Published in final edited form as:

Behav Processes. 2011 February ; 86(2): 222–229. doi:10.1016/j.beproc.2010.12.003.

Locomotor behaviors in zebrafish (*Danio rerio*) larvae

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Abstract

Locomotor behaviors were examined in two experiments using zebrafish (*Danio rerio*) larvae at 4, 5, 6 and 7 days post fertilization (dpf). Larvae were observed in individual wells of a 12-well plate for one hour a day. In Experiment 1, the same larvae were observed for four consecutive days beginning on post-fertilization day 4; in Experiment 2, different groups of larvae from the same egg collection were observed at 4, 5, 6 and 7 dpf. Automated images collected every 6 seconds were analyzed for information about larval location, orientation and general activity. In both experiments, 4 dpf larvae rested significantly more, used a smaller area of the well more frequently, and were generally less active than older larvae. All larvae exhibited a preference for facing away from the center of the well and for the edge of the well. However, prolonged exposure to the well influenced overall activity, orientation, and preference for the edge region. The implications of these results for understanding the development of larval behavior and for the design of procedures to measure the effects of experience in zebrafish larvae are discussed.

Keywords

activity; *Danio*; larval models; learning; orientation; thigmotaxis

1. Introduction

The zebrafish (*Danio rerio*) is a small cyprinid teleost fish native to the Ganges River of East India, Bangladesh, and Burma. Adults have been observed in abundance in silt-bottomed, well-vegetated shallow pools and rice paddies, and at the margins of slow moving streams with overhanging vegetation (Engeszer et al., 2007b; McClure et al., 2006; Spence et al., 2006). Zebrafish have a rich repertoire of natural behaviors (Spence et al., 2008). They are known to hunt and capture live prey (Borla et al., 2002; Budick and O'Malley, 2000; Gahtan et al., 2005; McElligott and O'Malley, 2005), develop dominance hierarchies (Gerlach, 2006; Larson et al., 2006; Spence et al., 2008), select and defend spawning sites (Spence et al., 2007), form shoals (Engeszer et al., 2007a; Kerr, 1963; Pritchard et al., 2001), and avoid predators (Dill, 1974; Domenici, 2002).

Locomotor behaviors play an integral role in the feeding, social, and defensive activities of the zebrafish throughout its lifespan. The kinematics and genetic architecture of the

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locomotor repertoire of larval zebrafish have been well characterized by evolutionary biologists (e.g., Bruste et al., 2003; Drapeau et al., 2002). Granato et al. (1996) described the normal development of locomotor activity across embryogenesis. At 18 hours post fertilization (hpf), spontaneous muscle contractions begin in the embryo, and at 24 hpf mechanical stimulation will cause the embryo to twitch. By 48 hpf, the embryo is able to perform a tail-flip response when the tip of the tail is touched as well as a fast escape response when it is touched on the head. Other studies have shown that locomotion in zebrafish larvae is comprised of a small set of distinctive motor elements, including C-starts (Kimmel et al., 1974), routine turns (Budick and O'Malley, 2000), J-turns (McElligott and O'Malley, 2005), O-bends (Burgess and Granato, 2007), slow scoots (Budick and O'Malley, 2000), burst swims (Budick and O'Malley, 2000; Gahtan et al., 2005; Muller and van Leeuwen, 2004), and capture swims (Borla et al., 2002).

The present experiments complement these studies of the developing microstructure of locomotor behavior with an examination of spontaneous locomotor activity. Much of the work examining spontaneous locomotor activity across development has focused on circadian-driven activity cycles and the effects of photic stimulation (Cahill et al., 1998; Hurd et al., 1998; Hurd and Cahill, 2002). Few studies have provided information about the developmental trajectory of spontaneous activity. Prober et al. (2006) continuously monitored locomotor activity of normal wild type (WT) larvae in 96 well plates for several days starting at 4 days post fertilization (dpf). They reported that larvae in a normal light-dark cycle spend about 30% of their time in one of three states, inactive (1 min period with no movement), low-active (1-min period with 1 s or less activity) and high-active (a 1 min-period with >1 s of activity), and that robust locomotor activity emerges at 5 dpf. Thirumalai and Cline (2008) taped the spontaneous swimming behavior of 3 and 5 dpf WT larvae in 5-cm wide circular dishes for 15 min at 20 frames/s. They found that the total distance covered by 5 dpf larvae was significantly greater than that covered by the 3 dpf larvae.

Detailed information about larval zebrafish locomotor activity is critical for biomedical and behavioral studies of development and genetic modification. Mutant strains of zebrafish typically have an acutely abbreviated lifespan. Consequently, if we are to understand the effects of gene mutations on behavior, it is very important to characterize the behavioral repertoire of normally developing zebrafish during the early developmental window. The reasons that the zebrafish has emerged as a suitable and highly attractive model organism for the development of pharmaceutical and large-scale genetic screens have been extensively reviewed elsewhere (Gerlai, et al., 2000; Winter et al., 2008; Zon and Peterson, 2005). Among the most important are that its genome has been sequenced and multiple genetic markers and gene chips are available commercially (http://www.sanger.ac.uk/Projects/D_zerrio/). Zebrafish are inexpensive to maintain in large numbers and are ideal for medium- to high-throughput genetic screening. Embryonic development is extremely rapid and *ex utero*. Moreover, neural processes can be observed visually in the developing embryo because of the transparent chorion.

Our studies focused on activity and space use in zebrafish larvae from 4 dpf to 7 dpf. In two experiments, images were digitally recorded (see Creton, 2009) of individual larvae in 12-well plates every 6 sec for one hour a day. In Experiment 1, using a repeated measures design, the same larvae were imaged for four consecutive days beginning at 4 dpf and ending at 7 dpf. In Experiment 2, using a between-subjects design, different larvae randomly selected from the same egg collection were imaged at 4, 5, 6 or 7 dpf. Larval position in each image was calculated in XY coordinates using image processing software. From these data, we obtained information about the overall activity level, orientation and spatial location of individual larvae.

2. Experiment 1

The main aim of Experiment 1 was to document any developmental trend in general activity levels in 4 to 7 dpf zebrafish larvae. Secondary aims of Experiment 1 were to determine any age-dependent preferences for spatial location and orientation during this period of larval development. Details about space use patterns have potential for developing pharmaceutical and genetic screens. Yet, surprisingly, there have been no systematic studies of how zebrafish larvae at different ages use the physical space in which they are tested. High-throughput studies typically use 96-well plates that clearly constrain variation in larval spatial distribution. In one of the few studies to use a relatively large test arena, an edge preference was evident in a plot of the swimming trajectories of 5 dpf but not 3 dpf WT larvae in a 5 cm circular dish (Thirumbalai and Cline, 2008). Another study, however, found no edge preference in 7 dpf AB or WIK larval strains tested in groups of 10 in a rectangular compartment $8 \times 6 \times 2$ cm ($l \times w \times h$) for 20 min (Lockwood et al., 2004). We measured proximity to the edge of the well and the frequency of quadrant use.

Newly hatched larvae (2.5 to 3 dpf) attach periodically to a hard surface using small secretory cells in the epidermis of the head (Laale, 1977). Through a series of attachments at successively higher levels, larvae eventually reach the air-water boundary where they gulp a bolus of air to inflate the developing swim bladder (Goolish and Okutake, 1999; Riley and Moorman, 2000). Attachment and vertical migration are commonly and readily observed in multi-gallon aquaria and in containers with water at least 3-4 cms deep but are not necessary for swim bladder inflation in extremely shallow water (Riley and Moorman, 2000). We assessed whether 4 dpf larvae would exhibit a greater propensity for facing outwards and being attached to the agarose rings in our modified multiwell plates. We also recorded larval orientation (clockwise or counterclockwise) to determine which eye (left or right, respectively) was used to view the edge of the well. Visual lateralization has been documented in both larval and adult zebrafish. Studies with adult zebrafish have shown that the left eye is used to assess the novelty of objects or scenes (Miklosi et al., 1997) whereas the right eye is used in the visual control of response such as prey capture (Miklosi and Andrew, 1999). Zebrafish larvae as young as 8 dpf have been reported to show a strong left eye preference for assessing novelty (Sovrano and Andrew, 2006; Watkins et al., 2004).

2.1. Materials and methods

2.1.1. Subjects—Fifty adult male and female wild type zebrafish (*Danio rerio*) for breeding were obtained from Carolina Biological Supply Co. (Burlington, NC). They were housed in a 20-gallon tank at 28°C and maintained on a 14h light/10h dark cycle. They were fed a combination of frozen or fresh brine shrimp and flake food once or twice a day. Embryos were collected from the tank ninety minutes after light onset and were raised in a culture medium containing 60mg/l sea salt (Instant Ocean) in deionized water and 0.25 mg/l methylene blue as a mold inhibitor. Embryos were grown at a density of approximately 45 embryos per 50 ml culture medium in a plastic 8.5 cm culture dish (Corning no. 430591) and kept in an incubator set at 28°C with a 14h light/10 hr dark cycle. Behavioral testing was conducted on 36 larvae from 4 through 7 days post-fertilization (dpf). During this period, food supplements were not provided because the larvae absorb nutrition from their yolk sac (Jardine and Litvak, 2003) and we wanted to avoid contaminating the agarose well water. Four larvae were excluded from the study: two did not survive until 8 dpf and two escaped from the agarose wells during one or more recording sessions.

2.1.2. Materials—Three 12-well plates (Corning Costar no. 3513) were prepared for behavioral testing by lining each well with a ring of agarose (1% agarose w/v in culture

medium) to create a 14mm × 3mm inner well (see Creton, 2009 for specifics of the agarose ring preparation and a discussion of the imaging benefits of the altered plates).

Details of the imaging system have been described previously (Creton, 2009). Briefly, a multiwell plate was illuminated from above with a thin lightbox (Electron Microscopy Sciences, Hatfield, PA, Cat no. 71649-5A) and imaged from below using a high-resolution digital camera (Canon PowerShot SX110 IS, Tristate Camera, NY). The camera was set up for time lapse recordings using Canon's ZoomBrowser EX 6.1 software. The following camera settings in ZoomBrowser's Remote Capture were used: 1.3× digital zoom, image quality = medium 1 normal (2816 × 2112 pixels), white balance = fluorescent, iso-speed = 100, aperture (Av) = 6.3, and exposure time (Tv) = 1/30 sec. With the 1.3× digital zoom, the field of view is 104 × 78 mm (one pixel equals 0.0369 mm). Acquired images were compressed as 0.6 MB JPEGs and stored on a standard desktop computer (Dell OptiPlex).

2.1.3. Procedure—At 4 dpf, 36 larvae were randomly selected from the culture dish and transferred individually by pipette, one to each well. To reduce any bias in the larvae assigned to a plate, one well in each plate was filled before another well in the same plate was filled. Enough culture medium was then added to create a surface that was level with the top of the agarose ring. Larvae remained in the 12-well plates until 8 dpf. When not being imaged, the plates were housed in the incubator. Culture medium was added to the wells as necessary to offset any evaporation and to maintain water quality.

Following a 5 min acclimation period, each plate was imaged for one hour a day for four consecutive days, beginning on 4 dpf. Each plate was imaged at the same time each day between 11 am and 3 pm. Timelapse recordings were collected at 6 second intervals and the 601 images for each session were stored as 360 MB files.

2.1.4. Image processing and statistical analysis—ImageJ software (<http://rsb.info.nih.gov/ij/index.html>) was used to process the collected images and to calculate the location in XY coordinates of each individual larva in each image (see Creton, 2009 for details). Briefly, an intensity threshold was used to separate the larva from the lighter colored background. The XY coordinates of the centroid of the selected pixels, corresponding to a point in the middle of the head behind the eyes, provided the larva's location in the well. The XY coordinates of the midpoint of a bounding rectangle drawn around the selected pixels, corresponding to a point located in the tail region, were used in conjunction with the XY co-ordinates of the centroid to derive the orientation of the larva in the well. The logs generated by ImageJ were saved as Microsoft Excel files for further analysis. 'IF' equations were used to calculate if a larva was facing up, down, left or right. A comparison of larval orientations scored by automated image analysis and by manual scoring revealed that the automated analysis was consistent with manual scoring in 99 of 100 cases. In the one case where there was disagreement, the larva was oriented at an approximate 45 degree angle and the automated image analysis was likely more accurate than the manual scoring. Repeated measures analysis of variance (ANOVA) and dependent t-tests were used to test for differences in measures of activity, orientation and space use across development. A Bonferroni correction procedure was used to adjust significance levels for multiple pairwise comparisons.

2.2. Results and Discussion

2.2.1. Activity—A measure of overall locomotor activity was derived by summing the magnitude of the displacements between pairs of consecutive images for each larva on each day of recording. These individual sums were subsequently expressed as the average distance moved in mm per minute. Means and standard error of the means (SEMs) are

shown in Figure 1A for post-fertilization days 4 through 7. Numerically, larvae moved less at 4 dpf than at 5, 6 or 7 dpf but an ANOVA found no significant main effect of age, $F(3, 93) = 1.6$, $p > .10$.

2.2.2. Resting—An index of percent inactivity or resting was calculated for each larva on each day of recording using the number of consecutive pairs of images with larval displacements of less than 5 pixels (.185 mm). Mean percent resting is plotted in Figure 1B for each dpf. The mean percent observations of resting declined significantly from 4 to 7 dpf, $F(3, 93) = 13.3$, $p < .01$. A series of pairwise comparisons revealed significantly more rests at 4 dpf than at 5, 6 or 7 dpf, $t(31) \geq 3.09$, $ps \leq .01$, but no significant differences in percent resting among 5, 6 and 7 dpf, $t(31) \leq 2.27$, $ps > .10$.

2.2.3. Orientation—Two measures of orientation were calculated. Figure 1C shows the mean percentage of observations that larvae faced away from the center of the well. This outward orientation was dominant at all ages tested and was significantly different from 50% (chance) at each dpf, $t(31) \geq 12.3$, $ps < .01$. An ANOVA revealed a significant main effect of age, $F(3, 93) = 4.4$, $p < .01$. Paired t-tests revealed significant differences in percent outward orientation between 4 and 5 dpf, $t(31) = 2.67$, $p < .05$ and between 4 and 6 dpf, $t(31) = 2.66$, $p < .05$. No other pairwise comparisons were statistically significant, $t(31) \leq 2.02$, $ps > .10$. Examination of which eye was used to view the edge of the well revealed no significant preference as a function of age ($F < 1$). The mean percent observations that larvae faced clockwise (left eye view of well edge) was 45.3 (SEM = 4.8) at 4 dpf, 49.7 (SEM = 3.8) at 5 dpf, 46.3 (SEM = 3.0) at 6 dpf, and 52.1 (SEM = 2.7) at 7 dpf. These means did not differ significantly from 50%, $t(31) \leq 1.25$, $ps > .10$.

2.2.4. Location preference—Two measures of space use were analyzed. For one analysis, we divided each well into an inner (center) and outer (edge) region that were matched for total area. For each larva, we calculated the percentage of observation points in the edge region (more than 4.69 mm from the midpoint of the well) on each of the 4 recording days. Means and SEMs are shown in Figure 1D for post-fertilization days 4 through 7. An edge preference was dominant at all ages tested and was significantly different from 50% at each dpf, $t(31) \geq 9.15$, $ps < .01$. An ANOVA revealed a significant main effect of age, $F(3, 93) = 4.6$, $p < .01$. Paired t-tests revealed that larvae were more likely to be located on the edge at 4 dpf than they were at 5, 6 or 7 dpf, $t(31) \geq 2.69$, $ps < .05$. No other pairwise comparisons were statistically significant, $t(31) \leq 0.21$, $ps > .10$. Visual inspection of the images confirmed analyses using the XY coordinates that, despite being in the edge region, the majority (28/32) of the 4 dpf larvae were never attached to the agarose ring during testing.

For the other analysis, we divided each well into quadrants and calculated the percentage of observations a larva was located in each quadrant during each recording session. Means and SEMs are shown in Figure 1E for the quadrant with the highest percent use at each dpf. The preference for this quadrant was significantly greater than for the quadrant with the next highest percent use on each test day, $t(31) \geq 5.6$, $ps < .01$. Over the 4 test days, larvae concentrated less of their time in the quadrant of highest use, $F(3, 93) = 13.7$, $p < .01$. A series of pairwise comparisons revealed more observations in the preferred quadrant at 4 dpf than at 5, 6 or 7 dpf, $t(31) \geq 3.35$, $ps < .01$. The difference between 5 and 6 dpf was not significant, $t(31) = 0.75$, $p > .10$ and the differences between the larvae at 7 dpf and the larvae at 5 and 6 dpf were marginally nonsignificant, $t(31) \geq 2.44$, $ps < .06$.

The main findings of Experiment 1 revealed four ways in which the behavior of larvae at 4 dpf could be distinguished from their behavior at 5, 6 or 7 dpf. Larvae at 4 dpf showed a stronger preference for an outward orientation than at 5 or 6 dpf, a stronger preference for

being in the outer region of the well than at 5, 6 or 7 dpf, a higher rate of resting than at 5, 6 or 7 dpf, and a more concentrated use of a single quadrant of the well than at 5, 6 or 7 dpf. However, Experiment 1 found no statistically significant age-related patterns in overall activity or visual lateralization.

The distinctive behavioral profile of 4 dpf larvae is consistent with their transitional developmental stage and their biological imperative to reach the water's surface to inflate the swim bladder. Although we did not observe the majority of 4 dpf larvae attached to the agarose ring, they were facing outwards in close proximity to the ring. Be this as it may, we cannot exclude the possibility that some, if not all, of the age-related differences we found may be a consequence of differential exposure to the well. Conversely, it is possible that differential exposure to the well may have obscured detection of age-related differences. For instance, the older larvae may have been less active in the well because of its increasing familiarity and poor prospects for foraging. To help disentangle the relative contributions of experiential and maturational factors, we replicated Experiment 1 using different larvae from the same egg collection on each day of testing.

3. Experiment 2

Experiment 2 was a between-subjects replication of Experiment 1. A total of ninety-six larvae were randomly selected for observation at one of four different ages (4, 5, 6 and 7 dpf). All larvae originated from the same egg collection and thus were tested on different but consecutive days. On each day of testing, 24 larvae were transferred to individual wells in 12-well plates. As in Experiment 1, larvae were imaged every 6 sec for one hour. Recording sessions occurred at the same time every day between noon and 3 p.m. The same six behavioral measures used in Experiment 1 were used here. If experience with the well had no impact on the behavioral outcomes of Experiment 1, we should obtain the same pattern of results in Experiment 2 that we observed in Experiment 1.

3.1. Materials and Methods

3.1.1. Subjects and Materials—Details of breeding, egg collection and embryo rearing were identical to those of Experiment 1. A total of 96 larvae were used. Data were excluded from two larvae tested at 6 dpf that escaped from the agarose wells during the recording session. Multi-well plates were prepared in the same way as described for Experiment 1. The same imaging system was used.

3.1.2. Procedure—Details of behavioral testing and image processing were the same as those for Experiment 1 with one exception. On each of the 4 days of testing, 24 randomly selected larvae were transferred from a culture dish to individual wells. Testing began on day 1 with 4 dpf larvae and ended on day 4 with 7 dpf larvae. All larvae were drawn from the same egg collection. Data were analyzed using a between-subjects one-way ANOVA. Significant main effects of Group (age in dpf) were explored using Tukey HSD pairwise comparison post hoc tests.

3.2. Results and Discussion

The behavioral measures were the same as those used in Experiment 1.

3.2.1 Activity—Figure 2A shows the mean distance moved in mm per minute on post-fertilization days 4 through 7. Larvae at 4 dpf showed less movement overall than larvae at 5, 6 or 7 dpf, $F(3, 90) = 8.98$, $p < .01$. Post-hoc comparisons revealed that Group 4 dpf moved significantly less than Groups 5 and 7 dpf ($ps < .05$) but the difference with Group 6 dpf was marginally nonsignificant ($p < .06$). No other group comparisons were statistically

significant although the differences between Group 5 dpf and Groups 6 and 7 dpf were marginally nonsignificant ($ps < .06$).

3.2.2. Resting—Figure 2B shows mean percent inactivity or resting at the four ages tested. Statistical analysis revealed a significant main effect of Group, $F(3,90) = 12.6$, $p < .01$. Post hoc comparisons revealed that mean percent resting was significantly higher in Group 4 dpf relative to the other three Groups, $ps < .01$. There were no significant differences among Groups 5, 6 and 7 dpf.

3.2.3. Orientation—Figure 2C shows the mean percent observations that larvae faced towards the periphery of the well. An outward orientation was preferred at all ages tested but did not vary with age, $F(3,90) = 1.4$, $p > .10$. Examination of which eye was used to view the edge of the well revealed no significant preference as a function of age, $F(3,90) = 1.7$, $p > .10$. The mean percent observations that larvae faced clockwise (left eye view of well edge) was 53.5 (SEM = 5.2) at 4 dpf, 47.6 (SEM = 3.3) at 5 dpf, 49.3 (SEM = 2.5) at 6 dpf, and 58.8 (SEM = 3.7) at 7 dpf. Paired t-tests revealed no significant differences from 50% in larvae aged 4 to 6 dpf but did show a significant difference for the 7 dpf larvae, $t(23) = 2.36$, $p < .05$.

3.2.4. Location preference—Figure 2D shows the mean percent observations that larvae were located in the edge region. Larvae were consistently more likely to be in the outer region than in the inner region regardless of age. An ANOVA found no significant main effect of Group, $F(3, 90) = 1.17$, $p > .10$. Figure 2E shows the mean percent observations larvae were located in the quadrant of highest use at each age. There was a significant main effect of Group, $F(3,90) = 12.7$, $p < .01$. Post hoc comparisons revealed that Group 4 dpf had a stronger preference for the quadrant of highest use than any other group, $ps < .01$. No other group comparisons were significant. As in Experiment 1, the quadrant preference was also significantly stronger than for the quadrant with the next highest percent use at each dpf, $ts(23) > 4.1$, $ps < .01$ at 4, 5, and 7 dpf, and $t(21) = 4.5$, $p < .01$ at 6 dpf.

The main findings of Experiment 2 revealed that larvae at 4 dpf were significantly less active than the 5 and 7 dpf larvae and rested significantly more than the 5, 6 and 7 dpf larvae. In addition, they used their preferred quadrant to a significantly greater degree than the older larvae. There were no statistically significant age-related differences in percent outward orientation, preference for the edge region, and percent clockwise orientation. However, we did find a clockwise orientation to be above chance in the 7 dpf larvae.

4. General Discussion

Zebrafish larvae were observed in individual wells of a 12-well plate for one hour a day at 4, 5, 6 and 7 dpf in two experiments. In Experiment 1, the same larvae were observed for four consecutive days beginning on post-fertilization day 4; in Experiment 2, different larvae from the same egg collection were observed at each of the 4 ages tested. Automated images collected every 6 s were analyzed for information about larval location, orientation and general activity. Three principal findings emerged from our studies. First, both experiments found significant behavioral differences in resting, strength of preferred quadrant and to a lesser extent in activity between 4 dpf and older larvae. Second, in both experiments, there were behavioral biases for the edge region and an outward facing orientation across all four ages tested. Third, there were differences between the two experiments in space use patterns of older larvae and in visual lateralization of 7 dpf larvae. Collectively, these findings reflect both maturational and experiential effects on larval zebrafish behavior.

Regarding maturational factors, our data suggest two ways in which the behavioral profile of 4 dpf larvae is clearly distinguishable from that of 5 to 7 dpf larvae. In both experiments, 4 dpf larvae rested significantly more than the 5, 6, and 7 dpf larvae, and they were located significantly more often in their highest use quadrant than older larvae. These results are consistent with other reports of high rates of resting in larvae prior to inflation of the swim bladder and the emergence of spontaneous swimming at 5 dpf (Granato et al., 1996; Thirumalai and Cline, 2008). The novel finding that this high rate of resting is associated with a reduction in overall spatial distribution has important implications both for the presentation of stimuli to larvae younger than 5 dpf and for the assessment of their effects in studies of learning, memory and cognition.

We also found evidence in line with previous reports that younger larvae are less active than older larvae. In both experiments, 4 dpf larvae moved less than older larvae although not all comparisons were statistically significant. Increasing the size of the testing arena might enhance detection of statistically significant age-related changes in activity. The small testing arenas used in our experiments may have limited the opportunity for exploratory behavior in the older larvae, especially when coupled with their extensive exposure and potential habituation to the wells in Experiment 1 where we found no statistically significant age-related effect in amount of movement. Development of the larvae in Experiment 1 may also have been affected by the size of the testing arena which in turn could have affected activity levels. The resolution of our imaging system was insufficient to provide precise measurement of larval length because of the transparent tail tip. Future research will need to address this issue. Alternatively, it is possible that features of our testing arenas artificially increased activity in the 4 dpf larvae as they tried to inflate their swim bladders. If the agarose ring was not sufficiently rigid to support climbing and the water in the wells was not deep enough for the larvae to hang naturally, the 4 dpf larvae may have made frequent lateral movements as they struggled to migrate vertically to the surface. Recent observations in our laboratory confirm that agarose is a more challenging surface for a newly hatched larva to navigate than the smooth plastic wall of a multiwell plate.

Complicating statistical comparisons further is the possibility that activity in 4 dpf larvae may be inherently more variable than at any other age. Larvae at 4 dpf are making the transition from an inactive swim bladder to a fully inflated one. The timing of this transition is not precisely synchronized but is complete by 5 dpf. Swimming is topographically distinct in the periods before and after inflation of the swim bladder as the transition is made from intact touch-evoked swimming responses but negligible spontaneous swimming to a free swimming, self-feeding larva with a functional beat and glide motor system and the ability to maneuver in all three dimensions (Brustein et al., 2003; Buss and Drapeau, 2001; Granato et al., 1996; Thirumalai and Cline, 2008). Despite identical egg collection and rearing protocols, there were fairly large behavioral differences between the 4 dpf larvae in our two experiments. For example, we had higher percentages of resting and lower rates of movement in Experiment 1 than in Experiment 2. Almost 20% of the 4 dpf larvae in Experiment 1 scored rest rates of 100% whereas this was true for only 5% of the 4 dpf larvae in Experiment 2. Among the factors known to affect speed of embryonic development are temperature and light (Laale, 1977). However, even when these external factors are controlled and egg fertilization is synchronous, there are still variations in time of hatching and larval development. In future studies, whenever feasible, it would be useful to screen 4 dpf zebrafish larvae after testing to stage inflation of the swim bladder. This can be done by immobilizing the larvae on their side in 2% methylcellulose or by anesthetizing the larvae in tricaine and examining them under a stereo microscope.

Two clear behavioral biases emerged in our studies that were shared by larvae of all four ages tested. One was a preference for an outward orientation and the other was a

consistently strong preference for the edge of the well; both were significantly above chance. The edge preference we observed confirms and extends a previous report of an edge effect in the swimming trajectories of 5 dpf but not of 3 dpf WT larvae (Thirumalai and Cline, 2008). Observations of wall seeking behavior (thigmotaxis) have also been reported for adult zebrafish (Anichtchik et al., 2004; Peitsaro et al., 2003). For example, Peitsaro et al. (2003) found that individually tested adult zebrafish tended to avoid the center of an unfamiliar circular tank (22 cm in diameter with water 8 cm deep) and to swim in a circular pattern by following the wall of the tank. For 4 dpf larvae, the observed behavioral biases may be related exclusively to their drive to reach the air-water boundary to inflate their swim bladder. On the other hand, we cannot discount the possibility that an explanation for thigmotactic behavior in adult zebrafish and larvae with fully inflated swim bladders may also apply to the 4 dpf larvae.

Three functional explanations that could account for thigmotactic behavior in older larvae and adult zebrafish are shelter seeking, foraging, and predator avoidance. Two results, however, suggest that wall seeking is unlikely to be either a shelter seeking strategy or a foraging strategy. One result is our novel finding that the edge effect was attenuated in larvae after 24 hours exposure to the test wells in Experiment 1. Specifically, we found that the mean percent observations in the center of the well showed a two-fold increase from approximately 10 to 20 per cent between 4 and 5 dpf in Experiment 1 but were consistently around 10 per cent at all ages tested in Experiment 2. The other result is Lockwood et al.'s (2004) finding that wall seeking was not observed in the presence of a group of 10 conspecifics. There is no obvious reason why shelter seeking or foraging would be attenuated by exposure to the test well or by the presence of other larvae. Instead, the effect of these manipulations favors a widely held view that thigmotactic behavior is indicative of anxiety and may function as a defensive strategy to avoid detection by predators. Familiar environments provide security and social groups afford protection against predation.

Thigmotactic behavior in rodents has generally been interpreted as evidence of anxiety because it is increased by anxiogenic drugs (dexamphetamine, pentylenetetrazole, yohimbine, idazoxan), reduced by anxiolytic drugs (buspirone, phenobarbital), and attenuated by familiarization to the novel testing arena or context (Choleris et al., 2001; Simon et al., 1994; Treit and Fundytus, 1989). Related effects have been observed in adult zebrafish. Peitsaro et al. (2003) found that adult zebrafish spent more time in the center of a tank after injection with α -fluoromethylhistidine, a chemical that lowers histamine levels in the brain. Reduced histamine production has been linked to an increase in anxiolytic behaviors in mammals (Frisch et al., 1998; Yanai et al., 1998). Peitsaro et al. (2003) also found that when control fish, treated with saline, were reintroduced to the tanks 3 days after their first exposure, they spent more time in the center of the tank than they did on the first day. They suggested that the adult fish recognized the environment and were less anxious because of reduced neophobia. Additional studies are needed to determine if that interpretation or one involving generalized state changes such as sensitization applies to our related finding that zebrafish larvae also spent more time in the center of a well with which they were familiar.

Prolonged exposure to the testing environment had one other effect aside from its influence on exploration of the center of the well and levels of overall activity. In Experiment 2, 7 dpf larvae unfamiliar with the wells displayed a significant preference for a clockwise orientation whereas 7 dpf larvae in Experiment 1 that had been exposed to the wells since 4 dpf did not show any such preference. A clockwise orientation of 7 dpf zebrafish larvae has previously been observed in a two-fish assay and was thought to reflect a right-eye preference in social interactions (Creton, 2009). However, the present finding of a similar clockwise orientation with just one fish per well suggests an alternative possibility that 7 dpf

larvae may simply have a left eye preference for evaluating the novel agarose edge, with or without another fish in the well. It will be of interest to evaluate the connection between visual lateralization and learning by examining the consequence of a left-eye preference for viewing the agarose ring on subsequent exploratory behavior in the well.

The results of our studies have three broad implications for zebrafish behavioral studies. First, based on our data, we strongly recommend using 6 or 7 dpf larvae for examining the effects of drug treatments and chemical mutagenesis on locomotor activity. In both experiments, between-subject variability was relatively minimal at these ages on all of our behavioral measures including activity. Second, our analysis suggests multiple ways to expand behavioral measures of space use that could be used to detect more subtle differences among pharmacological compounds in this simple testing situation. Third, our data suggest a relatively simple but powerful method and behavioral index for studying motivational and cognitive processes in individual larval zebrafish. Vertebrate studies of classical conditioning processes have used modulation of overall activity as an index of motivational states and cognitive expectancies (Bouton and Bolles, 1980; Grau and Rescorla, 1984; Mustaca et al., 1991). By supplementing activity measures with those of space use developed in the current studies, we have been able to develop medium- and high-throughput assays for measuring avoidance behavior (Pelkowski et al., in preparation) and recognition memory for contextual stimuli (Colwill et al., in preparation). Studies aimed at understanding the development of learning and memory in both wild-type and mutant zebrafish will be able to exploit the behavioral repertoire that we have established.

Acknowledgments

This research was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD, R01HD060647) and the National Institute of Environmental Health Sciences (NIEHS, R03ES017755). We thank Elena Carver and Ben Drapcho for assistance with data collection, all 2008-2010 members of our zebrafish lab for helpful discussions, and two anonymous reviewers for helpful suggestions. We presented these data at the March 2010 meeting of the Eastern Psychological Association in Brooklyn, NY.

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Figure 1.

Experiment 1. Behavioral measures of activity and space use in the same zebrafish larvae imaged every 6 sec for one hour a day at 4, 5, 6 and 7 dpf. A: mean distance moved in mm/min. B: mean percent observations of resting. C: mean percent observations facing away from the center of the well. D: mean percent observations located in the outer half of the well. E: mean percent observations in quadrant with highest use. Error bars show the standard error of the mean (SEM).

Figure 2.

Experiment 2. Behavioral measures of activity and space use in four different groups of zebrafish larvae imaged every 6 sec for one hour a day at 4, 5, 6 or 7 dpf. A: mean distance moved in mm/min. B: mean percent observations of resting. C: mean percent observations facing away from the center of the well. D: mean percent observations located in the outer half of the well. E: mean percent observations in quadrant with highest use. Error bars show the standard error of the mean (SEM).