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Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables

S. Padilla [a,*], D.L. Hunter [a], B. Padnos [a], S. Frady [b], R.C. MacPhail [c]

[a] Integrated Systems Toxicology Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U. S. Environmental Protection Agency, Research Triangle Park, NC, 27711, United States
[b] National Center for Computational Toxicology, Office of Research and Development, U. S. Environmental Protection Agency, Research Triangle Park, NC, 27711, United States
[c] Toxicity Assessment Division, National Health and Environmental Effects Research Laboratory, Office of Research and Development, U. S. Environmental Protection Agency, Research Triangle Park, NC, 27711, United States

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ABSTRACT

The U.S. Environmental Protection Agency is evaluating methods to screen and prioritize large numbers of chemicals for developmental toxicity. We are exploring methods to detect developmentally neurotoxic chemicals using zebrafish behavior at 6 days of age. The behavioral paradigm simultaneously tests individual larval zebrafish under both light and dark conditions in a 96-well plate using a video tracking system. We have found that many variables affect the level or pattern of locomotor activity, including age of the larvae, size of the well, and the presence of malformations. Some other variables, however, do not appear to affect larval behavior including type of rearing solution (10% Hank's vs. 1:3 Danieau vs 60 mg/kg Instant Ocean vs 1× and 1:10× EPA Moderately Hard Water). Zebrafish larval behavior using a microtiter plate format may be an ideal endpoint for screening developmentally neurotoxic chemicals, but it is imperative that many test variables be carefully specified and controlled.

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1. Introduction

Zebrafish is a popular and versatile experimental animal used for various types of laboratory investigations. In particular, zebrafish larvae less than two weeks of age are increasingly being employed as a model for investigating the effects of genetic manipulation or chemical exposure on physiology and behavior (Levin et al., 2004; Li et al., 2010; Neuhauss et al., 1999; Richards et al., 2008; Best and Alderton, 2008; Dowling, 2002; Fetcho et al., 2008; Fetcho and Liu, 1998; Guo, 2004; Guo, 2009; Rubinstein, 2006; Tropepe and Sive, 2003; Wolman and Granato, 2011). In fact, because the larval zebrafish nervous system exhibits developmental, structural and pharmacological conservation with the mammalian nervous system (Anderson and Ingham, 2003; Panula et al., 2006; Xi et al., 2010), zebrafish larval behavior is also being used as a rapid screen for neuroactive and neurotoxic drugs (Kokel and Peterson, 2008; Peitsaro et al., 2007; Rihel et al., 2010; Selderslaghs et al., 2010; Winter et al., 2008).

To date, zebrafish larval behavioral assays appear to be a powerful paradigm, but it is imperative that we understand the variables that affect behavior and whether these alter the effects of a treatment (e.g. a drug, genetic manipulation or environmental pollutant). At the very least one must be able to identify, specify, and control the variables that affect behavior or the measurement of larval behavior in order to insure consistency and reproducibility of results. In many cases, however, it is remarkably difficult to tell from the literature what were the exact conditions of a given assay. Often, few specifics are given regarding the testing conditions or the condition of the larvae, making it very difficult to compare the results with other reports or to reproduce the testing conditions in one's own laboratory. As our laboratory is involved in zebrafish larval behavioral testing, we endeavored to explore a number of variables, and to determine which did and did not affect the behavioral profile.

We chose five variables to assess: (a) two variables that are intrinsic to the larvae (age of the larvae, and presence or absence of malformations) and (b) three variables that are extrinsic (light intensity, size of rearing environment, type of rearing solution). Many of these variables are neither mentioned nor detailed in publications, and yet, as we found, they can have significant effects on zebrafish behavior.

2. Materials and methods

2.1. Experimental animals

Wild type adult zebrafish (*Danio rerio*; undefined, outbred stock obtained from Aquatic Research Organisms, Hampton, NH, 03842 or

* Corresponding author at: ISTD (B105-03), U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, United States. Tel.: +1 919 541 3956; fax: +1 919 541 0717.
E-mail address: Padilla.Stephanie@epa.gov (S. Padilla).

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EkkWill Waterlife Resources Ruskin, FL 33575) were housed in an AAALAC-approved animal facility with a 14:10 h light:dark cycle (lights on at 08:30 h). Adult fish (2–3 females per male; 15–20 fish per tank) were kept in one of several 9-liter (L) flow-through colony tanks (Aquaneering Inc., San Diego, CA) with a water temperature of 28 °C. For egg collection, all adults in a colony tank were placed in a 2-L (static) breeding tank (Aquatic Habitats, Apopka, FL) 1 h prior to light onset. Typically, adults from four colony tanks were mated on the same day. One hour after light onset the adults were returned to the colony tank. All embryos were gathered from the breeder tank and placed in a 26 °C water bath for 2 h, followed by 2 washes (Westerfield, 2000) with 0.06% bleach (v/v) in 10% Hanks' Balanced Salt Solution (13.7 mM NaCl, 0.54 mM KCl, 25 μM Na_2HPO_4, 130 μM $CaCl_2$, 100 μm $MgSO_4$ and 420 μm $NaHCO_3$, (hereafter referred to as 10% Hanks') for 5 min.

All studies were carried out in accordance with the guidelines of, and approved by, the Institutional Animal Care and Use Committee at the U.S. EPA National Health and Environmental Effects Research Laboratory.

2.2. General embryo rearing

After bleaching, fertilized eggs were individually placed into 10% Hanks' solution (except where noted below) in 96-well mesh microtiter plates (Multiscreen™ catalog #MANMN4050, Millipore Corp., Bedford, MA), and reared for 6 days. Each plate was sealed with a non-adhesive material (Type A, BioRad, Hercules, CA), covered with a lid, and wrapped in Parafilm™ to minimize evaporation. Each day, 250 μl of the aerated 10% Hanks' solution (except where noted below) in each well was completely renewed. Use of the mesh microtiter plates minimized disturbance of the embryos/larvae during the daily changes of Hanks' solution. The embryos/larvae (hatch on or before 4 dpf (days post fertilization)) were then returned to an incubator, where they were maintained on a 14:10 h light:dark cycle (lights on at 08:30 h) at 26 ± 0.1 °C.

2.3. Behavioral testing

All testing was performed on 6 dpf larvae (except where noted below) in the same 96-well plate (except where noted below). Recording fish behavior was essentially as described previously (MacPhail et al., 2009). Luminance measures were taken at the level of the recording platform using a photometer (model Dr-2250-1, 2B silicon detector, TC 284 photometric filter, Gamma Scientific, San Diego, CA). The luminance level for each study is detailed in each figure legend.

On the morning of testing, after the rearing solution was changed, plates were moved to a light-tight drawer in the behavioral testing room. Temperature in the testing room was kept at 26 °C. For all experiments, testing occurred between 12:30 and 16:30 h. For testing, the plate was transferred to a light box, and the movement of each fish was monitored using a behavior-recording system (Noldus Information Technology, Leesburg, VA [www.noldus.com]). The light box provided both infrared and visible light. After transfer to the light box, all larvae were allowed 20 min in the dark to acclimate before actual testing began.

2.4. Analysis of fish movement

Fish movement (locomotion) was tracked from videos using Ethovision (Noldus Information Technology) software Version 3.1. Tracking rate was 5 samples/s (i.e., an image was captured every 200 ms). A subtraction method was used to detect objects that were darker than the background, with a minimum object size of 10 pixels. Tracks were analyzed for total distance moved (cm). An input filter of 0.135 cm (minimum distance moved) was used to remove system noise.

2.5. Specific methods for each study

2.5.1. Age of larvae experiment

Embryos of 3 different ages were reared and tested on the same microtiter plate. This was achieved by placing, in every third column of a 96-well microtiter plate, an embryo every day for 3 consecutive days. In this manner, by the sixth day after the plating was begun, all three ages (i.e., 4, 5 and 6 dpf), in alternating columns, were present on the same microtiter plate. On the afternoon of the sixth day, locomotor activity was tested using alternating epochs of light and dark.

2.5.2. Size of well experiment

Three different size microtiter plates were used: (1) a 96-well plate (described above), (2) a 48-well plate (Costar 3548, Corning, Inc; Corning, NY) and (3) a 24-well plate (Costar 3524, Corning, Inc.; Corning, NY). The embryos were placed 1 embryo per well in all 3 types of plates. They were reared in 250 μl 10% Hanks' in the 96-well plate, 1 ml 10% Hanks' in the 48-well plate, and 2 ml of 10% Hanks' in the 24-well plate. Because the 48-well plate and the 24-well plate were not multiscreen mesh plates, the entire volume of the well could not be changed each day without stressing the embryos/larvae, so ½ of the solution in each well was renewed each day. On day 6, the animals were tested for locomotor activity in the well that they were reared in.

2.5.3. Presence of malformations experiment

The malformations were not induced by chemical exposure; in other words, only untreated embryos were used. Control embryos were reared in 96-well plates according to the general rearing protocol. Embryos were scored for malformation at 6 dpf by visual inspection under a dissection scope using a rating scale (Table 1). Briefly, a single observer assessed each larva for 1) spine (*e.g.* stunted skeletal growth, curved spine, kink in tail), 2) fins (*e.g.* malformed or stunted fins), 3) cranial/facial (*e.g.* abnormal head, eyes, or otoliths), 4) thorax (*e.g.* distension, heart malformations), 5) abdominal (*e.g.* edema, emaciation), and 6) position in the water column (*e.g.* floating, lying on side) (see Table 1 for details). Each of the categories contains a number of possible malformations that could occur; some malformations were scored as yes/no, while others were scored as degree (*i.e.,* 0 through 4). The scores for all of the categories were then summed, with higher scores denoting more severely deformed fish. To give an idea of the normal range of scores, 96% of historical control values range between 0 and 3; an obviously deformed larva would score in the 15 to 20 range, and the highest score ever noted (not in this study) was 34. The results of the present assessment agreed with the historical control data as 95% of the animals (344 out of a total of 361) scored between 0 and 3. Behavioral testing was conducted on 6 dpf as described above.

2.5.4. Different rearing solutions experiment

Embryos were reared in one of five solutions: 10% Hanks' Balanced Salt Solution (recipe given above), 60 mg/L Instant Ocean® (purchased at a local pet store), 1:3 Danieau (19.33 mM NaCl, 0.23 mM KCl, 0.13 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$, 1.66 mM HEPES, pH 7.2); 1× EPA Moderately Hard Reconstituted Water (MHW: 54 μM KCl, 0.5 mM $MgSO_4 \cdot 7H_2O$, 1.1 mM $NaHCO_3$, 350 μM $CaSO_4$), and 1:10 EPA MHW. Each solution was prepared the day before the experiment began. After the embryos were washed (as described above), they were placed in a 96-well microtiter plate (1 embryo per well, as described above) in their respective solution, with solutions organized by column. All 5 solutions were included on each plate. The solutions were renewed every day until Day 6, at which time behavioral testing was conducted as described above.

2.5.5. Statistical analyses

All data were analyzed using Statview© (SAS Institute, Inc., Cary, NC; version 5.0.1). In general, the data were first assessed using a repeated-