High-Throughput Analysis of Behavior in Zebrafish Larvae: Effects of Feeding

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Abstract

Early brain development can be influenced by numerous genetic and environmental factors, with long-lasting effects on brain function and behavior. Identification of these factors is facilitated by high-throughput analyses of behavior in zebrafish larvae, which can be imaged in multiwell or multilane plates. However, the nutritional needs of zebrafish larvae during the behavioral experiments are not fully understood. Zebrafish larvae begin feeding between 4 and 5 days postfertilization (dpf), but can live solely on nutrients derived from the yolk until at least 7 dpf. To examine whether feeding affects behavior, we measured a broad range of behaviors with and without feeding at 5, 6, and 7 dpf. We found that feeding did not have a significant effect on behavior in 5-day-old larvae. In contrast, fed 6- and 7-day-old larvae displayed increased avoidance responses to visual stimuli, increased swim speeds, and decreased resting in comparison to unfed larvae. In addition, the fed 7-day-old larvae displayed a decrease in thigmotaxis and a decrease in the distance between larvae in the presence of visual stimuli. Thus, feeding affects a range of behaviors in 6- and 7-day-old larvae. We conclude that 5-day-old larvae are well-suited for high-throughput analyses of behavior, since effects of feeding can be avoided at this time. For high-throughput analyses of behavior in older larvae, standard feeding protocols need to be developed.

Introduction

DEVELOPMENTAL DISORDERS, such as attention deficit hyperactivity disorder, intellectual disability, cerebral palsy, autism, seizures, hearing loss, blindness, and learning disorders, affect ~15% of children between the ages of 3 and 17 years.^{1,2} The etiology of these developmental disorders is often poorly understood and may include multiple genetic and environmental factors. A better understanding of the underlying factors can be facilitated by analyses of behavior in animal model systems. Behavioral analyses are noninvasive and can reveal subtle defects in neural signaling that are easily missed by morphological analyses. In addition, behavioral analyses can be used to examine neural function in a wide variety of organisms, ranging from nematodes in a culture dish to large mammals in their natural environment.

The zebrafish is an emerging model system in the behavioral sciences.^{3–6} The signaling pathways that regulate brain development and function are highly conserved in vertebrate species. Adult zebrafish colonies are relatively easy to maintain and are cost efficient. A mixed adult female and male population in a tank on a 10-h dark/14-h light cycle will spawn year round and hundreds of synchronously developing embryos can be collected from the bottom of a tank on a daily

basis. The embryos develop predictable neural patterns, which are amenable to genetic manipulation and can be imaged in living embryos using state-of-the-art molecular tools. Moreover, the behavior of zebrafish larvae can be examined in multiwell plates, which provides unique opportunities for high-throughput applications.^{7–10} Zebrafish embryos develop rapidly and the larvae emerge from their chorions between 2 and 3 days postfertilization (dpf). At 5 dpf, the larvae have inflated swim bladders and actively hunt for food.^{11–15} A broad range of behaviors have been analyzed in zebrafish larvae, including scoot swimming; burst swimming; routine turns; J-, C-, and O-bend turns; optokinetic and optomotor responses; prey tracking; phototaxis; thigmotaxis; escape and avoidance behaviors; nonassociative learning; and visual recognition memory.^{6,16,17} Due to the rapid development of larvae, these behaviors can be studied within the first week after fertilization.

Various feeding protocols are used when studying larval behavior. Zebrafish larvae have a fully developed digestive tract at 4 dpf and can effectively hunt for food at 5 dpf when the larvae have an inflated swim bladder and are able to swim freely.¹⁸ Unfed larvae can live solely on nutrients derived from the yolk until at least 7 dpf and as long as 14 dpf, indicating that nutrients from the yolk contribute to larval

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survival long after the larvae start feeding. Only a few studies indicate that the larvae are fed before behavioral testing.^{19–21} Withholding external food from larvae helps to prevent contamination of the culture medium and variability caused by differences in food intake. However, it is not known whether larval behavior is affected by feeding.

In the present study, we examined zebrafish larvae at 5, 6, and 7 dpf with and without feeding, using a custom-developed imaging system for automated analysis of behavior.^{8,9} The larvae were first imaged on a white background and were then imaged in the presence of visual stimuli. This experimental paradigm allows for an analysis of a broad range of behaviors, including spontaneous behaviors in a neutral environment and various behaviors that are only displayed in the presence of aversive visual stimuli. We found that feeding did not have a significant effect on behavior at 5 dpf, but did affect behavior at 6 and 7 dpf. Based on these results, we conclude that 5-day-old larvae are well-suited for high-throughput analyses of behavior, since effects of feeding can be avoided at this time.

Materials and Methods

Zebrafish larvae and feeding

Adult wild-type zebrafish were originally obtained from Carolina Biological and are maintained at Brown University as a genetically diverse outbred strain. The fish are kept in a mixed male and female population on a 14-h light/10-h dark cycle. Embryos were collected from the tanks between 10:30 and 11:30 AM and were raised at 28.5°C in egg water, containing 60 mg/L sea salt (Instant Ocean) in deionized water and 0.25 mg/L methylene blue as a fungal inhibitor. Embryos were grown at an approximate density of 200 embryos per liter in Aquatic Habitats 2-L breeder tanks. Unfertilized eggs were removed from the breeder tanks and 50% of the egg water was changed daily, at ~10 AM. Starting at 5 dpf, larvae in the “fed” group were fed daily, immediately after the water change, with 0.04 g of Zeigler AP100 LD50 larval diet (Pentair Aquatic Eco-systems) per 2-L tank. Thus, fed 7-day-old larvae had continuous access to food from 5 to 7 dpf.

The zebrafish imaging system

A high-throughput imaging system for automated analysis of behavior in zebrafish larvae was developed in our laboratory.⁸ Briefly, the system was built in a tall cabinet; the top shelf of the cabinet holds a 15-megapixel Canon EOS rebel T1i digital camera and the bottom shelf holds an Acer Aspire 5517 laptop with a 15.6-inch screen to provide visual stimuli to the larvae. The camera is controlled with Canon’s Remote Capture software and is connected to a second laptop computer to run the software and store the acquired images.

Imaging zebrafish larvae

Zebrafish larvae were imaged at 5, 6, and 7 dpf, 3 h after feeding. The larvae were imaged in five-lane plates, with lanes that are 70-mm long × 18-mm wide and have 60° sloping edges to reach a 66 × 14-mm² bottom at a 3.5-mm depth.⁹ The multilane plates were made using a one-well plate (Cat. No. 267060; Thermo Fisher Scientific), 50 mL of agarose (0.8% agarose in deionized water with 60 mg/L; Instant Ocean), and a custom-designed five-lane mold, as de-

scribed previously.⁹ Twenty minutes prior to the start of the experiment, the lanes were filled with egg water and five larvae were transferred to each lane. Four plates (two plates with fed larvae and two plates with unfed larvae) were placed on the screen of the laptop computer in the imaging cabinet. A plastic diffuser (Pendaflex 52345) was placed between the five-lane plates and the laptop screen in order to avoid moiré patterns on the images. Canon’s software has a “Remote Shooting” feature for computer-controlled image acquisition in interval mode. The camera was programmed to collect high-resolution images every 6 s for a half hour for a total of 300 images per experiment.

Visual stimuli

Visual stimuli are shown to the larvae as PowerPoint presentations on the laptop’s LCD screen. The presentation used in this study starts with a blank white background for 15 min, followed by 15 min of a moving red bar (Fig. 1). The red bar is 1.3-cm wide and moves up and down at a speed of 2 cm/s in the upper half of the lanes. Our laboratory has previously shown that zebrafish larvae display robust avoidance responses when exposed to these visual stimuli.⁹ The PowerPoint file is included in the Supplementary Data (Supplement S1; Supplementary Data are available online at www.liebertpub.com/zeb).

Automated image analysis in ImageJ

The acquired images were analyzed in ImageJ, which can be downloaded free of charge from the National Institutes of Health (<http://rsb.info.nih.gov/ij/>). Our laboratory developed an ImageJ macro that automatically measures the location and orientation of the zebrafish larvae in a large number of images, which can go beyond the available RAM of the image analysis computer.^{8,9} This macro, called *zebrafish_macro25k*, can be downloaded from the Supplementary Data (Supplement S2). The macro automatically removes the visual stimuli by splitting the color channels, subtracts the background, applies a threshold, detects the larvae by particle analysis, and repeats this process for subsequent images in a folder. We recently added a display of the larval centroid and center of the “bounding box,” the tightest box that can be drawn around a larva. In addition, we added a display for all possible distances between larvae (10 distances in a group of 5 larvae). The macro creates a “Results” file containing the image name; larval area in pixels; mean intensity; X, Y

FIG. 1. Imaging of zebrafish larvae in five-lane plates. (A) Larvae are first imaged for 15 min without visual stimuli. (B) Larvae are then exposed to a red bar that moves up and down in the upper half of the lanes. The images show 5-day-old larvae that were fed 3 h prior to imaging. Note that the larvae avoid the visual stimulus. Scale bar=1 cm. Color images available online at www.liebertpub.com/zeb

coordinates of the centroid; X, Y coordinates of the center of mass; X, Y, width, and height of the bounding box (the tightest box containing a larva); lane number; and X, Y coordinates of the midpoint of the lane. The “Results” file is opened in MS Excel for further analysis.

Data analysis in MS Excel

The coordinates of the “Results” file are copied in an MS Excel template, which calculates the location, orientation, and swim speed of the larvae, as described previously.^{22,23} The location of the larvae along the main axis of the lane is calculated by comparing the Y coordinates of the larval centroids to the Y coordinates of the lane’s midpoint. If the Y coordinate of a larva is smaller than the Y coordinate of the midpoint of a lane, then the larva is located in the upper half of the lane (“up”). If not, then the larva is located in the lower half of the lane (“down”) or away from the visual stimuli when such stimuli are presented. It should be noted that “up” or “down” is a measure in the horizontal plane. A larva is considered to be on the “edge” if the larval centroid is in the outer 3 mm of the 70×18-mm² swimming area. If not, then the larva is considered to be located in the “center” of the swimming area. The swim speed is calculated by comparing the XY coordinates of larval centroids to the XY coordinates of the larval centroids in the next image. Larvae that move < 1 mm in a 6-s interval are considered at rest. The “social distance” or average of all possible distances between larvae was measured by comparing the centroids of larvae that are together in a lane. Diagonal distances were calculated using the equation $a^2 + b^2 = c^2$. The “COUNTIFS” function in Excel was used to determine how often the larvae were located down in the well, were located on the edge of the well, or were at rest. Behavioral parameters of a single lane were averaged for the first 15 min without visual stimuli and the second 15 min with visual stimuli. The bar graphs display the average and standard deviation of 20 lanes per experimental group.

Statistical analyses

Statistical analyses were carried out using SPSS software. To assure that the measurements are independent, the data were analyzed on a per-lane basis (n = number of lanes). The effects of feeding (fed and unfed) and age (5, 6, and 7 dpf) on various behaviors were analyzed using a two-way analysis of variance (ANOVA). If the ANOVA showed a significant effect ($p < 0.05$), then specific groups were compared by *post hoc* analyses using a two-tailed *t*-test (fed vs. unfed) or Tukey HSD test with a correction for multiple comparisons (5, 6, and 7 dpf).

Results

Zebrafish larval behavior in multilane plates

The behavior of zebrafish larvae was measured in multilane plates. The multilane plates were designed to give the larvae ample space to avoid aversive stimuli and interact with other larvae, which allows for the automated analysis of a broad spectrum of complex behaviors.⁹ We found that the larvae display a clear avoidance response to a red moving bar (Fig. 1). During the first 15 min of the recording, the larvae swim on a white background and move freely throughout the

lane (Fig. 1A). The larvae are then exposed for 15 min to a moving red bar, which moves continuously up and down in the upper half of the lanes. The larvae move into the lower half of the lanes to avoid the stimulus (Fig. 1B). All experimental groups, including 5-, 6-, and 7-dpf larvae fed and unfed, displayed clear avoidance behaviors in response to visual stimuli. For example, the fed 5-dpf larvae shown in Figure 1 were located down in the lane 53% of the time without visual stimuli versus 75% of the time with visual stimuli (Fig. 2A). This avoidance response is significant ($p < 0.0001$, $n = 20$ lanes, two-tailed *t*-test). The substantial avoidance response and level of significance indicate that the multilane assay is a robust assay for measuring avoidance behaviors. Other behaviors were similar to the behaviors measured previously in multiwell plates.⁸ For example, the fed 5-dpf larvae shown in Figure 1 spent 60% of their time on the edge of the swimming area, displayed an average swim speed of 29 mm/min and rested 50% of the time ($n = 20$ lanes). The developed algorithms for calculating “social distance,” the average of all possible distances between the larvae, revealed that the larvae display a social distance ranging from 23 mm (7 dpf fed with visual stimuli) to 32 mm (6 dpf unfed without visual stimuli) in the 70×18 mm² lane. The effects of age and feeding were analyzed using a two-way ANOVA. This analysis revealed that none of the measured behaviors is significantly affected by age, between 5 and 7 dpf ($p > 0.05$). We then further examined the effects of age within their respective feeding groups by using a one-way ANOVA. This revealed that there were no behaviors significantly affected by age, between 5 and 7 dpf, except the resting behavior without bar when comparing 5- to 7-dpf larvae in the not-fed group ($p = 0.032$). There is a significant interaction between age and feeding for the swim speed without visual stimuli and resting with or without visual stimuli ($p < 0.05$). In addition, all behaviors, except social distance without visual stimuli, are significantly affected by feeding ($p < 0.05$).

Effects of feeding on avoidance behavior

At 5 dpf, fed and unfed larvae display similar avoidance responses. In contrast, feeding induced a significant increase in the avoidance response of 6- and 7-dpf larvae (Fig. 2A). The 6-dpf larvae, exposed to visual stimuli, were 68% down in the lane without feeding versus 82% down in the lane with feeding. This difference between the fed and unfed groups is significant ($p < 0.05$, *t*-test, $n = 20$ lanes). The 7-dpf larvae, exposed to visual stimuli, were 68% down in the lane without feeding versus 85% down in the lane with feeding. This difference between the fed and unfed groups is significant ($p < 0.01$, *t*-test, $n = 20$ lanes). Based on these findings, we conclude that feeding affects avoidance behaviors in 6- and 7-dpf zebrafish larvae.

Effects of feeding on thigmotaxis

Zebrafish larvae are known to display a preference for the edge of a circular well when imaged in a multiwell plate.^{24,25} This edge preference or thigmotaxis was also observed in the current experiments with rectangular lanes. For example, Figure 2B shows that the 5-dpf-fed larvae spent 60% ($n = 20$ lanes) of their time in the outer 3 mm of the swimming area. This outer area corresponds to 39% of the total swimming

FIG. 2. The behavior of 6- and 7-day-old zebrafish larvae is affected by feeding. **(A)** Avoidance behavior: the percentage of time that larvae are down in the lane, away from the visual stimulus. **(B)** Thigmotaxis: the percentage of time that the larvae are located in the outer 3 mm of the swimming area. **(C)** Swim speed: the average swim speed in mm/min. **(D)** Percent rest: the percentage of time that larvae move < 1 mm in a 6-s interval. White = measurements from the “not-fed” group, Gray = measurements from the “fed” group. * $p < 0.05$, ** $p < 0.01$ (t -test, fed vs. not fed, $n = 20$ lanes). Error bars indicate the standard deviation (SD).

area or only 29% of the total volume of water, since the lanes have sloping edges. Thus, the larvae display a clear preference for the outer edge compared with the center of the swimming area. Similar percentages were observed with and without visual stimuli. The 7-dpf larvae without visual stimuli were 69% on the edge without feeding versus 58% on the edge with feeding. This difference between the fed and unfed groups is significant ($p < 0.01$, t -test, $n = 20$ lanes). The 7-dpf larvae with visual stimuli were 73% on the edge without feeding versus 65% on the edge with feeding. Again, this difference between the fed and unfed groups is significant ($p < 0.05$, t -test, $n = 20$ lanes). Based on these results we conclude that feeding affects thigmotaxis at 7 dpf.

Effects of feeding on swim speed

The swim speeds of fed and unfed larvae were analyzed at 5, 6, and 7 dpf with and without visual stimuli (Fig. 2C). At 5 dpf, the fed and unfed larvae did not display significant differences in swim speed. In contrast, fed larvae swam significantly faster than the unfed larvae at 6 dpf, both without visual stimuli ($p < 0.01$, t -test) and with visual stimuli ($p < 0.05$). Similarly, fed larvae swam significantly faster than the unfed larvae at 7 dpf, both without visual stimuli

($p < 1 \times 10^{-8}$, t -test) and with visual stimuli ($p < 1 \times 10^{-5}$, t -test). Based on these results, we conclude that feeding leads to an increase in larval swim speeds at 6 and 7 dpf.

Effects of feeding on resting behavior

Resting behavior of fed and unfed larvae was analyzed at 5, 6, and 7 dpf with and without visual stimuli (Fig. 2D). A larva was considered to be “resting” if the larva moved < 1 mm in a 6-s interval. At 5 dpf, the fed and unfed larvae did not display significant differences in resting. In contrast, fed larvae rested less than unfed larvae at 6 dpf. This effect of feeding was observed without visual stimuli ($p < 0.01$, t -test) and with visual stimuli ($p < 0.05$). Similarly, fed larvae rested less than unfed larvae at 7 dpf. Again, this effect of feeding was observed without visual stimuli ($p < 1 \times 10^{-6}$, t -test) and with visual stimuli ($p < 0.0001$, t -test). Based on these results, we conclude that fed larvae rest less than unfed larvae at 6 and 7 dpf.

Effects of feeding on social distance

The social distance of fed and unfed larvae was analyzed at 5, 6, and 7 dpf with and without visual stimuli (Fig. 3). At 5

FIG. 3. The fish-to-fish distance for 7-day-old zebrafish larvae is affected by feeding. (A) Updated algorithms in our ImageJ macro automatically measure the larval centroid (red dot) and the center of the bounding box (yellow dot) and plot the distance between larvae in all possible combinations (blue lines). (B) Feeding decreases the larval distance at 7 days postfertilization in the presence of visual stimuli ($*p < 0.05$, t -test, fed vs. not fed). White = measurements from the “not-fed” group, red = measurements from the “fed” group. $*p < 0.05$, (t -test, fed vs. not fed, $n = 20$ lanes). Error bars indicate the SD. Color images available online at www.liebertpub.com/zeb

and 6 dpf, feeding did not induce significant differences in social distance. In contrast, feeding did induce a significant decrease in social distance in 7-dpf larvae exposed to visual stimuli. Thus, the fed larvae swim closer together than the unfed larvae ($p < 0.05$, t -test). Based on these results, we conclude that feeding can affect the average distance between larvae at 7 dpf.

Discussion

The present study examined zebrafish larvae at 5, 6, and 7 dpf with and without feeding, using a custom-developed imaging system for automated analyses of behavior. We found that feeding did not have a significant effect on behavior in 5-day-old larvae. In contrast, fed 6- and 7-day-old larvae displayed increased avoidance responses to visual stimuli, increased swim speeds, and decreased resting in comparison to unfed larvae. In addition, the fed 7-day-old larvae displayed a decrease in thigmotaxis and a decrease in the distance between larvae in the presence of visual stimuli.

The observed changes in behavior may be explained, in part, by an increase in available energy when additional nutrients are provided by feeding. This increased energy level corresponds well with the increased swim speeds and reduced resting times observed in fed larvae, as compared with unfed larvae. In addition, avoidance responses may depend on available energy, since larvae need to swim down the lane to avoid the aversive visual stimuli. While the feeding-induced changes in swim speed, resting, and avoidance behavior may be explained by a general increase in available energy, these behaviors are not automatically linked. For example, unfed larvae display a substantial decrease in swim speed between 6 and 7 dpf, without a corresponding change in the avoidance response. Possibly, the unfed 7-dpf larvae swim as little as needed to save their energy for avoidance responses, which may be an effective adaptation for predator avoidance in nature. Feeding may also change behaviors that are unrelated to activity. For example, thigmotaxis, a preference for the edge of the swimming area, was affected at 7 dpf with or without visual stimuli. Thigmotaxis is used as a measure of

anxiety in various organisms, including zebrafish larvae.^{24,25} Thus, the feeding-induced decrease in thigmotaxis could reflect a reduced level of anxiety in well-fed larvae. Finally, the feeding-induced decrease in social distance at 7 dpf may be a direct result of the increased avoidance response to visual stimuli. When larvae display a strong avoidance response to visual stimuli in the upper half of the lane, they would be expected to end up close together in the lower half of the lane. In future experiments, it may be interesting to look in more detail at social behaviors during the first 15 min, before visual stimuli are presented to the larvae.

Zebrafish larvae are used in various high-throughput assays that screen for behavioral defects.^{7,10,26} These assays can provide a wealth of information on the genes, pharmaceuticals, and environmental toxicants that affect brain development and function. There is no consensus on feeding in such behavioral assays. In studies that do not pursue behavior, larvae are typically fed at 5 dpf and beyond to ensure that energy needs are met during development.^{27–29} Food intake and energy expenditure can be measured quantitatively^{30,31} and effective feeding protocols have been developed to improve larval survival.^{32,33} However, feeding protocols have not been standardized in behavioral studies. There have been several different feeding protocols used in previous studies that examined behavior of 5–7-dpf zebrafish larvae. These protocols include feeding with paramecia,¹⁹ feeding with a diet of TetraMin baby fish food and *Artemia*,^{20,21} or without feeding,^{8,10,24,34} demonstrating the wide variety of feeding protocols used during behavioral experiments. Our study is the first to directly examine the effect of feeding on larval behavior. Feeding can be problematic in behavioral assays, especially when raising larvae in multiwell plates. First, left-over food can quickly lead to bacterial or fungal contamination of the water. Second, feeding protocols need to be fast and consistent to avoid variability in food intake. Third, feeding will add another layer of complexity in the interpretation of the results; that is, behavioral defects may be a direct result of changes in neural function or may be caused indirectly by changes in food intake. A possible solution is to examine behavior in 5-day-old zebrafish larvae without

feeding. The 5-day-old larvae have a large yolk sac filled with nutrients and the results of the present study show that 5-day-old larvae do not display significantly different behaviors with or without feeding. Thus, the practical issues of feeding in a high-throughput setting can be avoided at this time. However, some behaviors can only be studied later in development. For example, shoaling behaviors develop as zebrafish age.³⁵ When studying such behaviors in a high-throughput setting, it will be important to develop protocols for consistent feeding while avoiding a detrimental decrease in water quality. Ultimately, the development of methodologies for automated analyses of behavior will provide the high-throughput tools that are needed to better understand the genetic and environmental factors that cause developmental brain disorders.

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Disclosure Statement

No competing financial interests exist.

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