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Short communication

Shuttle box learning in zebrafish (*Danio rerio*)

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ABSTRACT

Zebrafish is used in forward genetic and drug screening and is gaining popularity in behavioral brain research but high throughput learning paradigms are lacking. The sight of conspecifics has been shown to be rewarding in zebrafish. Here, in a novel paradigm, subjects learn to respond to alternating presentation of computer-animated zebrafish images. The simplicity and computerization of the paradigm will make it useful for high throughput screening.

Zebrafish has been an important laboratory study species in developmental biology and over the past three decades a large amount of genetic information has been accumulated and numerous genetic tools have been developed for this fish [8]. Zebrafish is also gaining popularity in behavioral brain research partly due to its strong genetics but also to some practical advantages of this species [18]. Zebrafish is easy to maintain and produces a large number of offspring (2–500 eggs per spawning per female). It is small (4 cm long when adult) and can be kept in large numbers in small tanks due to its shoaling (social) nature. The latter characteristics have made this species particularly appropriate for forward genetic studies in which mutations are randomly induced and the resulting thousands of mutants are phenotypically screened [2,8 and references therein]. The ability to screen thousands of fish cost effectively offers the chance to identify specific genes (or molecular targets [2]) involved in the phenotype of interest and drugs with particular effect profiles [21]. Massive screens have been performed most successfully for developmental alterations (Tubingen and Boston screens) but more recently such complex target phenotypes as cocaine induced place preference have also been successfully screened [5]. It is expected that other complex behaviors, including learning and memory may also be utilized as screening criteria [1,9].

Although learning and memory have been much better characterized in the classical laboratory rodent species, some learning tasks have already been employed in zebrafish too. For example, active avoidance learning [22], appetitive reinforcement-based learning in a spatial alternation task [20], and visual discrimination learning [4] have been demonstrated in zebrafish. Zebrafish has been found to acquire social preference during early development, an imprinting-like phenomenon [7]. Nicotine has been shown to significantly affect learning in zebrafish in a manner also found in mammalian species [11]. L1, a cell adhesion molecule, has been shown to play crucial roles in memory consolidation in zebrafish, similarly to its known roles in mammals [15,16]. Reinforcing properties of drugs of abuse have been demonstrated [14] and the genetic aspects of rewarding properties of food and opiates have been investigated in this species [10]. Automated computerized analysis of learning performance has also been described for zebrafish [9].

Zebrafish is a highly social species and is expected to prefer staying close to its conspecifics both in nature and the laboratory [6]. When an individual zebrafish is alone in a tank, upon presentation of a group of conspecifics it is expected to approach and stay in the proximity of the group [17]. Recently an associative learning task has been developed in our laboratory that was based on this observation [1]. The sight of conspecifics turned out to have reinforcing properties and was able to support the acquisition of an associative learning task [1]. We have also shown that live zebrafish can be substituted with animated (moving) images of zebrafish [17], i.e., experimental zebrafish will show strong preference toward animated images possessing certain species-typical

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characteristics, while images with atypical or abnormal features are either ignored or avoided. These results are noteworthy. They imply that a rewarding stimulus, images of zebrafish, can be precisely presented in a particular location and at a particular time (computer controlled animation) in a learning task for zebrafish. However, the temporal responses of experimental zebrafish to the appearance or disappearance of computer-animated zebrafish images have not been characterized and the reinforcing properties of computer-animated zebrafish images have not been experimentally demonstrated. In the current paper we present animated images of a group of six zebrafish to the experimental subject and investigate whether these images attract the experimental fish and whether they can support acquisition of a learning task.

The ease with which this stimulus can be delivered in a spatially and temporally controlled manner allowed us to first quantify the reactions of experimental zebrafish, and second to develop a simple shuttle box learning paradigm. In this latter paradigm a group of conspecific images are first shown on one side of the experimental tank and after a no-stimulus break period (Inter-Stimulus Interval, or ISI) the images appear on the other side, an alternating sequence that repeats itself 30 times. Zebrafish are expected to learn the alternating presentation sequence and during the Inter-Stimulus Interval should move away from the side where the stimulus was just presented and cross over to the side where the stimulus is going to appear. Below we describe the methodological details of this task and present evidence demonstrating significant acquisition of this task in zebrafish.

Thirty-four and 53 zebrafish (approximately 50/50 male/female) of the AB strain were used in the two parts of this study, the initial characterization of group preference induced by animated images of zebrafish and the subsequent learning experiment. All experimental fish were bred and maintained in our vivarium (University of Toronto at Mississauga, Ontario, Canada). The parents of the fish were obtained from the ZFIN Center (Eugene, OR, USA). At the time of behavioral testing the fish were 3.5 cm long and 4 months old. All subjects were housed in 2.8 L Plexi-glass tanks (10 fish per tank) on a recirculating system (Aquaneering Inc., San Diego, CA, USA) with multi-stage filtration including a mechanical filter, a fluidized glass bed biological filter, activated carbon filter, and a UV light sterilizing unit. The fish remained in their home tank until behavioral recording, i.e. they were not isolated before the experiment. Each day, 10% of the water was replaced with fresh system water (de-ionized oxygenated water supplemented with 60 mg/L Instant Ocean Sea Salt, Big Al's Pet Store, Mississauga, Ontario, Canada). The water temperature was controlled by a thermostat and was kept at 27 °C. The light cycle was also controlled with fluorescent lights on the ceiling turned on at 07:00 h and off at 19:00 h. Fish were fed three times a day (at 9:00, 13:00 and 17:00 h) with a mixture (50–50%) of ground freeze-dried krill and flake food (Tetramin Tropical Flakes, Tetra, USA).

The experimental fish were transported from a holding tank to the testing tank with a net that was submerged in a beaker containing system water. Each experimental fish was tested once individually in a 38 L experimental tank (50 cm × 25 cm × 30 cm, width × depth × height) which was illuminated from above by a 50 cm long Aquarium Spectrum fluorescent lamp (15 W). The tests were conducted between 10:00 and 16:00 h. A digital hard disk video-camera (JVC Everio GZ-MG37U) was placed in front of the tank to record the subjects' behavior. The recordings were later replayed and analyzed. A 2.5 cm thin, grey, corrugated, plastic frame was mounted onto the side and the bottom glass inside of the experimental tank at the center so that fish could not swim from one side of the tank to the other while close to the glass (Fig. 1). Two

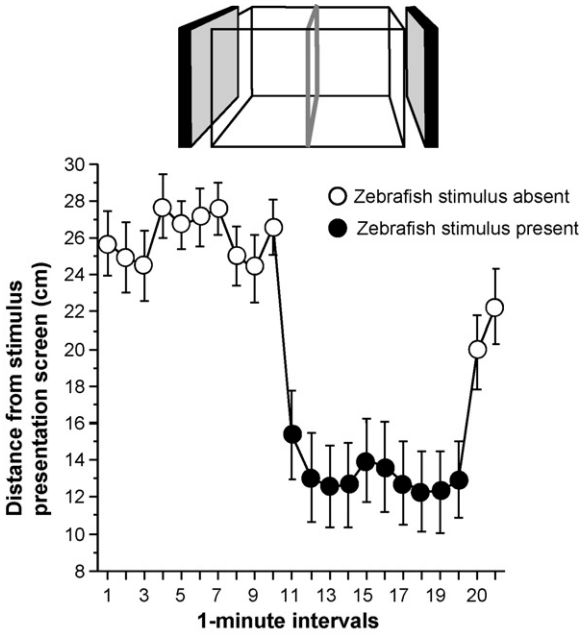

Fig. 1. Presentation of a set of animated images of zebrafish elicits a significant approach in experimental zebrafish. Distance (cm) from the stimulus presentation screen is shown for 1 min intervals of a 22 min session. Open circles represent the behavioral response during blank screen (black) and the black filled circles the behavioral responses during the period of stimulus presentation (six moving zebrafish images). Mean ± S.E.M. are shown. Sample size (n) is 34. The experimental tank with a stimulus presentation screen on each side and a center frame divider is also shown.

monitors (Samsung Syncmaster 732N) covered the two opposite sides of the test tank from the outside (Fig. 1). Each monitor was connected to a laptop computer (Dell Vostro 1000) that ran a custom software application [17], which allowed the presentation of computer-animated stimulus fish images. Zebrafish show evidence of shoaling with computer-animated conspecifics [17]. The stimulus fish were a group of six individually "swimming" zebrafish. The images were the picture of wild type zebrafish and their size was similar to that of the experimental zebrafish. They were shown on a black background. All images were females because this is expected to elicit the most consistent preference in male as well as female experimental zebrafish [19]. The animated fish images moved in random directions with speeds varying between 1.5 and 4 cm/s, mimicking a natural zebrafish shoal.

In the first experiment (analysis of the effect of animated stimulus presentation), the experimental subjects were shown a blank (black) screen for 10 min. This was followed by the presentation of the images of six moving zebrafish for 10 min and a subsequent 2 min period during which the black screen was shown again. The images were presented only on one side of the tank but which side it was changed randomly across different experimental zebrafish. The recordings were later replayed and analyzed using the video-tracking system Ethovision Color Pro (Noldus Info Tech., Wageningen, The Netherlands) as described elsewhere [3]. Briefly, the distance from the stimulus presentation screen was quantified for each 1-min interval of the 22 min recording session. The results (Fig. 1) show that upon presentation of the animated zebrafish images, the experimental zebrafish moved closer to the image presentation screen (decreased distance) a response that disappeared upon removal of the images (the last two intervals) (repeated measures ANOVA, interval $F(21, 693) = 14.083$, $p < 0.0001$). Briefly, experimental zebrafish showed a strong preference for staying in close proximity to the animated images.

In the second experiment (the learning study), all experimental fish were shown the stimulus fish for 20 s (Stimulus Presentation Interval) using the same set up as in the first study (Fig. 1). The stimulus presentation was followed by a 90 s long interval during which no stimulus fish were presented, the Inter-Stimulus Interval or ISI. The Stimulus Presentation and Inter-Stimulus Intervals repeated for a total of 30 times. Experimental zebrafish were divided randomly into three experimental groups. In the "Alternating side" group ($n = 18$) zebrafish received the stimulus fish on consistently alternating sides (left vs. right computer screen). In the "Same side" group ($n = 20$), the experimental fish were shown the stimulus fish always on the same side. That is, some experimental fish in this group received the stimulus on the left throughout the 30 Stimulus Presentation periods while others always on the right. In the "Random side" group ($n = 15$), the side of presentation of the stimulus fish followed a random sequence. In both experiments, the experimenter left the room during testing and the order of experimental fish was randomized according to group designation.

Digital video recordings were stored in AVI format and were transferred to an external hard-drive (500 GB LaCie hard drive by F.A. Porsche) connected to a Desktop computer (Dell Dimension 8400). The recordings were replayed and analyzed for experiment 2 using the Observer event recording software application (Version 3, Noldus Info Tech., Wageningen, The Netherlands). We decided to use this manual, human observation-based, method here because of the pilot nature of the learning study: we wanted to be able to observe potentially unexpected behavioral responses (motor and posture patterns) that would have remained undetected when using an automated video-tracking method. However, no unexpected or aberrant behaviors were seen. The following behaviors are reported and quantified in this paper: Location (stimulus presentation side vs. opposite side), Swimming (locomotion with the movement of caudal fin), and Thrashing (swimming against the glass wall). The duration of time relative to interval length was calculated for these measures and was analyzed using the statistical software application SPSS (version 14.1) for the PC. One-way repeated measures analyses of variance (ANOVAs) were employed to examine the effect of experimental condition (between subject factor with three levels: alternating-side, same-side, random-side) and the effect of interval (the repeated measure factor).

We expected zebrafish under the alternating side experimental condition to learn to move away from the stimulus presentation side as soon as the stimulus fish disappeared on the screen and to spend increasing amount of time on the opposite side during the interval when no stimulus fish were shown on either side (ISI). Fish in the "Same side" group on the other hand were expected to continue to stay close to the side where the stimulus fish were presented in the ISI, whereas fish in the "Random side" group were expected not to change their behavior with training.

Fig. 2 shows the performance of the experimental fish during the Inter-Stimulus Intervals (ISI) quantified as percent of time the fish spent on the side of the experimental tank where the stimulus fish used to be shown just before the ISI. The data shown represent the averages of three successive ISI (i.e. the data for the 30 ISI's are shown as 10 data points, each point representing the average of 3 ISI's). Gender had no significant effect or interaction with other factors and the data are pooled for the sexes. Fig. 2 suggests that fish in the Alternating side group reduced the time they spent near the computer screen that used to present the stimulus fish, whereas the fish in the Same side group increased it. ANOVAs partially confirmed this observation and showed a non-significant Interval effect ($F(9, 441) = 0.513$, $p > 0.85$), a non-significant Experimental condition effect ($F(2, 49) = 1.396$, $p = 0.257$) but a significant Interval × Experimental condition interaction ($F(18, 441) = 1.698$, $p < 0.05$). The latter suggested that the change of performance across intervals depended upon the experimental condition, therefore we examined the Interval effect separately for each experimental group. ANOVA revealed a significant Interval effect for the Alternating side group ($F(9, 153) = 2.561$, $p < 0.05$), confirming that these fish indeed reduced the time spent on the side of the tank where the stimulus fish used to be shown as training proceeded. However, the apparent trend seen in the Same side group was found non-significant (ANOVA, Interval $F(9, 162) = 1.087$, $p > 0.05$). The Interval effect for the Random side group was found, as expected, to be also non-significant (ANOVA $F(9, 126) = 1.124$, $p > 0.05$).

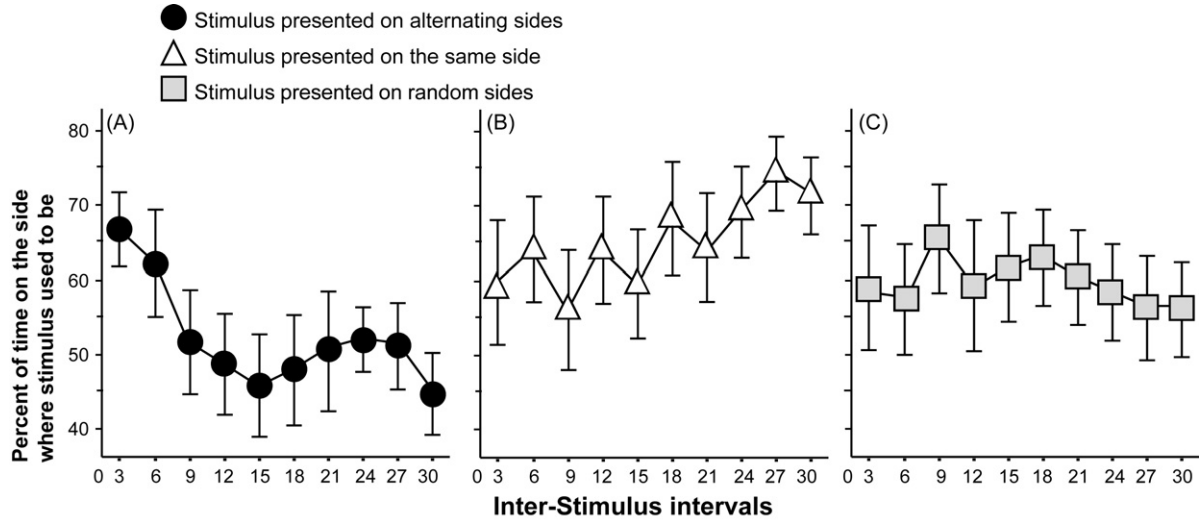

Fig. 2. Performance during the Inter-Stimulus Intervals (ISI): percent of time experimental fish spend on the side of the experimental tank where the stimulus fish images used to be shown. Graphs show the performance of experimental fish averaged over three successive ISI's (for a total of 30 ISI's). (A) Stimulus fish presented in a systematically alternating manner "Alternating side"; (B) stimulus fish presented always on the same side, "Same side"; (C) stimulus fish presented sometimes on the same and other times on the opposite sides in a randomly varying sequence, "Random side". Mean ± standard error of the mean are shown. Sample sizes (n) are specified in the text. Note that during the ISI (90 s each) no stimulus fish are shown on either side. Note the results suggest that experimental zebrafish under the alternating side condition learned to reduce the time spent on the side of the tank where the stimulus was shown before and increased the time spent on the opposite side where the stimulus was going to appear.

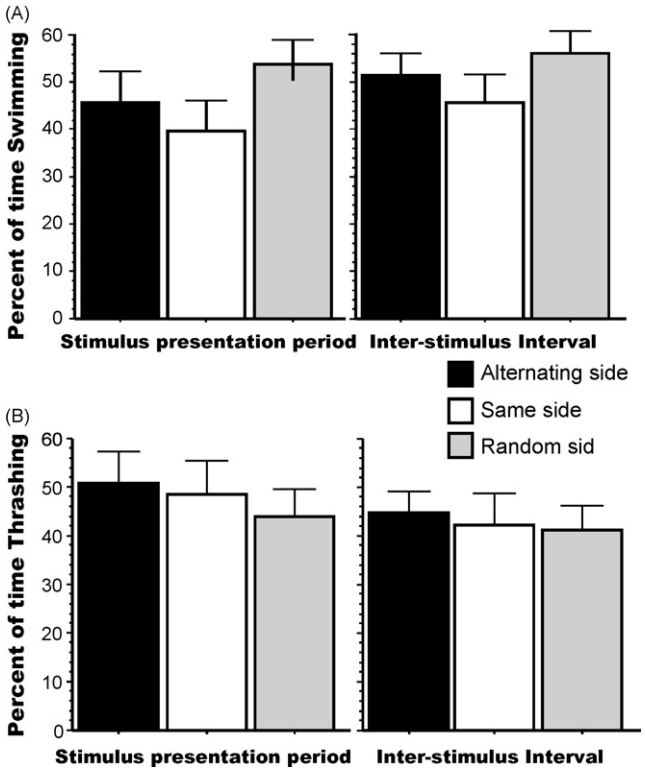

Fig. 3. Motor performance of experimental zebrafish during the Stimulus presentation period (left side of the graphs) and during the Inter-Stimulus Interval (right side of graphs) Panel A (upper two graphs): swimming. Panel B (lower two graphs): thrashing. Mean ± standard error of the mean are shown. Sample sizes are indicated in the text. Note that statistical analysis demonstrated a modest but significant increase upon disappearance of the stimulus fish (i.e. during the ISI) in swimming and a modest but significant decrease in thrashing, but the experimental condition had no significant effect in either behavior, i.e. all fish showed similar level of activity during stimulus presentation and changed this activity similarly in response to the removal of the stimulus fish. Also note that no abnormal or unexpected motor or posture patterns were observed and fish were generally active most of the time.

It is possible that the above findings are due to altered motor performance, and even perhaps differential fear responses leading to immobility. Fish may be freezing or may increase or decrease their activity levels, which in principle, could translate into differential learning performance. However, our observations revealed no modifications in motor and posture patterns. Fish appeared to be moving normally. Also, analysis of the motor patterns swimming and thrashing did not confirm the above speculations. For this analysis we calculated the average of the relative duration of time fish spent with the corresponding behavior for the 30 Stimulus Presentation periods and also for the 30 Inter-Stimulus Intervals. Fig. 3 shows that fish in all experimental groups were active and essentially spent all their time either swimming or thrashing. ANOVA revealed a significant Interval (Stimulus Presentation period vs. Inter-Stimulus Interval) effect for both swimming ($F(1, 50) = 5.641$, $p < 0.05$) and thrashing ($F(1, 50) = 5.713$, $p < 0.05$) indicating a modest but significant increase in swimming and a modest but significant decrease in thrashing during the Inter-Stimulus Interval as compared to the values during the Stimulus Presentation period. ANOVA demonstrated no significant Experimental condition effect (swimming $F(2, 50) = 1.203$, $p > 0.30$; thrashing $F(2, 50) = 0.196$, $p > 0.80$) or Interval × Experimental condition Interaction (swimming $F(2, 50) = 0.293$, $p > 0.70$; thrashing $F(2, 50) = 0.305$, $p > 0.70$). These results, together with the fact that swimming and thrashing make up approximately 95% of the time suggest that all fish were indeed active and experimental conditions did not alter their motor performance. The modest but significant changes in the activity patterns from the Stimulus Presentation Period to the Inter-Stimulus Interval are also noteworthy. Increased swimming during the ISI is likely to represent enhanced exploratory behavior elicited by the disappearance of the stimulus fish. Furthermore, as thrashing (trying to swim through the glass) is often observed on the wall adjacent to the stimulus fish (a form of shoaling response)[17], reduction of this behavior during ISI is an expected response to the disappearance of the stimulus fish.

Returning to the main learning performance measure, time spent near the stimulus side, the results suggest that zebrafish are capable of acquiring a shuttle box-type learning task well within 30 trials and can alter their location according to the expected appearance of the image of a shoal on the opposite side. This is notable for several reasons. First, learning performance of the experimental zebrafish in this task was supported by the motivation to join a conspecific group, a finding that confirms our previous result where this phenomenon and its utility for learning tasks was first described [1]. Second, in the current paper the stimulus fish used were not live conspecifics but a set of animated zebrafish images. This is the first time computer-animated images have been demonstrated to support acquisition of a learning task in zebrafish. Notably, computerization of stimulus presentation allows complete temporal and spatial control of stimulus delivery, which increases precision of the task. Furthermore, this method of stimulus presentation is also expected to facilitate running the paradigm in a massively parallel manner (multiple test tanks running at the same time), which is necessary for high throughput applications. Third, the main performance parameter measured, the location of fish, is easy to quantify in an automated manner [3], which again implies that the paradigm has utility for high throughput screening.

Numerous questions remain unresolved, however. Perhaps the most interesting one concerns the cognitive aspects of the task. The question is whether the significant performance improvement in the Alternating side group was due to the ability of zebrafish to learn the temporal aspects of the task. That is, did the fish improve because they learned to anticipate the future appearance of the reward on the opposite side, or was this improvement the result of associative learning whereby the experimental fish associated the location of the reward with the disappearance of the stimulus on the opposite side? These alternative hypotheses will be explored in the future.

Another question concerns why fish in the Same side group showed an improvement that was only apparent but not significant. At this point, the answer to this question is speculative. Given the pilot nature of our study, we suspect that several parameters of the learning task could be better optimized. Although we experimented with the temporal aspects of these parameters and came up with the applied Stimulus Presentation and Inter-Stimulus Interval period lengths and number of trials, we have not explored such factors as the dimensions of the apparatus or the characteristics of the stimulus fish. For example, zebrafish are very active and can traverse long distances quickly. It is possible that our experimental tank was not long enough to sensitively detect side preferences in all groups. In the 50 cm long experimental tank zebrafish can effortlessly cross over from one side to the other quickly. The distance between nearest neighbors in small zebrafish shoals is expected to be within, 1–1.5 body lengths, approximately 4–6 cm [13,12,17]. Therefore, although experimental zebrafish may be motivated to stay within a few centimeters from their images, they can easily move away and quickly rejoin the group in the small tank, an oscillatory behavior already described in freely moving zebrafish shoals [12]. Increasing the length of the apparatus may force the experimental zebrafish to decide whether to “join” the shoal presented on one side or to join the shoal that is going to appear on

the other side. Thus, lengthening the experimental tank may enable increased precision in quantifying the responses of zebrafish. Similarly, characteristics of the stimulus fish images such as their size, color, speed, number and even gender composition may have to be optimized to make them elicit a maximal preference from experimental zebrafish [17].

In summary, although numerous questions will have to be explored and test parameters will have to be optimized, our results demonstrate significant acquisition of memory in the shuttle box alternation learning task in zebrafish, and also suggest that the newly developed task will be appropriate for high throughput screening.

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