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Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol☆,☆☆

R.C. MacPhail [a,*], J. Brooks [b], D.L. Hunter [a], B. Padnos [a], T.D. Irons [a,c], S. Padilla [a]

[a] Neurotoxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
[b] Division of Science and Mathematics, Brevard College, Brevard, NC, USA
[c] Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

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ABSTRACT

The increasing use of zebrafish (*Danio rerio*) in developmental research highlights the need for a detailed understanding of their behavior. We studied the locomotion of individual zebrafish larva (6 days post-fertilization) in 96-well microtiter plates. Movement was recorded using a video-tracking system. Time of day results indicated locomotion, tested in darkness (infrared), decreased gradually from early morning to a stable level between 13:00 and 15:30 h. All further studies were conducted in early-to-late afternoon and lasted approximately 1 h. Each study also began with a period of darkness to minimize any unintended stimulation caused by transferring the plates to the recording platform. Locomotion in darkness increased initially to a maximum at 4 min, then decreased steadily to a low level by 20 min. Locomotion during light was initially low and then gradually increased to a stable level after 20 min. When 10-min periods of light and dark were alternated, activity was low in light and high in dark; curiously, activity during alternating dark periods was markedly higher than originally obtained during either extended dark or light. Further experiments explored the variables influencing this alternating pattern of activity. Varying the duration of the initial dark period (10–20 min) did not affect subsequent activity in either light or dark. The activity increase on return to dark was, however, greater following 15 min than 5 min of light. Acute ethanol increased activity at 1 and 2% and severely decreased activity at 4%. One-percent ethanol retarded the transition in activity from dark to light, and the habituation of activity in dark, while 2% ethanol increased activity regardless of lighting condition. Collectively, these results show that locomotion in larval zebrafish can be reliably measured in a 96-well microtiter plate format, and is sensitive to time of day, lighting conditions, and ethanol.

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1. Introduction

Studies on zebrafish as a model system have steadily increased (Dowling, 2002; Miklosi and Andrew, 2006; Orger et al., 2004; Sison et al., 2006). Zebrafish are now used in many fields of biomedical science, including toxicology, for studying processes related to human disease susceptibility and risk (Alestrom et al., 2006; Guo, 2004; Hill et al., 2005; Lieschke and Currie, 2007). Zebrafish have also become widely used in the pharmaceutical industry, where literally thousands of chemicals can be screened rapidly *in vivo* for therapeutic and toxic potential (Jacobs, 2006; Zon and Peterson, 2005).

The use of zebrafish in developmental research is of particular interest. Zebrafish offer a number of advantages in studies on development (Westerfield, 2000). Their size and relatively simple husbandry requirements allow maintenance of large stocks of fish. Mating results in a large number of eggs. Development occurs rapidly, and progresses through well-defined stages (Kimmel et al., 1995). Transparency of the embryos and larvae allows detailed non-invasive observation of organ-system development. Numerous tools are also available to probe physiological functions including vital dyes, fluorescent probes, and several strains and transgenic lines of zebrafish (Feldman, 2001; Hill et al., 2005; Peterson et al., 2008). A notable advantage for developmental toxicity studies is that exposure to chemicals can take place directly, thereby avoiding the maternal compartment that can confound similar studies in mammals.

Assessment of the behavior of developing zebrafish is also becoming popular. Behavior represents the unique interface between intrinsic and extrinsic forces that determine an organism's

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* Corresponding author at: Neurotoxicology Division (B105-04), National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, 109 TW Alexander Drive, Research Triangle Park, NC 27711, USA.
Tel.: +1 919 541 7833.
E-mail address: macphail.robert@epa.gov (R.C. MacPhail).

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health and survival (MacPhail, 1990; Little et al., 1990). Several studies have focused on the behavioral development of sensory and motor functions (Easter and Nicola, 1996; Saint-Amant and Drapeau, 1998; Budick and O'Malley, 2000; Drapeau et al., 2002; Burgess and Granato, 2007). A number of studies have also explored the development of reflexes, learning and memory (Kimmel et al., 1974; Levin et al., 2004; Orger et al., 2004; Weber, 2006; Zeddies and Fay, 2005). Relatively few studies, however, have evaluated behavior during development in the type of small testing environment (e.g., a multi-well microtiter plate) that would be required for large-scale chemical screening in toxicology and pharmacology.

We report the results of several studies on the behavior of 6-day-old zebrafish larvae. Larvae were individually raised and tested in 96-well microtiter plates. The small environments (250 μl) of the microtiter plate greatly limit the types of behavioral responses that can be studied. We therefore focused on general locomotion of the larvae. Most previous studies have evaluated larval behavior in light using optical tracking devices (e.g., Lockwood et al., 2004; Orger et al., 2004). Infrared image analysis, however, has permitted studies of locomotion in both light and darkness (Cahill, 2007; Emran et al., 2007; Prober et al., 2006; Burgess and Granato, 2007). The current studies therefore evaluated locomotion under both light and dark conditions. Initial studies determined the optimal time of testing during the work day. Subsequent studies measured locomotion during extended periods (several minutes) of dark, and light, and during shorter alternating cycles of light and darkness. Further studies determined the effect of manipulating light- and dark-period durations. A final study determined the effects of a prototype centrally acting drug, ethanol, on locomotor activity. These experiments employed testing durations (ca. 1 h) that allowed repeated manipulation of lighting conditions, thereby permitting detailed investigation of the role of environmental influences on locomotion, ethanol effects, and their interaction.

2. Materials and methods

2.1. Experimental animals

All studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at the U.S. EPA National Health and Environmental Effects Research Laboratory. Wild type adult zebrafish (*Danio rerio*) were maintained in an AAALAC-approved animal facility at 28 °C with a 14:10 h light:dark cycle (lights on at 08:30 h). Adult fish were housed in 9-l flow-through colony tanks (Aquaneering Inc., San Diego, CA) with approximately eight females and four males per tank. On the day of embryo collection, and 1 h prior to light onset, all adults in a colony tank were placed in a 2-l (static) breeding tank (Aquatic Habitats, Apopka, FL). Usually, adults from two to three housing tanks were mated on the same day. One hour after light onset the adults were returned to the colony tank, and embryos were collected from the breeder tank. All embryos were then pooled from the breeding tanks and placed in a 26 °C water bath for 2 h, followed by washing with 1% bleach (v/v) in 10% Hanks solution for 1 min. Embryos were distributed randomly, one per well, into 96-well mesh-bottom (40-μm nylon) microtiter plates (Multiscreen™ catalog #MANMN4050, Millipore Corp., Bedford, MA), where they were reared and later tested.

2.2. Embryo rearing

Embryo rearing was essentially as described by Westerfield (2000). All embryos and larvae were maintained in microtiter-plate wells in 10% Hanks Balanced Salt Solution (13.7 mM NaCl, 0.54 mM KCl, 25 μM Na_2HPO_4, 44 μM KH_2PO_4, 130 μM $CaCl_2$, 100 μM $MgSO_4$, 420 μM $NaHCO_3$) hereafter referred to as Hanks solution. Each microtiter plate consisted of a bottom tray, buffer tray, mesh tray, and a lid. A photograph and schematic of the 96-well microtiter plate can be seen in Oxendine et al. (2006, Fig. 2). A piece of Plexiglas (10.5 cm long, 7.0 cm wide and 0.3 cm thick) was placed between the bottom and buffer trays to stabilize the buffer tray and avoid the possibility of spillage between wells. Individual wells within the buffer tray contained a single embryo within its own environment. The mesh-bottom tray permitted rapid solution replacement by transferring it to a new buffer tray. Each day, 150 μl of fresh aerated 10% Hanks solution was added to individual wells of the buffer tray of a new plate. The mesh tray (with embryos) was removed from the old plate, quickly blotted dry in a matter of seconds, and inserted into the new buffer tray. An additional 100 μl of 10% Hanks solution was added immediately to each well. Each 96-well microtiter plate was sealed with a non-adhesive material (Type A, BioRad, Hercules, CA), covered with the lid, and wrapped in Parafilm® to minimize evaporation. All embryos and larvae were kept in a 26 ± 0.7 °C incubator with a 14:10 h light:dark cycle (with lights on at 08:30 h).

2.3. Recording fish behavior

All testing was performed on 6-day post-fertilization (dpf) larvae in the same 96-well plate in which they were reared. At 6 dpf, larvae still feed from their yolk sac, obviating the need to introduce food to the wells. They also have a sufficient level of locomotion for study. Larvae were housed individually for ease of tracking and statistical analysis.

On the day of testing, larvae were placed in fresh 10% Hanks solution, the plate was covered, and then transferred into a light-tight drawer in the behavioral testing room. Temperature in the testing room was kept at 26 °C. For all experiments, except the time of day experiment, testing occurred after 13:30 h. Movement of each fish was monitored with a Noldus behavior recording system [Noldus Information Technology, Leesburg, VA (www.noldus.com)], consisting of a Pentax CCDIR XC E150 camera with a Pentax TV lens, a platform (light box containing LEDs) on which the plates were placed, with a baffle that blocked extraneous light. The light box provided infrared (800–950 nm with a peak at 860 nm) or visible (430–700 nm) light as measured with a wideband spectroradiometer (RPS900, International Light Technologies, Peabody, MA). The illuminance provided by the visible light was measured at 69.5 lx using a photometer (model DR-2550-1, 2B silicon detector, TC284 photometric filter, Gamma Scientific, San Diego, CA). The camera output was fed into a standard PC system, and Canopus Mediacruise MVR1000 software (Canopus Corporation, San Jose, CA) was used to create videos of fish movement. All recordings were stored as MPEG-2 files for later analysis.

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). The 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); to remove system noise, an input filter of 0.135 cm (minimum distance moved) was used (i.e., filtered data). Locomotion data are presented as mean ± S.E.M. distance (cm) moved per unit time under each condition that was arranged during a test session.

2.4.1. Procedure

Studies were conducted to determine the influence of several variables on the locomotor activity of 6-dpf zebrafish larvae. Details of each study are presented below.

2.4.1.1. Time of day effects. The first study determined the optimal time for testing, that is, when activity did not vary from hour to hour. Embryos were reared for 6 days in 10% Hanks solution, which was changed daily. On the morning of the sixth day, larvae were transferred to fresh solution, and the plate was moved to the behavioral testing room. After the plate was placed on the recording platform, the infrared light was turned on for the duration of the session. Throughout, infrared illumination is referred to as darkness, because zebrafish do not see infrared (Dowling, 2002). The baffle was lowered over the plate and the fish were given 20 min of acclimation to minimize any disturbance related to handling and transport. Beginning at 10:00 h, fish movement was recorded under infrared light for 5 min every half hour, for a total of 5.5 h.

2.4.1.2. Activity in light and dark. These studies were designed to assess the general pattern of larval activity in visible light and in darkness (infrared illumination). Embryos were reared in 10% Hanks solution for 6 days with solutions changed daily. Between 09:00 and 09:30 h on the sixth day, larvae were transferred to fresh solution, and the plate was moved to a light-tight drawer in the darkened behavioral testing room. Plates were tested between 13:30 and 15:30 h. In the darkened room, the plate was removed from the drawer, the lid removed, the sealer removed, the lid replaced and the plate placed on the testing platform (light box) under the baffle. Following transfer to the light box, locomotion was recorded in dark for 10 min of acclimation. After acclimation, activity was recorded in dark or in visible light for 40 min. One plate was used for testing in darkness, and the other plate was used for testing in light. To avoid confusion in subsequent studies, these conditions are described as extended dark or extended light, recognizing, of course, no reference to circadian rhythms of activity.

2.4.1.3. Effects of alternating light and dark periods. The larvae were reared and handled exactly as described above. After 10 min of acclimation in dark, the larvae were exposed to three cycles of alternating 10-min light and dark periods (for a total of 60 min).

2.4.1.4. Effects of varying duration of the dark period. This experiment was designed to determine the role of the initial dark (acclimation) period on subsequent locomotion in light and in dark. Acclimation in dark lasted for either 10 or 20 min, after which a 20-min period of light was followed by a return to darkness for 10 min.

2.4.1.5. Effects of varying duration of the light period. A 10-min acclimation in dark was arranged for larvae in each of two plates. Next, larvae on one plate were exposed to 5 min of light, while larvae on the other plate were exposed to light for 15 min. A 20-min period of darkness followed, then either 15 or 5 min of light (respectively), and, finally, another 20 min of darkness. The light-period durations were arranged differently in the two plates to counterbalance order of presentation.

2.4.1.6. Ethanol effects. Ethanol (95% purity) was prepared in concentrations of 1, 2, and 4% (v/v). All ethanol concentrations were non-lethal, and were selected on the basis of pilot studies. Group sizes were $n = 24$ per concentration, including a control (10% Hanks solution) group. The larvae were placed into the ethanol solutions shortly before transfer to the recording platform. Ethanol doses were distributed diagonally because pilot work with control larvae occasionally showed an effect of well location in the columns or (even more infrequently) rows of the plates. A 20-min period in darkness was arranged for acclimation and drug uptake. Activity was next recorded for 10 min in the dark and was followed by a 10-min period of light, then 20 min of dark, and then another cycle of light (10 min) and dark (20 min). Ethanol dose-effect determinations were carried out in two plates in which doses were similarly distributed.

2.4.1.7. Inferential statistics. Details for each analysis can be found in the figure legends. All data were analyzed using Statview© (SAS Institute Inc., Version 5.0.1). In general, data from each study were compared using a repeated-measures ANOVA. Activity throughout a test session was the dependent variable, whereas time of day, lighting condition (light vs. dark) or chemical treatment (ethanol dose) was the independent variable. Significant interactions between time within a session and an independent variable were followed by step-down ANOVAs to assess lower-order effects. When appropriate, Fisher's PLSD test was used to evaluate between-group contrasts.

3. Results

Fig. 1 shows the results of testing locomotion across day-time hours (from 10:00 to 15:30 h). Activity was evaluated in darkness (infrared) for 5 min at 30-min intervals. Locomotor activity was highest in mid-morning when testing began, and then decreased to a stable level by early afternoon. No further change in activity was noted between 13:00 and 15:30 h. Variability in activity between larvae was also lowest during this time span; standard errors were approximately 5% of the mean. All further testing occurred during the afternoon hours.

Locomotion was next tested in darkness during a 50-min session. Locomotion was also tested in light for 40 min, after an initial 10-min period of darkness. Results are shown in Fig. 2. During the initial dark period, activity for both groups of larvae quickly increased to a maximum and then decreased. In larvae that remained in darkness, activity reached a stable low level at approximately 20 min into the session. For larvae that were switched to light (after 10 min in darkness), activity decreased

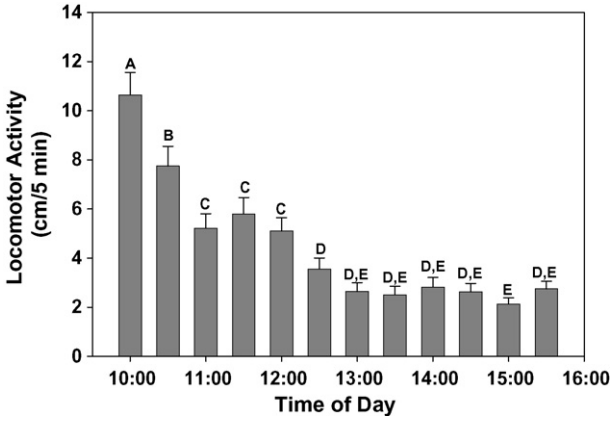

Fig. 1. Effect of time of day on baseline locomotion in the dark (infrared). Data are presented as mean distance moved (in cm) ± S.E.M. during 5-min tests occurring every 30 min. Activity was highest in the morning then decreased to a stable level that was reached by 13:00 h. Results are based on a total of 183 larvae from two plates. Repeated-measures ANOVA indicated a significant effect of time ($p < .0001$). Fisher's PLSD test was used to compare test times; common letters indicate no significant difference.

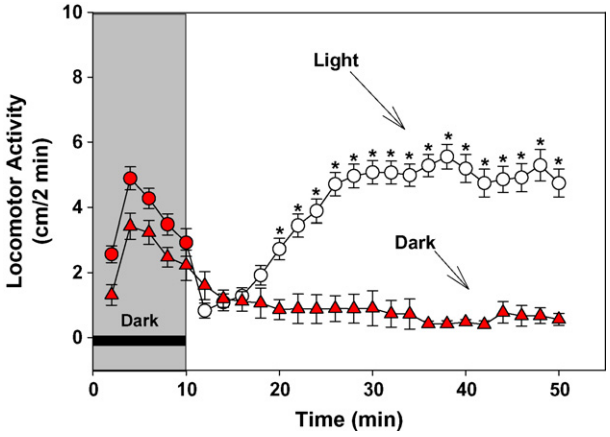

Fig. 2. Effects of darkness and visible light on locomotion in larval zebrafish. Data are presented as mean distance moved (in cm) ± S.E.M. in 2-min intervals during 50-min sessions. For both groups, activity increased and then decreased during the initial dark period (solid symbols in shaded area). Extended dark further decreased the level of activity (solid triangles). Switching to light (open circles) produced an initial decrease followed by a gradual increase in activity to a stable level. Results are based on a total of 89–91 larvae from each of two plates, one plate for evaluating extended light and the other for extended dark. Repeated-measures ANOVA for data from 10 to 50 min indicated a significant interaction ($p < .0001$) between lighting condition (light vs. dark) and time. Asterisks indicate locomotion in light significantly ($p < .05$) different from locomotion in dark (Fisher's PLSD test).

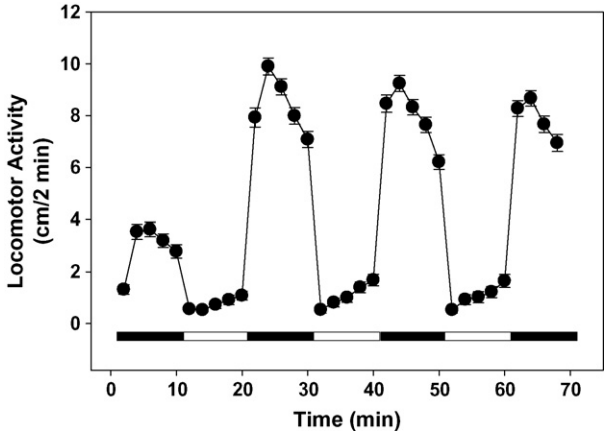

Fig. 3. Effect of alternating light–dark periods on locomotion in larval zebrafish. A 10-min period of darkness was followed by three alternating cycles of 10-min light and 10-min dark. Black and white bars at the bottom signify dark and light conditions, respectively. Data are presented as mean ± S.E.M. distance moved (in cm) in 2-min intervals throughout a 70-min session. Results are for a total of 191 larvae. Based on activity summed within each 10-min period, ANOVA indicated a significant effect of light and dark conditions. Post hoc comparisons indicated activity in each light period did not differ from one another, but was significantly different ($p < .05$) from the activity in all four dark periods. Activity in the first dark period was also significantly lower ($p < .05$) than the activity in all subsequent dark periods.

precipitously, but was followed by a gradual increase to a maximum sustained level after approximately 20 min in light (Fig. 2).

In the next study, locomotor activity was assessed under alternating light–dark conditions. Testing began in darkness, followed by three cycles of light (10 min) and dark (10 min). As reported above, activity in initial dark increased to a maximum and then began to decrease (Fig. 3). Switching to light precipitously decreased activity. On return to darkness, however, activity rapidly increased to a level that was substantially higher than that obtained during the initial dark period. Additional cycles of alternating light and dark reliably produced alternating levels of low and high activity, respectively (Fig. 3). This pattern of activity is in stark contrast to what was obtained during extended periods of light or dark. The next two studies explored the conditions under which this "paradoxical" pattern of activity occurred.

Duration of the initial dark period (10 or 20 min) was varied in the next study (Fig. 4, left panel). For both durations, activity in the initial dark period quickly increased to a maximum and then decreased (data not shown). Switching to light for 20 min resulted in an initial low level of activity that was followed by a gradual increase. Duration of the initial dark period did not affect either the low initial level of activity in light or the subsequent gradual increase (Fig. 4, left panel). Moreover, under both conditions the return to darkness produced a substantial increase in activity, and to levels that exceeded the maximum obtained initially in dark. Thereafter, activity in dark progressively decreased. Fig. 4 (left panel) shows that the levels of activity when dark was reinstated were identical regardless of the duration of the initial dark period.

Duration of the light period was next manipulated (Fig. 4, right panel). Following 10 min of dark, visible light was presented for either 5 or 15 min, and then followed by a return to darkness. Order

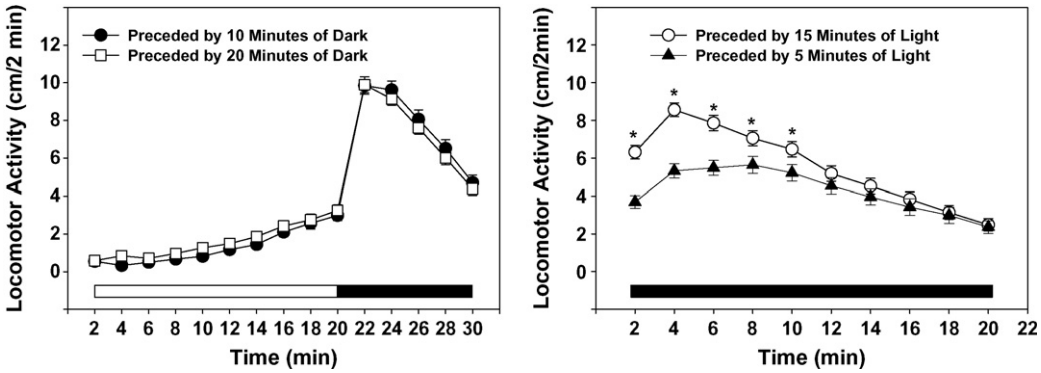

Fig. 4. Left panel: Effects of duration of the initial dark period on locomotion in larval zebrafish. Data are presented as mean ± S.E.M. distance moved (in cm) in 2-min intervals. White and black bars signify activity recorded in light and dark, respectively. Results are based on a total of 183 larvae. The curves are displaced in time to allow direct comparison of activity in light and during return to darkness. Duration of the initial dark period did not affect activity in either light or subsequent dark. Right panel: Effects of duration of visible light on locomotion. Data are presented as mean ± S.E.M. distance moved (in cm) in 2-min intervals. Following an initial dark period, light was presented for either 5 or 15 min. Activity data are presented during the return to dark for 20 min (denoted by the bottom black bar) after each light period. Results are based on a total of 188 larvae. Light period durations were counterbalanced between plates. Repeated-measures ANOVA indicted a significant interaction ($p < .0001$) between activity during the dark period and length of the preceding light period. Asterisks indicate significant difference in activity ($p < .05$) between larvae that had been exposed to 15 min of light and those that had been exposed to 5 min of light.

of presentation of the light-period durations was counterbalanced between plates. Fig. 4 (right panel) shows that the return to darkness reliably increased activity, and that the magnitude of the increase was greater following 15 min than 5 min of light. The activity in darkness (when reinstated) decreased progressively after the peak was reached, and the rate of decrease was approximately equal regardless of the duration of the preceding light period; a 50% decrease occurred approximately 10 min after the peak in activity.

In the last study, shown in Fig. 5, activity was recorded during acute exposure to 1, 2 or 4% ethanol. Control larvae were exposed to 10% Hanks solution. The lowest concentration of ethanol (1%) increased activity during the dark periods. At this concentration (1%), the increase in activity on return to darkness was greater than during the initial dark period, which was also apparent for the control larvae. Fig. 5 shows, however, that the decline in activity during reinstatement of the dark periods did not reach the level that was obtained in the control group at the end of the period. Interestingly, larvae exposed to 1% ethanol also showed a delayed decrease in activity during the transitions from dark to light. On the other hand, the rapid increase in activity during transitions from light to dark was similar to that obtained in vehicle-control larvae. The intermediate concentration of ethanol (2%) produced substantial increases in activity regardless of lighting condition, although transient drops in activity were noticeable immediately following the switch from dark to light. The highest concentration (4%) abolished activity throughout the test session; at this concentration the larvae were motionless but still alive.

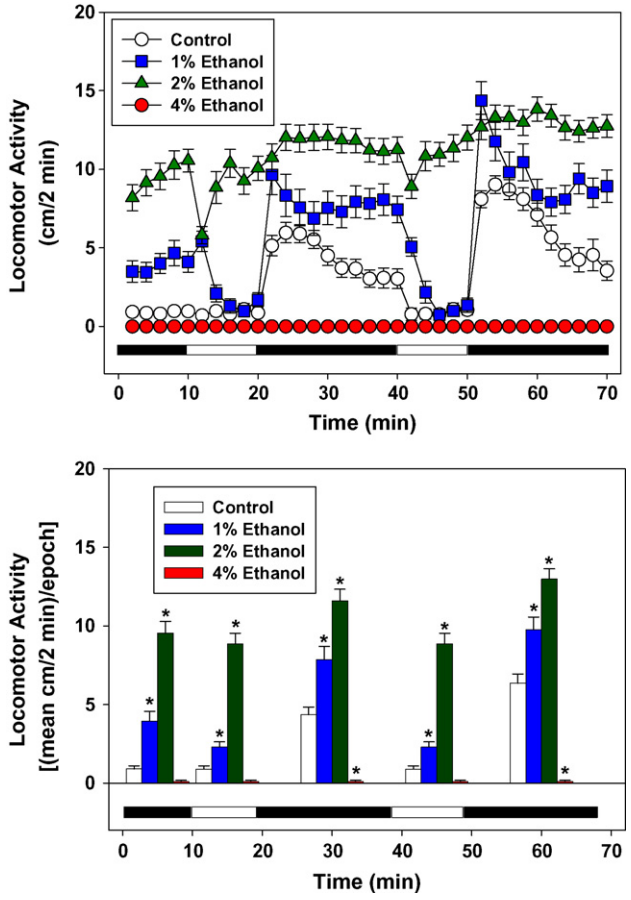

Fig. 5. Effects of ethanol on locomotion in larval zebrafish. Larvae were exposed to ethanol (1–4%, v/v) or vehicle (10% Hanks solution) for 90 min. Locomotion was recorded for the last 70 min. Data in the upper panel are presented as mean ± S.E.M. distance moved (in cm) in 2-min intervals during each lighting condition. Repeated-measures ANOVA indicated a significant interaction ($p < .0001$) between ethanol dose and time. Lower panel shows ethanol effects on activity averaged across each lighting period. Repeated-measures ANOVA indicated a significant interaction between ethanol dose and time ($p < .0001$). Step-down ANOVAs indicated a significant effect of ethanol dose ($p < .0001$) on activity in each lighting period. Asterisks indicate significant ($p < .05$) differences from control (Fisher's PLSD post hoc test). In both panels, black and white bars signify dark and light periods, respectively. Results are based on a total of 46–47 larvae per dose, from two plates, with each plate containing all doses.

4. Discussion

The increasing use of zebrafish in developmental toxicology highlights the need for a detailed understanding of their behavior. This challenge is difficult when the necessary testing environment is a multi-well microtiter plate suitable for the rapid screening of chemical compounds. Under these conditions the range of behavior for investigation appears limited. Behavior, however, has both spatial and temporal properties, and while the microtiter-plate wells restrict the spatial range of behavior, they do not necessarily constrain investigation of the time-dependent dimensions of behavior.

We began investigating the locomotion of larval zebrafish in 96-well microtiter plates in preparation for a series of studies on identifying toxicants that could potentially disrupt development of the nervous system in humans. Test-session durations allowed repeated manipulation of lighting conditions and a detailed characterization of larval locomotion. When locomotion was tested in extended dark, activity was high at first and then decreased to a low level (Fig. 2). When tested in extended light, activity gradually increased to a stable level (Fig. 2). These time-dependent patterns of activity are similar to those reported by Burgess and Granato (2007) for groups of larvae in a larger test environment. When shorter light and dark periods alternated within a test session, however, activity was low in light and considerably higher in dark (Fig. 3). Dark activity was also higher than activity in extended light. While this pattern of activity appeared paradoxical, we have found it to be a highly reliable finding under a variety of conditions. Additional experiments showed that the pattern of alternating activity did not depend on duration of the initial dark period (Fig. 4, left panel). Duration of the light period did, however, influence the magnitude of the increase in activity when darkness was reinstated (Fig. 4, right panel).

Recent reports (D'Amico et al., 2008; Emran et al., 2007; Prober et al., 2006) have identified a similar pattern of activity in larval zebrafish with switches in lighting conditions. Prober et al. (2006) arranged alternating 30-min periods of light and dark (infrared), during night-time hours, and obtained gradually increasing activity during the light periods, followed by spikes in activity early in the dark periods that decayed rapidly. Emran et al. (2007) showed that a switch from light to dark (infrared) produced a sudden spike in activity and then a higher level than in light. A switch from dark to light also produced a sudden spike in activity, but then a lower level than what had occurred in dark. The time frame (several seconds) in which data were presented for the transitions was, however, considerably shorter than what we used (i.e., several minutes) to study larval behavior patterns and transitions. Finally, D'Amico et al. (2008) recently presented alternating larval activity patterns, under alternating lighting conditions, that are identical to what we report.

In the present studies, substantially higher levels of activity occurred when the larvae were returned to darkness than when they were tested initially in darkness. This result indicated that the light period played an important role in enhancing activity when darkness was reinstated. The greater increase in dark activity after 15 min than 5 min of light (Fig. 4, right panel) further indicates the importance of the light period on the subsequent increase in dark

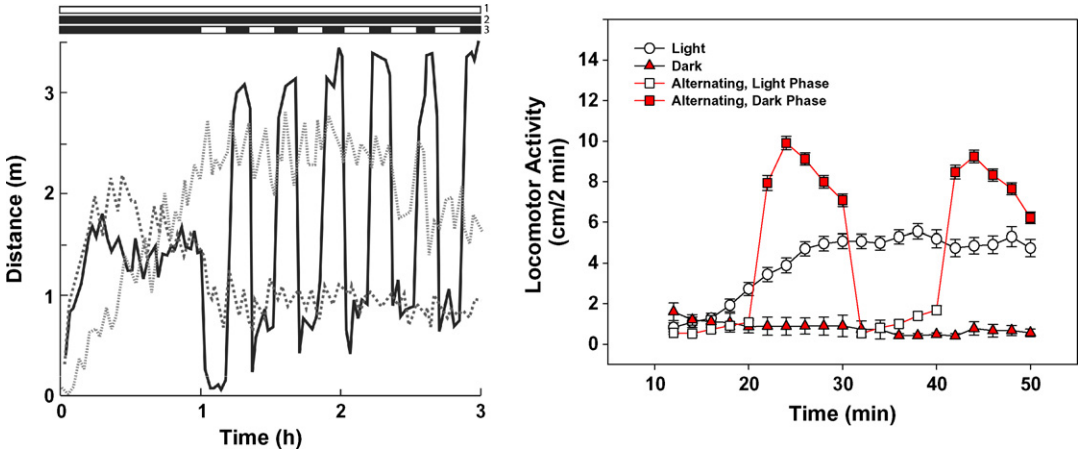

Fig. 6. Comparison of the effects of alternating light–dark cycles on locomotion in locusts and in larval zebrafish. Left panel shows results from Moorhouse et al. (1978) for locomotion in locusts under varying lighting conditions (indicated by top bars). Group 1 (fine dotted line) was tested in extended light; Group 2 (coarse dotted line) was tested in extended dark; Group 3 (solid line) was tested in dark for 1 h then alternating light and dark periods. Each line represents mean activity for 10 locusts. Right panel summarizes results from the present experiments for locomotion of larval zebrafish in extended light, extended dark, and alternating light and dark (data from portions of Figs. 2 and 3 are combined). Note difference in time scales between panels. Results from Moorhouse et al. are reproduced with permission of the Company of Biologists.

activity. The results could be interpreted as a rebound activity increase in dark following exposure to a period of light that was aversive to the larvae. This interpretation suffers from the fact that when either light or dark was presented for an extended period, activity was higher in light than in dark. Interestingly, a similar pattern of activity was reported for locusts by Moorhouse et al. (1978) that are presented here in Fig. 6 (left panel). When tested in dark, the activity of locusts initially increased to a maximum and then gradually decreased. When tested in light, activity increased more slowly and to a maximum that was both higher and occurred later than in dark. When light and dark periods alternated, activity was substantially lower in light and substantially higher in dark. Moorhouse et al. (1978) interpreted the paradoxical activity pattern as a case of antagonistic induction (Sherrington, 1906), where in their case light created an inhibitory effect on activity that was slow to dissipate, dark created a more rapid-acting excitatory effect, and alternating light–dark conditions produced a rebound excitation of activity in dark (see Staddon, 1983, for further discussion). Fig. 6 also presents results from the current studies (right panel). In comparing the two panels, the similarity of results is clear; higher levels of activity occur in extended light than in extended dark, yet alternating light and dark periods produces the opposite pattern of activity. Together, these results suggest a common mechanism that may underlie the effects of light–dark alternation on activity in both species.

In the present studies, the initial dark period was included to allow acclimation and minimize any disturbance accompanying transfer of the microtiter plate to the activity recording platform. During this period, activity increased quickly to a maximum and then slowly decreased. When light followed, activity decreased rapidly. The return to darkness produced a rapid and substantial increase in activity. The peak of activity on return to darkness exceeded the maximum level obtained during the initial dark period, and was greater when preceded by a long period of light (15 min) than a shorter (5 min) one. The results are consistent with light being aversive. Following the peak, activity during reinstatement of dark decreased steadily. Interestingly, the rate of decrease in dark was approximately equal regardless of the duration of the preceding light period. These results suggest the peak in activity and the subsequent decay may be separable phenomena. Further studies are warranted on the effects of a broader range of light-period durations on activity in both light and subsequent dark.

The decrease in activity during dark may represent habituation, which is considered an ancestral form of learning. Habituation is often referred to as non-associative learning, and occurs in a wide range of species and experimental conditions (e.g., Thompson and Spencer, 1966). A recent study demonstrated habituation of activity in larval zebrafish with repeated auditory-tone bursts (Best et al., 2008). The present experiments were not specifically designed to study habituation or the variables that influence it. It is interesting to note, however, that activity during light periods gradually increased, especially during the longer durations. These results may also reflect habituation or, alternatively, the gradual dissipation of some aversive properties of light onset. Further experiments are needed on whether the time-dependent activity changes in each lighting condition are due to habituation (see Thompson and Spencer, 1966, for a review of habituation and its parameters).

Exposure to ethanol produced several effects on larval locomotion that were concentration-dependent (Fig. 5). The lowest concentration (1%) increased activity in both light and dark periods. This concentration of ethanol also delayed the transition in activity when darkness switched to light. This finding may be related to the results of Matsui et al. (2006), using the optokinetic response, that acute ethanol exposure reduced visual sensitivity in 5-dpf zebrafish larvae. Ethanol did not, however, affect the transition in locomotion from light to dark. These results suggest the effect of ethanol on activity during transitions in lighting conditions may not due to a common process. The lowest concentration also delayed the reduction in activity during the dark periods, suggesting an additional action of ethanol on transitions in activity, and perhaps habituation. The intermediate concentration (2%) greatly increased activity regardless of lighting condition. It is notable, however, that activity decreased briefly during the transitions from dark to light. This result indicates the overall increase in activity was not entirely a general effect, and that larval activity was still partially sensitive to a change in lighting conditions. The highest concentration (4%) almost completely abolished activity regardless of lighting condition. The present results are similar to those of Lockwood et al. (2004), in studies of ethanol's effects on the activity of groups of larval zebrafish that were tested in a larger, rectangular environment. In both experiments, low concentrations of ethanol increased activity, and high concentrations decreased activity. The present

results show, however, how changes in lighting conditions can modulate the effect of ethanol on locomotor activity in larval zebrafish, and reveal the complexity of its actions.

In summary, the present results show the behavior of larval zebrafish in microtiter-plate wells is reliable, quantifiable and sensitive to time of day, lighting conditions and ethanol. By testing individual larva for extended durations, the current paradigm allowed multiple and repeated manipulation of lighting conditions to characterize the locomotor activity patterns and the effects of ethanol. As a result, these studies have established a complex and highly reproducible, three-phase pattern of activity involving (1) moderate initial activity in dark, (2) decreased activity in light, and (3) elevated activity on return to darkness. Screening studies using this paradigm, as an integral part of a test battery, can provide considerably more information regarding the effects of toxicants, and their behavioral selectivity, than is currently available.

Conflict of interest

None.

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