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Induction of Social Behavior in Zebrafish: Live Versus Computer Animated Fish as Stimuli

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Abstract

The zebrafish offers an excellent compromise between system complexity and practical simplicity and has been suggested as a translational research tool for the analysis of human brain disorders associated with abnormalities of social behavior. Unlike laboratory rodents zebrafish are diurnal, thus visual cues may be easily utilized in the analysis of their behavior and brain function. Visual cues, including the sight of conspecifics, have been employed to induce social behavior in zebrafish. However, the method of presentation of these cues and the question of whether computer animated images versus live stimulus fish have differential effects have not been systematically analyzed. Here, we compare the effects of five stimulus presentation types: live conspecifics in the experimental tank or outside the tank, playback of video-recorded live conspecifics, computer animated images of conspecifics presented by two software applications, the previously employed General Fish Animator, and a new application Zebrafish Presenter. We report that all stimuli were equally effective and induced a robust social response (shoaling) manifesting as reduced distance between stimulus and experimental fish. We conclude that presentation of live stimulus fish, or 3D images, is not required and 2D computer animated images are sufficient to induce robust and consistent social behavioral responses in zebrafish.

Introduction

The zebrafish has been gaining popularity among behavioral neuroscientists.[1] This is partly due to the fact that zebrafish has been in the forefront of genetics and by now numerous powerful reverse and forward genetic tools have been developed for this species.[2] Another reason why zebrafish is preferred is that it appears to strike the right balance between system complexity and practical simplicity: it is a vertebrate species, but it is also highly prolific, easy to keep in large numbers, and its maintenance is cheap. Recent years have seen an upsurge of behavioral studies conducted with zebrafish.[3,4] These behavioral studies often utilized visual stimuli to induce or modify behavioral responses. Zebrafish are diurnal and thus vision is an important modality for this species.[5] Visual stimuli are also some of the easiest to control, which is partly due to the fact that our own species is also highly visual and thus consumer grade products developed for the everyday human user, such as TV screens and video cameras, can be readily utilized in experiments with zebrafish.

Several recent behavioral studies have focused on social behavior of zebrafish. In nature[6,7] and in the laboratory,[8–12] zebrafish aggregate and swim in groups, a behavior called shoaling. This behavior is not associated with coordinated directional movement of individuals. It only represents social cohesion leading to distances among shoal members smaller than what would be expected in case of random or stochastic distribution of the individuals.[13,14] We have also shown that the original assumption of having two distinct forms of group forming, shoaling, without coordinated directional movement, and schooling, coordinated directional movement of individuals within the group, is indeed correct.[15] These are distinct forms of group forming behavioral responses, at least in zebrafish, with very little overlap between them.[13] In this article , we focus on shoaling and do not assume nor quantify coordinated directional swimming, that is, schooling.

Shoaling may be elicited and quantified in the laboratory in primarily two different ways. One of these methods is to allow freely moving individuals to aggregate spontaneously and to measure their behavior, for example, by quantifying the inter-individual distances of shoal members.[9,10,16] The other method is to provide social stimuli in a controlled manner, for example, present the sight of conspecifics to individual experimental fish and measure the response of this focal fish, for example, by quantifying the distance it keeps from the shoaling stimulus.[12,17–20] The advantage of the former is severalfold. Analysis of freely moving shoals

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may allow unprecedented insights into the dynamic changes that occur in the group and thus it may help us understand the behavioral mechanisms that drive these changes.[10,13,21] However, the disadvantage of this method is that it is not easily applicable to the neurobiological or genetic analysis of the mechanisms of social behavior, which is usually better achieved by the manipulation and analysis of the responses of single individuals. For this reason, we and others have attempted to induce social behavior in fish tested singly by providing social stimuli to the experimental animal.[8,12,22,23]

We employed two different methods of presentation of social stimuli, both utilizing conspecifics: (1) we presented the experimental subject with live stimulus fish;[12,17,18,20,24] or (2) we presented computer animated (moving) images of zebrafish.[12,25,26] Both presentation methods induced robust behavioral responses that resembled shoaling. The experimental fish quickly approached the social stimuli and remained close to them. It is notable that this response was easy to distinguish from that induced by non-social stimuli. Images of scrambled zebrafish (containing the same pixels as a photo of a zebrafish but presented in a scrambled manner within a moving rectangle whose length and height was identical to that of the zebrafish image) did induce a robust approach but without subsequent maintenance of close proximity to the moving objects.[27] This transient approach we interpreted as exploratory behavior. Thus, according to our prior findings, social behavioral responses are characterized by a robust initial approach and subsequent maintenance of the reduced distance between the focal fish and the stimulus, and they can be induced by the sight of moving conspecifics but not by moving inanimate objects.

Although both live and animated stimulus fish have been employed successfully, the effectiveness of these two methods of stimulus presentation has not been systematically compared. Such comparison is important because each method may have its own advantages and disadvantages, idiosyncrasies that become particularly important when these stimuli are used in high throughput screening.[28] Live stimulus fish may be more attractive as they move naturally and may also respond to and interact with the experimental fish. Thus, they may induce the most robust natural shoaling reaction in the experimental fish. However, live stimulus fish may not behave consistently within and across test sessions and thus their use comes inherently with a potentially increased error variation. Animated images of conspecifics may be delivered in a user-defined consistent manner and thus their presentation may reduce experimental error variation but they may not be realistic enough to induce the maximum level of attainable social reaction in the experimental fish.

We emphasize that development of appropriate social behavior inducing test methods and paradigms would be an important achievement for translational research with zebrafish. There are a number of human central nervous system disorders associated with abnormal social behavior, including depression,[29,30] schizophrenia,[31] autism spectrum disorders,[32] and drug abuse related disorders such as alcoholism.[33] The zebrafish has been proposed as a potentially useful model organism for the analysis of all of these disorders[1,8,34,35] and thus proper induction and evaluation of social responses in zebrafish may have translational relevance.

In the past, we utilized a custom software application developed in house, the General Fish Animator (GFA) that allowed us to custom design and control the delivery of animated zebrafish images[12] and thus induce social responses in a controlled and consistent manner. However, this software application had some drawbacks, which we will discuss below, and thus we developed a new application we call Zebrafish Presenter (ZFP). In this article, we compare the effectiveness of these software applications and measure the behavioral responses in experimental fish induced by animated conspecific images presented using them. In addition, we also explore how these software applications measure up against other methods of stimulus delivery, including the playback of previously video-recorded live stimulus fish (fish moving in 3D), presentation of actual live stimulus fish outside the tank (providing realistic natural stimuli but only in the visual domain), and presentation of live stimulus fish inside the tank (separated by a perforated thin plexiglass divider from the experimental fish, providing a full spectrum of stimuli of all modalities). In addition to presenting our behavioral findings, we also describe in detail the new ZFP program and how it compares in terms of functionalities to the other methods.

Materials and Methods

Animals and housing

Adult zebrafish (*Danio rerio*) were obtained from a local commercial supplier (Big Al's Aquarium, Vaughan ON). This ''wild-type'' population is not genetically characterized but is expected to be genetically heterogeneous.[36] The advantage of using such a genetically heterogeneous population is that these fish may not suffer from genetic drift-induced idiosyncrasies, unique strain-specific features, and thus may be regarded as more typical of zebrafish as a species.[36] The fish used in all our experiments were young adults, 6–8 months old (50%–50% males and females). The fish were housed in 10 L tanks (groups of 30 per tank) in a high density recirculating water rack system (Aquaneering, Inc., San Diego, CA) that employed mechanical, biological, and activated carbon filters in addition to a UV sterilizing unit. The system water used on the rack was reverse osmosis purified and the salt concentration was reconstituted to the desired salinity by adding 60 mg/L of sea salt (Instant Ocean; Aquarium Systems, Inc., Mentor, OH). The temperature of the water in the rack system was maintained by thermostat-controlled internal heaters at 27°C. All fish were given 3 weeks to acclimatize in our facility before experimental habituation trials and test sessions began. Five female fish were randomly chosen and were housed separately. These fish served as stimulus fish for all experimental fish. Images and video recordings of female zebrafish were also used as stimuli. Further, the female fish used in the video recordings were the same as those employed for live shoal presentation. The rationale for the presentation of all female shoals as stimulus is that females have been found attractive to both male and female zebrafish, whereas males induce differential responses from females and males.[37] The fish holding room (same as the experimental room) was illuminated by fluorescent light tubes from the ceiling with lights turned on at 08:00 h and off at 19:00 h. The fish were fed commercial flake food twice a day (Tetramin Tropical fish flake food; Tetra Co, Melle, Germany).

Behavioral apparatus

All fish were tested singly in a 40-L glass tank ($50 \times 25 \times 30$ cm, length × width × height) whose bottom and back side was coated with green Plexiglass to reduce glare (for video-tracking) and to provide a more naturalistic background for the fish. The front and sides of the tank were transparent glass. The experimental tank was illuminated from above by a fluorescent aquarium light fixture (13 W). For all conditions a computer monitor (17 inch Samsung 732N) displayed a black screen for the duration of the trial. Another monitor of the same type was connected to a Dell Vostro 1520 laptop and was placed flush against the other short side of the tank. This monitor displayed stimuli (animated images of zebrafish) using the GFA or ZFP software applications or a previously created video recording of live fish. In addition, live fish were also used as a stimulus. For a subset of experimental subjects, these stimulus fish were placed outside of the experimental tank in a Plexiglas container ($27 \times 9 \times 30$ cm, length × height × width) that replaced one of the side monitors. The side of the Plexiglas container facing the experimental tank was clear, and the remaining sides and bottom of the container were black. This set up was chosen to mimic the overall appearance of the stimulus monitor used for the software driven animated stimulus presentation, and thus to make the experimental set ups used for the live fish versus animated fish image presented fish as similar as possible. Live fish could also be placed inside the experimental tank. For this, a perforated clear Plexiglas divider was lowered into the experimental tank 5 cm away from the side wall of the testing tank. Thus, live stimulus fish placed into this compartment could be seen, smelled, and detected by the lateral lines of the experimental fish (low frequency vibrations, a modality analogous to tactile cues for fish). The last habituation session and the stimulus presentation sessions, each lasting for 15 min, were recorded using a SONY AVCHD Handycam video camera. The video recordings were later transferred to the hard drive of computers, replayed, and analyzed using the Ethovision Videotracking software application (Version 8.5; Noldus Info Tech, Wageningen, The Netherlands).

Behavioral testing procedure and experimental design

First, all experimental and stimulus fish were habituated to the testing environment by placing them into the experimental tank for 30 min in groups of 10 for a total of four occasions, a procedure successfully employed in the past.[24,38] This procedure was followed to make sure that fish behavior stabilized and potential habituation status-dependent between-session changes were minimized. Subsequently, each experimental fish was randomly assigned to one of five stimulus presentation conditions: live stimulus fish inside the experimental tank, live stimulus fish outside the experimental tank, pre-recorded video of live experimental fish, animated zebrafish images presented by the software application GFA, animated zebrafish images presented by the software application ZFP, a between subject experimental design. Experimental fish of these five stimulus treatment groups were subsequently placed into the experimental tank individually in a randomized order for a 15-min habituation session during which no stimuli were presented. The behavior of the experimental fish was recorded and later analyzed. Twenty-four hours later, fish were individually placed in a randomized order into the same experimental tank but this time they were presented with the social stimulus according to their group designation for the entire 15 min of the session as described below. The stimulus was presented continuously for the stimulus session on the same side of the experimental tank for a given experimental fish but the side of presentation randomly varied across experimental subjects. The behavior of experimental fish during this stimulus presentation session was also recorded and later analyzed. Notably, all experimental conditions were identical between the last habituation and the subsequent stimulus presentation sessions except that during the latter social stimuli were presented. It is also notable that the only difference across fish of different stimulus presentation groups was the type of stimulus presented, that is, all other experimental factors remained identical.

Stimulus treatment conditions

All fish were tested only once and stimuli were presented in a randomized and blind manner. The GFA is a custom software application that was developed in our lab and was previously utilized.[12] This application allows us to present the images of conspecifics that move in a realistic manner similar to live zebrafish. It allows us to control the speed of the images (which in this study was set to range between 1.5 and 4 cm/s), the number of fish in the shoal (which here was set to 5), the timing and duration for which the stimuli are displayed (which was continuous for 15 min in the current study), and the area of swim where the images are shown (which precisely corresponded to the size of the side wall of the experimental tank, i.e., it was 25×30 cm, width × height). Briefly, GFA presented the moving images of zebrafish in a way that these images moved on a random trajectory and each image moved with varying speeds keeping the speed within the desired range. Visual Basics 6 was used to write the code for this software, and the moving image of each zebrafish was composed of a series of still images that were then masked as new pictures were added. This software design had some disadvantages, however. For example, we noticed that the fish images flickered and also as they moved across the screen a faint trail was left behind them. Further, after repeated trials delays began to occur and as the delays accumulated, the timing of the presentation versus no presentation of fish the stimulus fish images became inaccurate and did not conform to the user-defined time periods. Last, this software does not work with more modern operating systems. For example, with an operating system of Windows Vista or later, GFA will not execute due to a dynamic-link library that was deleted. It is possible to manually add the library to the newer Windows operating system but the program may work unreliably under these conditions.

To circumvent the above issues, the ZFP application was designed. In terms of functionality, this application is very similar to GFA. The parameters (speed, number of fish, location of stimuli presented, etc.) used in the current study were identical to what was set with the GFA. Further, the very same image used for GFA was also used for ZFP (Fig. 1). However, unlike GFA, ZFP utilizes Visual Basics .NET, which is a newer language than VB6, as the coding language, and this software now includes additional options compared to GFA. ZFP allowed the user to change the size of the fish displayed, enabling the displayed fish to precisely match the

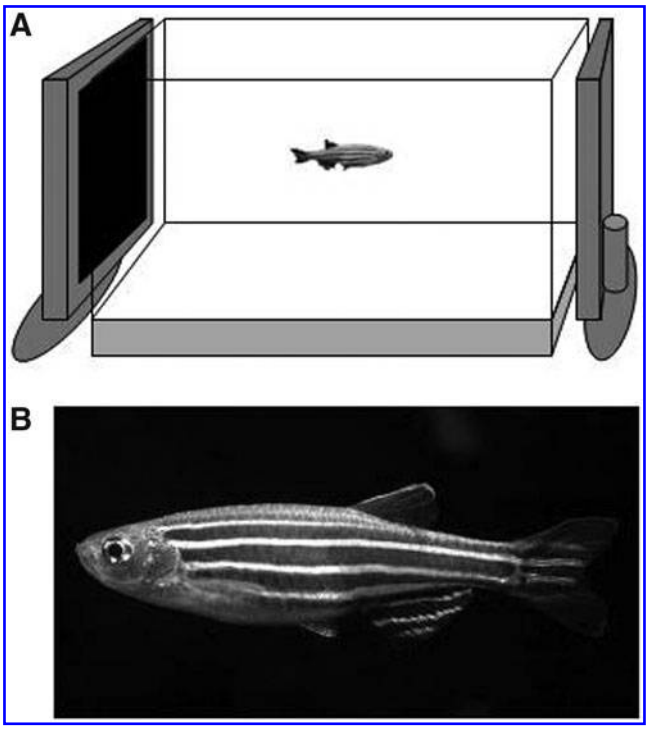

FIG. 1. The experimental apparatus **(A)** and the zebrafish image **(B)** employed for animated image presentation. Note that two computer monitors were employed to deliver the animated (moving) images of zebrafish using the software applications General Fish Animator (GFA) or Zebrafish Presenter (ZFP). These monitors were also used to replay video recordings of live zebrafish. Also note that for any given experimental fish, the stimulus was shown on only one side of the tank, but the side of presentation varied across the experimental subjects. The stimulus utilized for animated image delivery was a photograph of an adult sexually mature female zebrafish showing the characteristic wild type color and stripe pattern. A female was chosen because females are known to be preferred by both males and females and thus possible sex differences associated with territoriality or dominance status in males were minimized. For further details of experimental procedures and methods of presentation of live stimulus fish, see Materials and Methods section.

size of the test fish for each trial (our current study), or to appear smaller or larger than the test fish if required. The background color of the presentation could also be specified and changed and the program automatically saved all entered preferences without the user having to manually enter the information each time a stimulus delivery session was initiated. The moving image in ZFP was created by using one stationary image per fish and moving it around the screen. This arrangement eliminated the trail and flickering caused by successive masking as seen in GFA. Last, no delays have been found following repeated trials using ZFP. In summary, the ZFP is able to display multiple images of the chosen fish photograph. The number of fish, average fish speed, size of fish, duration, and background color of each instruction is customizable. A set of instructions can be re-ordered, added to, or deleted from. The set of instructions along with the display area can be saved and loaded. A log of the instructions that was executed is automatically generated and available for the researchers to confirm that the correct set of instructions was executed. The animated fish are programmed to move between 0× and 2× the average fish speed determined by the user on the x-axis, with a 20% chance of changing speed per cycle. The animated fish have a 5% chance of changing speed on the y-axis. The algorithm is programmed such that the animated fish have a tendency to move toward the center on the y-axis, rather than stay at the edges of the screen. The algorithm also dictates that the animated fish will turn around when they reach an outside edge and the fish also have a collision algorithm, which makes one of the fish turn around when running into another.

In addition to the computer animated images, we also explored the effect of presentation of stimuli via video recording and with the use of live fish. Five female stimulus fish were placed in the black Plexiglas tank, which was also used for live stimulus presentation. After 30 min of undisturbed habituation, the stimulus fish were videotaped using a high definition camcorder (Canon VIXIA HF G20). The digital recording was transferred to a computer and it was later replayed for the stimulus presentation period for the corresponding experimental fish.

To test the effect of live fish outside of the experimental tank five female zebrafish were placed into the black Plexiglas tank described above prior to each experimental session. These stimulus fish were allowed to habituate to the stimulus tank for 30 min prior to the introduction of the experimental fish into the experimental tank and remained visible to the experimental fish for the entire 15 min of the stimulus presentation session.

It is possible that visual stimuli alone are not sufficient and will not induce a maximal social behavior response (shoaling) in the experimental zebrafish. To test this possibility, we placed live stimulus fish inside the experimental tank behind a thin clear and perforated Plexiglas divider that separated the stimulus fish from the experimental fish. This set up mimicked the conditions employed in case of the presentation of live stimulus fish outside of the tank in the dimensions of the stimulus area, and thus the total space in which the stimulus fish could move were identical to those of the black Plexiglas stimulus tank. However, the transparent and perforated Plexiglas divider allowed the perception of the stimulus fish not only via vision but also via all other modalities.

The differences and similarities in practical and functional aspects among the five stimulus presentation methods are summarized in Table 1.

Quantification of behavior

The behavior of experimental fish was analyzed using the video-tracking software application Ethovision (Noldus Info Tech). Several behavioral parameters were extracted from the swim paths of the fish. For example, distance to stimulus is defined as the distance (expressed in cm) between the center of the body of the experimental fish and the glass or Plexiglas wall behind which the stimulus is presented. This is the most important behavioral measure of the current study. It has been shown to reflect social cohesion, a measure of shoaling tendency in zebrafish.[11] The distance to the stimulus is sampled 30 times per second. The average of these samples is calculated for each 1-min interval of the 15 min recording sessions. In addition, the variability standard error of the mean (SEM) of the distances obtained at every sample time

Table 1. Comparison Between Stimulus Presentation Methods Employed

	Zebrafish presenter	*General fish animator*	*Live fish videotaped*	*Live fish outside the experimental tank*	*Live fish inside the experimental tank*
Programming language	Visual Basic .NET 4.0	Visual Basic 6	n/a	n/a	n/a
Instruction—fish available	Zebrafish Predator fish Blank screen Scrambled zebrafish	Zebrafish Predator fish Blank screen No	Any species	Any species	Any species
Instruction—attributes available	Speed of fish Number of fish Duration of instruction	Speed of fish Number of fish Duration of instruction	Variable Number of fish Manually controlled	Variable Number of fish Manually controlled	Variable Number of fish Manually controlled
	Size of fish Background color	No No	Difficult Manually controlled	Difficult Manually controlled	Difficult Manually controlled
Instruction—logs	Running log automatically generated	No	n/a	n/a	n/a
Algorithm	Each fish is a picture boxed moved by the program at time intervals	Each fish is image drawn on background and masked over at each time intervals	n/a	n/a	n/a
Known problems	Fish cannot overlap They will bounce and move away from each other	Flickering of screen A white trail behind animated images Delays after many switches Incompatible with Windows 7 without manually adding a DLL from XP Untested on Vista and Windows 8	Inconsistent within and between session behavior	Inconsistent within and between session behavior	Inconsistent within and between session behavior

point is also calculated and expressed for each 1-min interval. Importantly, this measure of variability is not between individual variance referring to the group of fish, but rather a measure of within individual variance representing the temporal changes (inconsistencies) in the distance of the particular fish from the stimulus.

Distance to the bottom of the tank (expressed in cm) is also quantified. This behavioral parameter is thought to be important as it has been argued to be dependent upon fear.[8] Fish placed in aversive situations or exposed to aversive stimuli often show what is referred to as the diving response,[35,39] that is, a decrease of distance from the bottom. Here, both the average and the temporal within individual variability (SEM) of distance to bottom are calculated and expressed for each 1-min interval of the recording session.

Angular velocity (speed of turning) represents the magnitude of change in the direction of movement as calculated between two consecutive time samples. Angular velocity is a cumulative measure and is expressed in degrees/second (°/s). Its value ranges from 0°/s to a maximum value of 180×30 (180° direction change $\times$ 30 samples per second) = 5400°/s. This measure has been used to quantify active fear responses including erratic movement that is seen in fish placed in highly aversive or fear inducing situations.[39] The average and the within individual temporal variability (SEM) of this parameter was calculated for 1-min intervals.

Statistical analysis

Data were found normally distributed and variances were homogeneous; therefore, parametric statistical tests were employed. Also notably, the parametric tests employed in the current study are insensitive to the violation of homogeneity of variance and normality of distribution criteria especially in case of equal sample sizes of groups compared, which is the case here. Repeated measure variance analysis (ANOVA) was performed to analyze time-dependent changes within a recording session (Interval, the repeated measure factor with 15 levels), the difference between habituation versus stimulus delivery session (Session, a non-repeated measure factor with 2 levels) and to test the effect of stimulus delivery method (Stimulus, a non-repeated measure factor with

5 levels). Session was treated as a non-repeated measure between subject factors because we could not identify individual fish and follow their behavior from the habituation to the stimulus presentation session. This was because we did not want to employ invasive marking methods, and we also decided not to house fish individually between the habituation and stimulus presentation sessions to avoid stress. It is notable that multiple-comparison *post hoc* statistical tests capable of reducing type I error are not appropriate for repeated measure experimental designs. To solve this problem and to be able to compare our five stimulus presentation groups with each other, we calculated the average of the 15 intervals for the habituation session and for the stimulus delivery session and conducted a non-repeated measure ANOVA (with factors Session and Stimulus) and a subsequent Tukey honestly significant difference (HSD) test for each session separately. We report significance when the probability of null hypothesis is $<5\%$, that is, when $p<0.05$.

Results

During the habituation session all fish were active, swam around the experimental tank, and appeared to show no preference for either side. Figure 2A also suggest that the distance they swam from the side where the stimulus would be shown the next day was on average around 25 cm, which is the midpoint of the 50 cm long experimental tank. However, the distance to the stimulus during the stimulus presentation session showed a robust decrease in all stimulus groups, and these groups did not appear to differ from each other (Fig. 2B). These observations were confirmed by statistical analyses. Using ANOVA, we found a significant Interval effect [$F(14, 1932)=1.825$, $p<0.05$], and a significant Interval × Session interaction [$F(14, 1932)=2.357$, $p<0.01$], but other interaction terms were nonsignificant. Importantly, the effect of Session (i.e., the difference between the habituation and the stimulus presentation sessions) was found significant [$F(1, 138)=60.912$, $p<0.001$]. Also notably, the effect of Stimulus (i.e., the difference among the five stimulus delivery methods) was nonsignificant. To further examine the effect of stimulus presentation and whether the five stimulus groups differed from each other we analyzed the data averaged for each of the 15 min recording sessions, habituation, and stimulus presentation sessions (Fig. 2C, D). ANOVA confirmed that the stimulus groups did not differ during the habituation session as expected (Fig. 2C). Importantly,

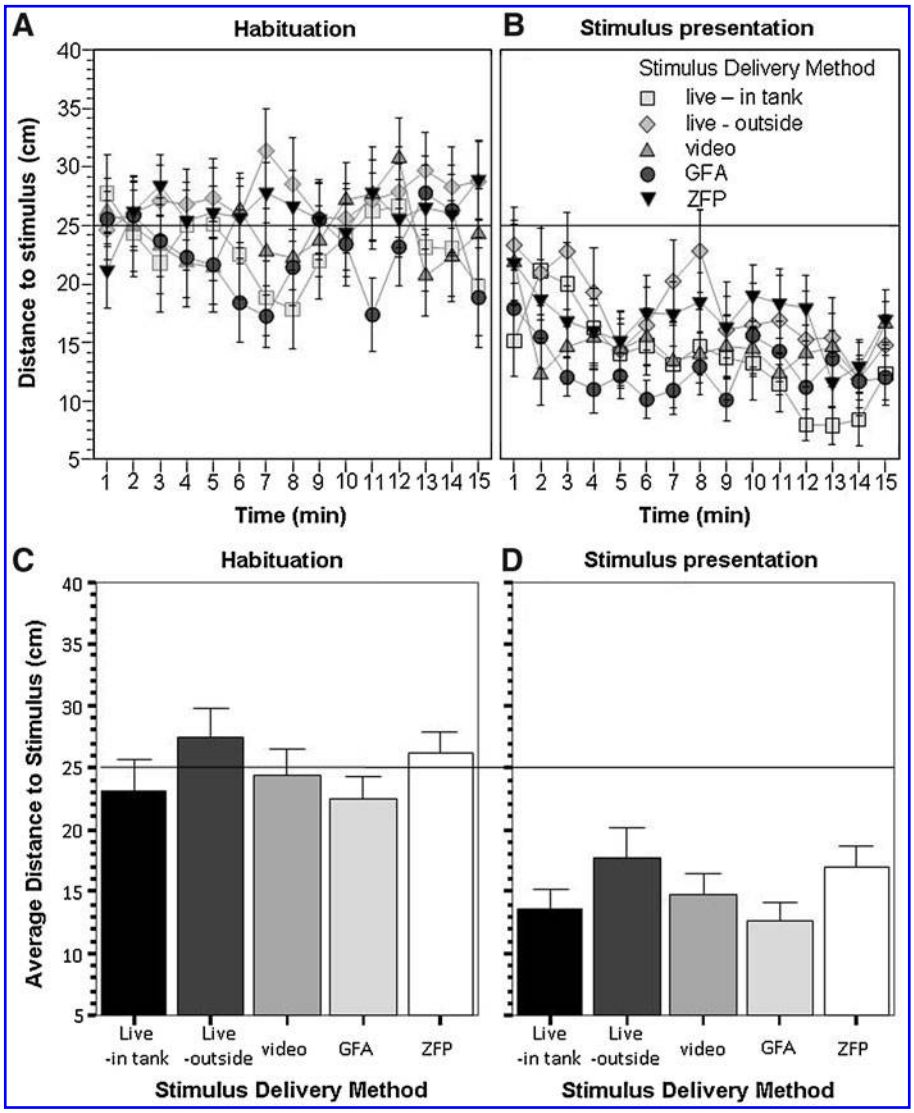

FIG. 2. The distance to stimulus presentation side (cm) is significantly decreased upon presentation of the stimulus. Mean ± SEM are shown. Each stimulus group had an $n=30$ experimental fish except the one shown live stimulus fish inside the experimental tank, for which $n=28$. The horizontal line across the upper two graphs **(A, B)** represent the midpoint (25 cm distance from stimulus side). **(A, B)** Show the distance values obtained for each 1 min interval of the 15 min habituation session (no stimulus presented, **A)** and the stimulus presentation session **(B)**. **(C)** Habituation session and **(D)** stimulus presentation session show results averaged over the fifteen 1-min interval. Note the robust reduction of distance from the stimulus side during stimulus presentation compared with the habituation session **(B** vs. **A** and **D** vs. **C)**. Also note the lack of significant difference among the five stimulus presentation groups. SEM, standard error of the mean.

ANOVA also demonstrated that these groups did not differ even during the stimulus delivery session (Fig. 2D) despite receiving different social stimuli.

The temporal within individual variability of the distance to the stimulus side appeared to be stable during the habituation session with no apparent differences among the five treatment groups (as expected given that none of the fish received any stimulus presentation during this session, Fig. 3A). The five stimulus groups also did not appear to exhibit robust differences during the stimulus presentation session (Fig. 3B). Using ANOVA, we found only one term significant, the Interval × Session interaction term [$F(14, 1932) = 1.904$, $p < 0.05$] but all main factors and other interaction terms were nonsignificant. Analysis of the data averaged for each 15 min session (the habituation and the stimulus delivery sessions) showed that the stimulus groups did not differ during the habituation session, but a significant stimulus effect was found for the stimulus presentation period [$F(4, 74) = 2.761$, $p < 0.05$]. Tukey HSD test revealed that fish that were shown the live fish presented inside the experimental tank exhibited significantly ($p < 0.05$) smaller variability in their distance to the stimulus compared with fish that were shown animated images of zebrafish using the ZFP software application. Other group differences were nonsignificant ($p > 0.05$).

Fish during the habituation session appeared to start swimming closer to the bottom of their tank, a response that quickly habituated during the habituation session and remained apparently stable after the first 4 min (Fig. 4A, B). ANOVA confirmed this observation and found the effect of Interval significant [$F(14, 1932) = 4.514$, $p < 0.001$], and also showed that no other main factors or interaction terms were significant. Analysis of the data averaged over the 15 min of the habituation and for the stimulus presentation sessions (Fig. 4C, D) showed a significant stimulus group difference for the habituation session [unexpected because no stimuli were delivered during this period, ANOVA $F(4, 68) = 2.524$, $p < 0.05$]. Tukey HSD showed that this effect was due to the difference ($p < 0.05$) between the fish that would be receiving the video-recorded images and the animated images using the software ZFP, while other group differences were nonsignificant. However, for the stimulus delivery period ANOVA found no significant stimulus effect.

The temporal within individual variability of the distance from bottom showed a robust increase during the first 4–5 min of the habituation session and also during the 4–5 min

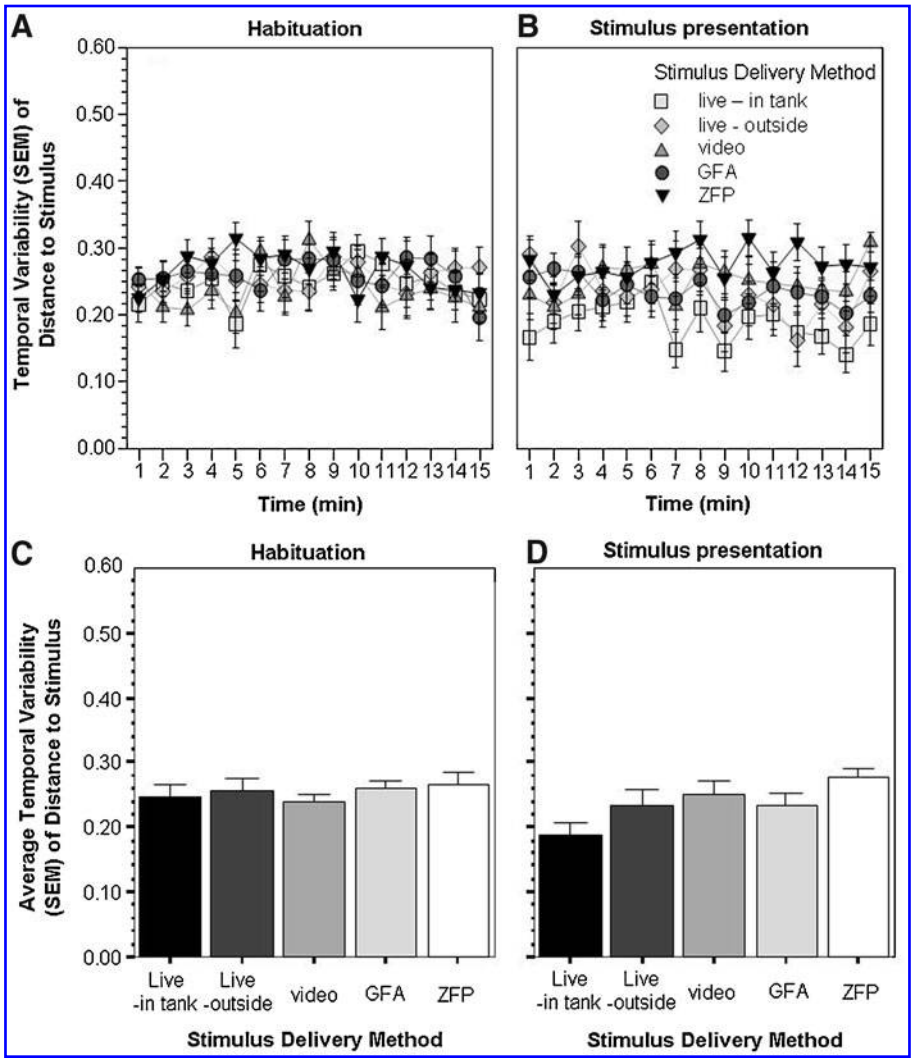

FIG. 3. The temporal within individual variability (expressed as SEM) of distance to stimulus presentation side remains stable across both the habituation and the stimulus presentation sessions. Mean ± SEM of this variability are shown. Each stimulus group had an $n = 30$ experimental fish except the one shown live stimulus fish inside the experimental tank, for which $n = 28$. **(A, B)** Show the values obtained for each 1 min interval of the 15 min habituation session (no stimulus presented, **A)** and the stimulus presentation session **(B)**. **(C)** Habituation session and **(D)** stimulus presentation session show results averaged over the fifteen 1-min interval. Note the lack of significant difference among the five stimulus presentation groups.

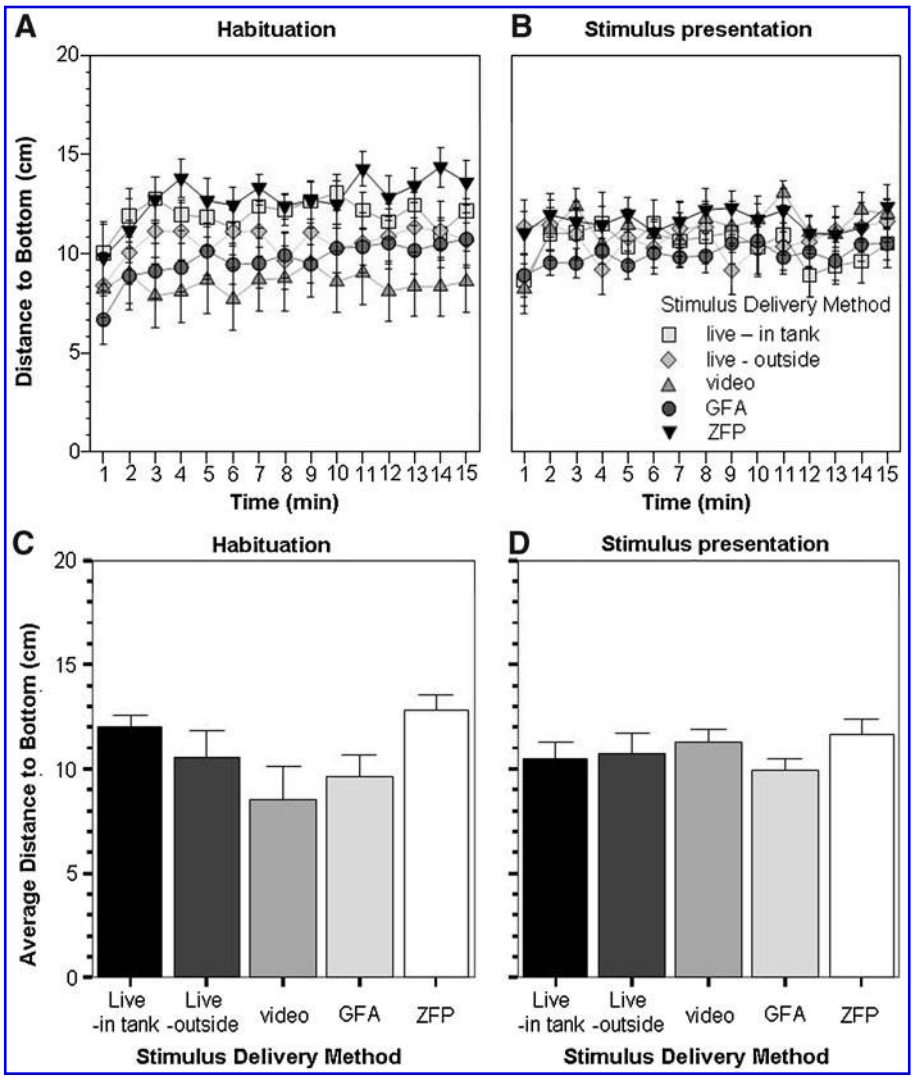

FIG. 4. The distance to bottom slightly increases during the first 3 min of the sessions. Mean ± SEM are shown. Each stimulus group had an $n = 30$ experimental fish except the one shown live stimulus fish inside the experimental tank, for which $n = 28$. **(A, B)** Show the distance values obtained for each 1 min interval of the 15 min habituation session (no stimulus presented, **A)** and the stimulus presentation session **(B)**. **(C)** Habituation session and **(D)** stimulus presentation session show results averaged over the fifteen 1-min interval. Note the lack of significant difference among the five stimulus presentation groups.

of the stimulus delivery session (Fig. 5A, B). ANOVA confirmed a significant Interval effect [$F(14, 1932) = 39.753$, $p < 0.001$]. The significance of the effect of Session was borderline [$F(1, 138) = 3.869$, $p = 0.051$] and the effect of Stimulus was nonsignificant. The Interval × Session interaction term [$F(14, 1932) = 2.752$, $p < 0.001$] and the Stimulus × Session interaction term [$F(4, 138) = 4.732$, $p < 0.01$] were found significant, but other interaction terms were nonsignificant. The results for the temporal within individual variability of distance from bottom averaged for the 15 intervals of the habituation and of the stimulus delivery sessions showed (Fig. 5C, D) again an unexpected stimulus effect during the habituation session [ANOVA $F(4, 68) = 2.657$, $p < 0.05$]. However, this time Tukey HSD found the fish to be receiving the live stimulus fish inside the tank to differ ($p < 0.05$) from fish to be receiving the video-recorded zebrafish. However, again, for the stimulus presentation session ANOVA did not find any stimulus group to significantly differ from another ($p > 0.05$).

Angular velocity showed no consistent interval dependent or obvious stimulus presentation-induced effects (Fig. 6A, B). ANOVA did find a significant Interval effect [$F(14, 1932) = 2.660$, $p < 0.01$] but no other main factor or interaction terms were found significant. Analysis of data averaged for the 15 intervals of the habituation session and of the stimulus recording session showed no significant stimulus group differences during either session (Fig. 6C, D).

The temporal variability of angular velocity also did not show any apparent differences across stimulus groups during the habituation session (Fig. 6A) but during the stimulus presentation session fish receiving the animated zebrafish images via the ZFP software application appeared to exhibit somewhat lower values (Fig. 7B). Using ANOVA we found a significant Interval effect [$F(14, 1932) = 3.652$, $p < 0.001$] and a significant Stimulus effect [$F(1, 138) = 3.378$, $p < 0.05$], but the effect of Session was nonsignificant. All interaction terms were also found nonsignificant. The analysis of the data averaged for the 15 intervals of the habituation and of the stimulus delivery sessions (Fig. 7C, D) showed that during the former no stimulus group differences were significant, while in the case of the latter, the stimulus groups did differ [ANOVA $F(4, 70) = 2.811$, $p < 0.05$]. Tukey HSD revealed that this effect was due to the significant difference ($p < 0.05$) between fish that received the animated zebrafish images by the GFA and the ZFP software applications, the latter exhibiting smaller values.

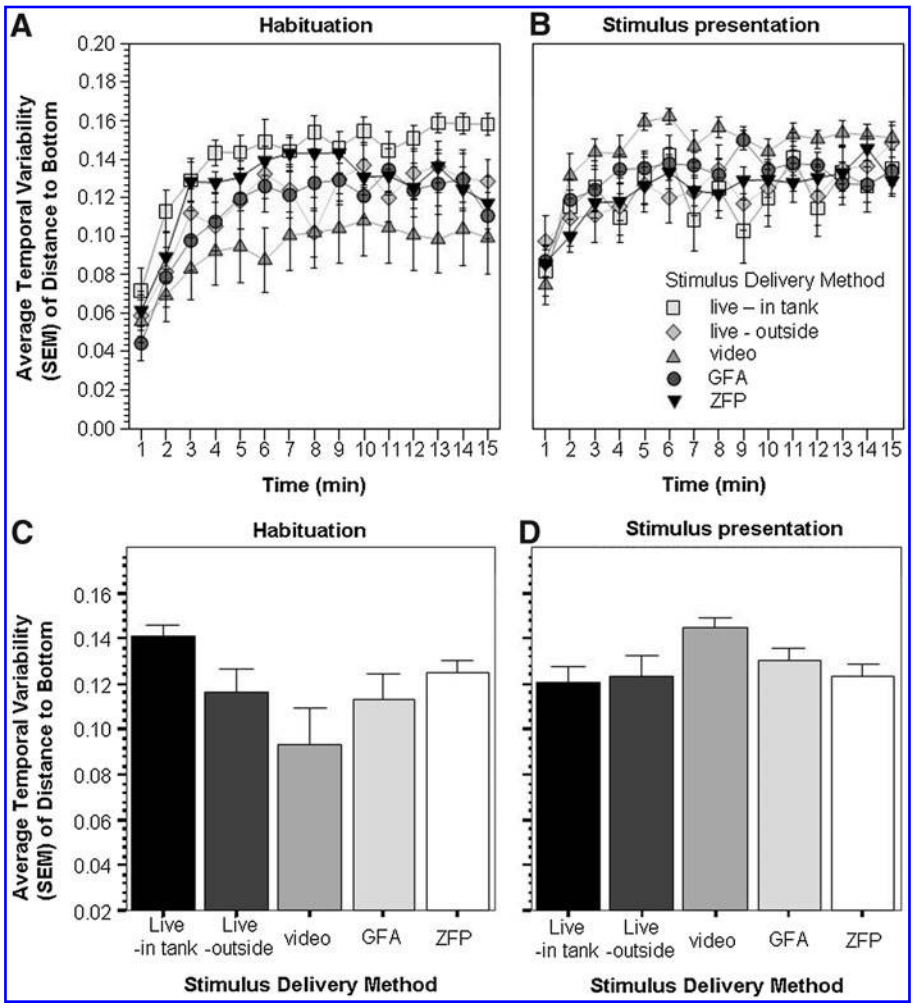

FIG. 5. The temporal within individual variability (expressed as SEM) of distance to bottom robustly increases during the first 4–5 min of the sessions. Mean ± SEM of the variability are shown. Each stimulus group had an $n = 30$ experimental fish except the one shown live stimulus fish inside the experimental tank, for which $n = 28$. **(A, B)** Show the distance values obtained for each 1 min interval of the 15 min habituation session (no stimulus presented, **A)** and the stimulus presentation session **(B)**. **(C)** Habituation session and **(D)** stimulus presentation session show results averaged over the fifteen 1-min interval. Note the lack of significant difference among the five stimulus presentation groups during stimulus presentation.

Discussion

A potentially important advantage of the zebrafish over traditional laboratory rodent species is that unlike these nocturnal mammals, the zebrafish is diurnal and thus has excellent vision. Our own species is also diurnal and thus this similarity may help achieve better face validity of zebrafish models of human brain disorders. Further, visual cues are easier to manipulate than olfactory or auditory cues, and numerous consumer grade products, including computer and TV monitors and cameras can be utilized in zebrafish research in a cost-effective manner. The visual system of the zebrafish has been very well studied from a mechanistic standpoint.[5] However, behavioral responses to visual cues have not been well characterized and zebrafish behavioral studies utilizing visual cues are still rare.

We have started to employ a range of visual cues in the form of computer animated images to induce a variety of responses including fear responses with the use of sympatric predator images[39,40] and social behavior with the use of conspecific images.[12,25,26] In this article we have focused on the latter.

Although the effectiveness of conspecific images has been demonstrated by several studies, the question of how they compare to the presentation of live stimulus fish has not been systematically tested. The question of what constitutes an effective visual cue in the context of shoaling is also controversial. For example, 3D images that mimic a natural view of conspecifics moving in their environment may be effective[41] and may favorably compare to images moving back and forth on a flat monitor. Last, it is also not known whether visual stimuli alone are sufficient to induce social responses.

Our current study showed that live stimulus fish that were placed just outside the experimental tank were equally as effective compared to stimulus fish that were placed inside the tank behind a perforated divider. This divider was transparent and also allowed sound and lower frequency vibrations to pass through (lateral line detection). Because stimulus fish under these two conditions attracted the experimental fish similarly (leading to a robust reduction of distance between experimental and stimulus fish), we conclude that visual stimuli alone are sufficient to induce the social response at least in this test paradigm. The lack of difference between video-recorded and live stimulus fish induced social behavioral responses of our experimental fish also suggests that interaction between the stimulus and the experimental fish is not really a requirement for strong social responses to be induced in the experimental fish (the video-recorded fish obviously do not respond to the experimental fish). We also conclude that 3D representation of images (video-recorded live fish) does not present an advantage, at least in the context of the current paradigm, as to its ability to

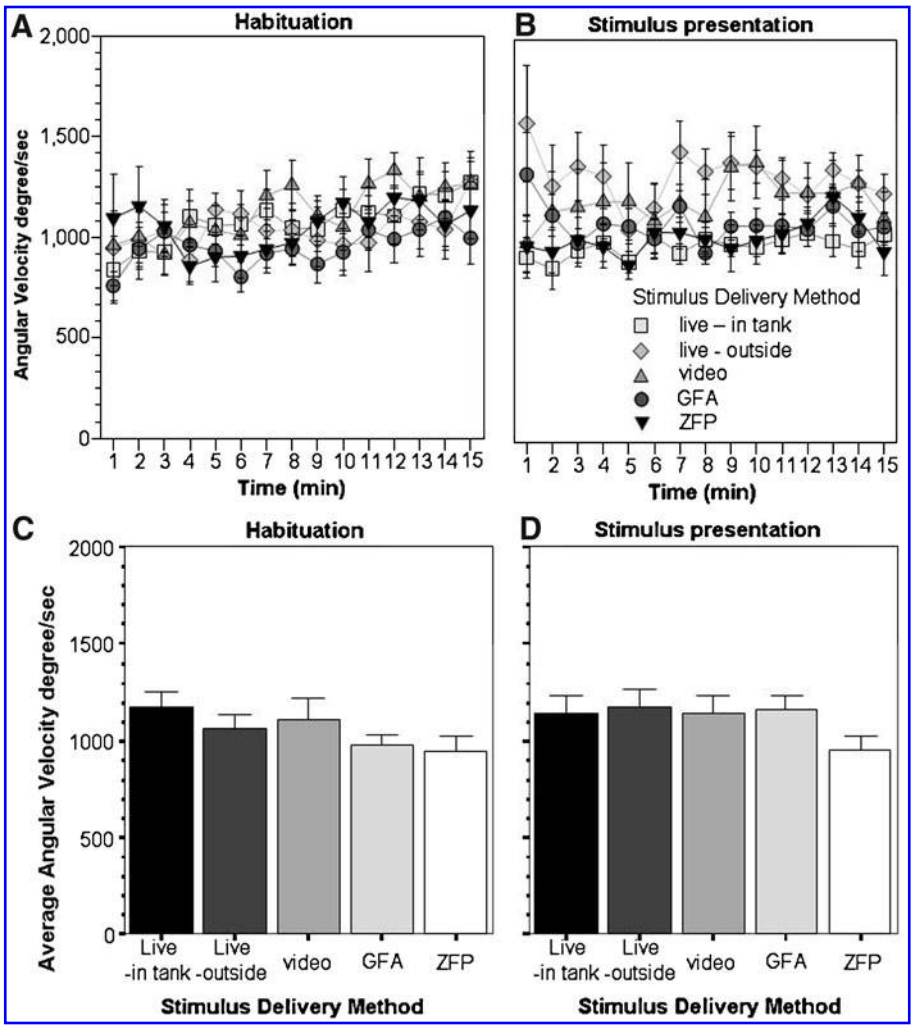

FIG. 6. Angular velocity remains stable across both sessions. Mean ± SEM are shown. Each stimulus group had an $n = 30$ experimental fish except the one shown live stimulus fish inside the experimental tank, for which $n = 28$. **(A, B)** Show the angular velocity values obtained for each 1 min interval of the 15 min habituation session (no stimulus presented, **A)** and the stimulus presentation session **(B)**. **(C)** Habituation session and **(D)** stimulus presentation session show results averaged over the fifteen 1-min interval. Note the lack of significant difference among the five stimulus presentation groups.

attract experimental fish and induce their social responses as compared to 2D animated images. In summary, we emphasize that the computer animated images presented by our two software applications (GFA and ZFP) were effective and the shoaling response they elicited was statistically indistinguishable from that induced by live stimulus fish.

Perhaps due to our extensive habituation procedure, we observed no signs of fear responses in the current paradigm. This is reflected by the angular velocity values that remained consistently stable across the intervals of both the habituation and the stimulus presentation sessions. The temporal variability of angular velocity was also found stable and not different between the habituation and stimulus presentation sessions. Video-tracking measures, such as angular velocity, which quantify change in the direction of swimming have been shown to correlate well with motor patterns erratic movement and leaping (also called jumping or darting).[39] These latter motor patterns have been shown to appear in response to fear inducing stimuli or in aversive contexts.[35,39,40,42–45]

Diving, or remaining close to the bottom, has also been shown to appear under aversive conditions and has been argued to be a measure of fear or anxiety.[46] However, we have found this response somewhat variable in our prior research occasionally being present[42,47,48] but often also absent[39,45,49,50] even under fear inducing conditions. The controversies are likely due to the fact the fear responses of zebrafish are complex and context dependent.[50] Depending on the strength and the type of aversive cues, zebrafish may respond with specific behavioral strategies that may or may not involve diving. In the current study, we found a mild reduction of distance to the bottom only during the first 3 min of placing the fish in their respective test environment, a response that is likely to be due to human handling. Interestingly, unlike the hardly detectable change in the distance to bottom, the variability of this response did show a robust increase after the fish was placed in their experimental tank, a response that has been described before.[39,51] Notably, this variability reflects the temporal within individual variance, that is, the changes in the vertical position of the fish during each interval. These changes were very small for the first minute of the recording sessions and subsequently and gradually increased, which we argue represents a steady increase in the vertical exploration of the environment. Briefly, we argue that the temporal variability of the distance to bottom is a more reliable measure of fear and that fear (likely induced in our case by human handling) is associated with reduction of vertical exploration of the environment. Last, we note that none of the above potentially fear-associated behavioral measures distinguished our stimulus groups and thus

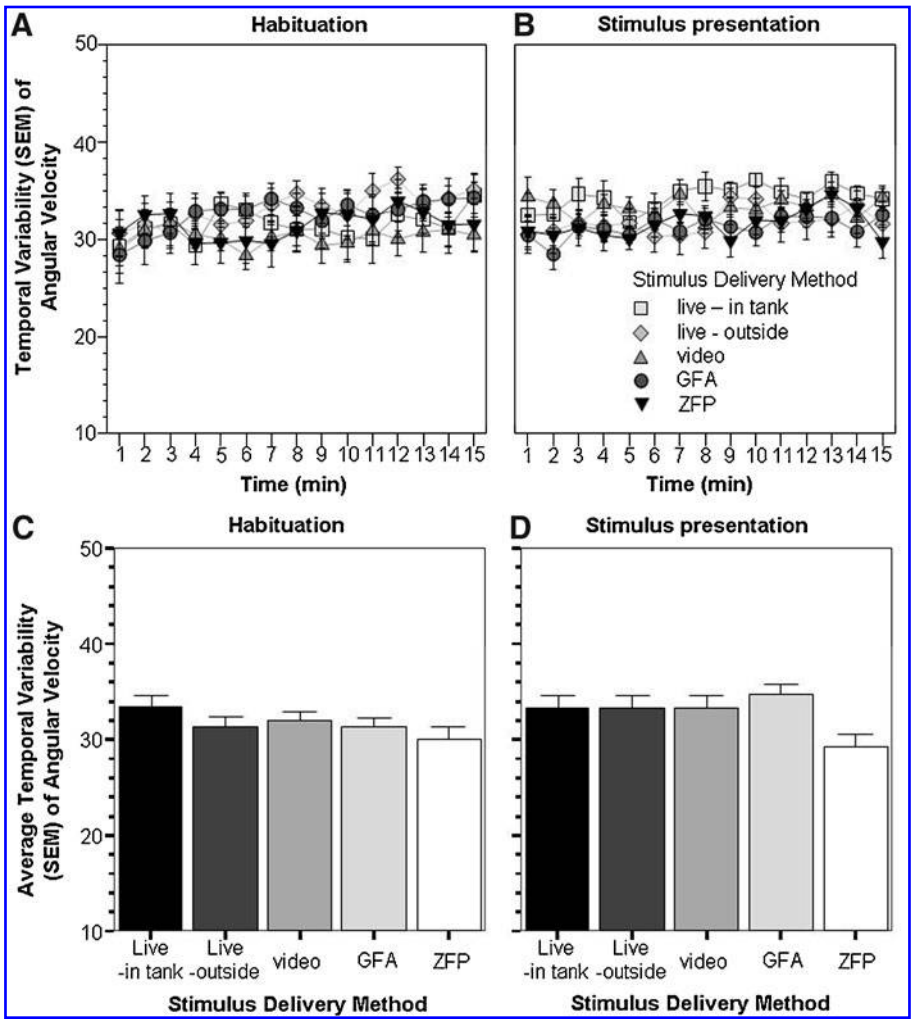

FIG. 7. Temporal within individual variability of angular velocity remains stable across both sessions. Mean ± SEM are shown. Each stimulus group had an $n = 30$ experimental fish except the one shown live stimulus fish inside the experimental tank, for which $n = 28$. **(A, B)** Show the angular velocity values obtained for each 1 min interval of the 15 min habituation session (no stimulus presented, **A)** and the stimulus presentation session **(B)**. **(C)** Habituation session and **(D)** stimulus presentation session show results averaged over the fifteen 1-min interval. Note that the within individual variability of angular velocity is reduced in zebrafish receiving animated images by ZFP compared with those receiving the images delivered by GFA.

we conclude that all stimuli employed in the current study had similar effects and did not differentially increase fear or anxiety in the experimental zebrafish.

In the current study we only focused on social attraction, shoaling, and did not explore several other aspects of social behavior. For example, we did not examine aggressive or agonistic responses and we also did not study reproductive behavior. At this point it is unknown whether the effectiveness of 2D animated conspecific images presented using our software applications would extend to these domains of social behavior. Further, numerous parametric aspects of the social stimuli (the presented conspecific images) their size, speed, number, color, length frequency of presentation, and so on may all need to be systematically varied to investigate what may constitute an optimal image presentation. Despite all these unknowns, however, the current study presents an optimistic outcome. Two-dimensional animated images of conspecifics are effective stimuli that induce robust and reliable social responses in zebrafish. These images are now deliverable by our ZFP software in a reliable and consistent manner across several operating systems and hardware platforms. However, notably, such images may also be presented by using the custom animation function of a number of commercially available slide presentation software applications, albeit with simpler functionality and more limitations in the animation. Thus, we conclude that visual stimulus based behavioral paradigms will be a cheap and simple approach to a range of behavioral studies with zebrafish.

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Disclosure Statement

No competing financial interests exist.

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