantify differences in behavior associated with exposure to metals, organics, pesticides, harmful algal bloom toxins and agricultural waste. Behavioral alterations, as identified by this movement tracking hardware and software system, also provides a potential tool for the quantification of differences in reproductive behaviors, predator-prey interactions, behavioral changes across environmental gradients, and differences in swimming behavior between species. Analysis of behavioral responses to a variety of stimuli can provide quantitative measures of neural and mechanical disruption, reflecting biochemical and physiological alterations (Brewer et al., 2001). These measures ultimately provide sensitive, non-invasive, and broadly applicable endpoints for the description of behavioral changes associated with exposure to a wide variety of stressors.

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