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High fidelity: Assessing zebrafish (*Danio rerio*) responses to social stimuli across several levels of realism

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ABSTRACT

Behavioral assays of zebrafish shoaling have recently been employed to investigate social behavior in zebrafish models of psychiatric disease. Many studies have developed simulated models of conspecifics to serve as alternatives to live shoals in order to examine specific cues that contribute to shoaling behavior. However, no studies have investigated the extent to which zebrafish prefer one stimulus over another when given the choice between two conspecific alternatives (live or simulated). In the present study, we employed a new, four-quadrant choice preference task that allowed zebrafish to swim freely between a live shoal and a motorized mobile shoal, a live shoal and playback of a video-recorded shoal, or a motorized mobile shoal and playback of a video-recorded shoal. Behavior tracking software was used to track subjects' movements in upper and lower quadrants on either side of the test arena. Subjects spent more time near the live shoal, especially in the lower quadrant, and exhibited different swim patterns in response to each simulated conspecific alternative, suggesting that zebrafish prefer a live shoal over models of lower fidelity.

1. Introduction

Zebrafish (*Danio rerio*) disease models are becoming increasingly powerful tools in behavioral research. Zebrafish express similar developmental, cognitive, and behavioral processes to other experimental vertebrate models, such as rodents (Williams et al., 2002; Miklósi and Andrew, 2006; Sison and Gerlai, 2010). Specifically, zebrafish are capable of complex learning processes, social behaviors, affective responses, and they exhibit an array of ethologically relevant behaviors (Gerlai, 2017b). Further, zebrafish models for motor neuron disease (Babin et al., 2014), anxiety (Stewart et al., 2012), drug addiction (Miller et al., 2013; Khan et al., 2017), Alzheimer's disease (Newman et al., 2014), and autism spectrum disorder (Maaswinkel et al., 2013) have greatly contributed to our understanding of these disorders. Importantly, many psychiatric diseases, such as autism and anxiety, include a social component, making social behavior in zebrafish a vital area of investigation in the pursuit of functional models of psychiatric disease. In order to increase confidence in the use of zebrafish models of social behavior, it is necessary to establish standards based on zebrafish preference for one stimuli versus another, with the goal of choosing experimental stimuli that elicit natural social behavior. Our findings indicate that zebrafish are able to differentiate between shoal models of varying fidelity and prefer to interact with live shoals over motorized or video shoals when given the choice between two shoal types. These data suggest that the choice of stimuli in models of zebrafish social behavior can affect the interpretation of the results.

1.1. Zebrafish shoaling

Shoaling behavior in social organisms allows for the transmission of social cues between group members, reducing the risk of predation and increasing overall foraging success (Buske and Gerlai, 2011). Laboratory-derived tests on social fish have shown that shoaling also likely reduces risk of parasite infection by first detecting the parasite, sending an alarm signal throughout the shoal, and then initiating a behavioral response to avoid the parasite (Stumbo et al., 2012). Group living provides protection from predators, enabling members of a shoal to act more boldly to gain fitness benefits (Guayasamin et al., 2017; Kareklas et al., 2018). The development of neurobehavioral assays has established reliable protocols to quantify shoaling behavior in zebrafish (Miklósi and Andrew, 2006; Miller and Gerlai, 2011). For example, past research has demonstrated systematic variations in zebrafish shoaling behavior related to age (Buske and Gerlai, 2011), shoal size (Gleason et al., 1977), biological sex (Snekser et al., 2010) and size of

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conspecifics (Bartolini et al., 2016). Further, zebrafish determine a shoal preference depending on similar phenotypes (Snekser et al., 2006), sex ratios (Etinger et al., 2008), predator factors (Oliveira et al., 2017), and food availability (Maaswinkel et al., 2013). Zebrafish shoaling behavior is innate, but experience within the shoal over time allows zebrafish to learn to identify conspecifics (Spence et al., 2008), a phenomenon that develops around three weeks of age and is driven primarily by visual cues (Dreosti et al., 2015).

In contrast to the development of a robust social response, a desolate social environment is detrimental to zebrafish fitness. Zebrafish anxiety has been investigated through pharmacological, behavioral, and genetic manipulation (Stewart et al., 2012). As a result, their prototypical fear-responses, such as freezing, thrashing, and diving, have been well described (Fox et al., 1997; Champagne et al., 2010; Cachat et al., 2011). Social isolation during zebrafish development leads to decreased shoal cohesion, dysfunctional dopamine transmission, chronic anxiety (Shams et al., 2017), and blunted cortisol responses to acute stressors (Giacomini et al., 2015). Anxiety can be caused by context or a cue and is independent of zebrafish phenotype; however, shoaling during development precludes many of the deleterious behavioral and physiological effects of stress (Quadros et al., 2016; Franks et al., 2018). Anxiety in zebrafish can be characterized by diving, freezing, and erratic movement (Kalueff et al., 2013). Clearly, a social environment is necessary for healthy zebrafish maturation and the transmission of important cues between conspecifics, thereby improving the fitness of individuals within shoals. Shoaling is complex and ethologically relevant, making zebrafish social interaction a critical area of study.

1.2. Alternative conspecific models

Researchers have attempted to isolate specific aspects of visual shoaling cues, such as inter-fish distance, swimming profile, and conspecific morphology by using alternative conspecific models, as zebrafish rely heavily on these visual cues (Butail et al., 2014; Qin et al., 2014). Zebrafish can distinguish between robotic models of varying fidelity. Robotic stimuli of lower fidelity do not elicit a shoaling response, even if multiple robots are designed to mimic a shoal; the presence of a free swimming robotic conspecific alters overall group cohesion and coordination in a small shoal of zebrafish (Butail et al., 2014). Higher fidelity models can attract single zebrafish and small shoals, but this attraction is weaker than the social response observed towards live conspecifics (Polverino et al., 2012; Ruberto et al., 2017). 3D-printed hyperrealistic models achieve an approach response similar to live conspecifics, but also evoke more freezing and more erratic swimming (Bartolini et al., 2016). These findings suggest visual cues on a robotic model of a conspecific must be finely tuned to match the efficacy of a live conspecific. Otherwise, robots may unintentionally elicit fear and avoidance responses in experimental zebrafish.

In contrast to robotics, screen-based conspecific presentation creates the opportunity for enhanced experimental manipulations and controls, allowing the researcher to eliminate tactile, aural, and chemical stimuli while only presenting visual stimuli to focal fish (Gerlai, 2017a). Using computer-animated images of fish, Saverino and Gerlai (2008) found color and shape influenced shoaling behavior of zebrafish whereas stripe pattern did not. Further research found that an image of a conspecific is rewarding to zebrafish (Al-Imari and Gerlai, 2008), and revealed no differences in the capabilities of live conspecifics, a video of conspecifics, and two moving images created by computer software to evoke approach responses (Qin et al., 2014). Based on these findings, more recent research has investigated zebrafish social behavior in response to animated conspecifics. Researchers have characterized the neurochemical changes associated with differential exposure to an animated conspecific (Saif et al., 2013) and used animated shoals to investigate the effects of different pharmacological substances on zebrafish social behavior (Fernandes et al., 2015). Chouinard-Thuly et al. (2017) describe the many parameters that affect the fidelity of animated zebrafish images presented alone and in tandem with rotoscoping or virtual reality techniques. Studies in other social fish have highlighted incremental changes that may hinder an animation's effectiveness (Gierszewski et al., 2017; Nakayasu et al., 2017). These studies underscore the utility of alternative conspecifics, but stress the care that must be taken when relying on them in lieu of live animals.

Previous investigators have examined a number of factors that affect shoaling tendency in zebrafish, but no systematic examination of zebrafish responses to stimuli of varying degrees of fidelity have been reported. Given that shoaling tendency appears to be affected by activity level, the dynamic responses of the stimulus fish, the number of stimulus fish in the shoal, as well as the potential interaction of stimulus fish with subjects, more investigation is needed to determine what social stimuli can be used in a laboratory setting to best elicit a natural shoaling response. Exploring the degree to which zebrafish respond to various stimuli provides an opportunity to further explore the nature of shoaling behavior in zebrafish. Although some research has suggested that the use of video generated models, 3D printed models, or computer generated animations can be used as an adequate substitute for live conspecifics while studying the social behavior of zebrafish, the present study reveals that zebrafish can distinguish between live conspecifics and other moving stimuli, and that zebrafish prefer live conspecifics over these lower-fidelity options.

2. Materials & methods

2.1. Animals

Male and Female (˜50:50 ratio) 6–12 month old EKK wild-type zebrafish ($N = 100$) were obtained from Aquatica Tropicals, Inc. and placed in a pre-trial 79.4 l (20 gal) home tank (61 × 42 × 31 cm). Each housing tank had a biofilter, heater, gravel substrate, environmental enrichment, and was maintained on a 12 L : 12 D cycle with a water temperature of 28 °C. Fish were fed commercial flake food once daily. Chemical quantification of ammonia, nitrite, and nitrate concentrations in the water were performed weekly to ensure zebrafish were kept in a healthy environment. All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at Christopher Newport University under protocol #2016-4.

2.2. Experimental setup and procedure

Fish were observed using the three-chamber social choice task similar (but not identical) to the apparatus described by Ariyasiri et al. (2018). Briefly, experimental fish were observed in a 9.5 L (2.5 gal.) experimental tank (30.5 cm wide × 20.3 cm high × 15.3 cm long) flanked by two identical tanks in which the test stimuli were placed. All three tanks were filled with conditioned 25C water to a depth of 16 cm so that experimental fish had the same range of vision for both stimuli presented. Three adjustable white barriers were placed around the tanks and the camera, increasing the amount of diffused light on the apparatus and reducing shadows in the arena. The barriers also reduced external visual stimuli that might influence subjects.

Three fish stimulus models of varying fidelity were employed: 1) One live shoal of four zebrafish of mixed sex. 2) MP4 video recording of the live shoal was taken and played on a 6 min loop with a frame size of 21 cm x 20 cm on an LCD screen (Dell Model P2314HF, full HD resolution of 1920 × 1080, refresh rate = 60 Hz with an opaque border to prevent glare. 3) An active mobile of flexible plastic models resembling zebrafish was suspended by thin wires from a motor-driven disk that rotated within the stimulus tank at a rate of 2 cm/s, the typical swim speed of wild zebrafish (Tierney et al., 2011). The live shoal was replaced halfway through the experiment due to the death of one of the

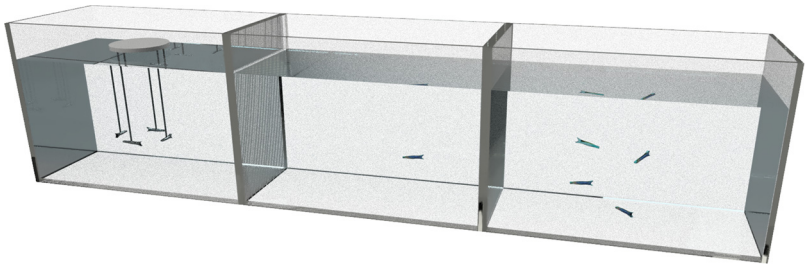

Fig. 1. Diagram of test apparatus illustrating a test between the Mobile and the Live Shoal. The setup consists of three identical 9.5 L (2.5 gal.) glass aquaria (each 30.5 cm wide × 20.3 cm high × 15.3 cm long; total width = 91.5 cm) filled with water to a depth of 16 cm. When video playback was tested, the associated tank on that side was replaced with the LCD monitor.

shoal fish. All four fish in the live shoal were replaced, and the video recording of the live shoal was re-recorded with the new fish.

Two conspecific stimuli were presented simultaneously in the conspecific tanks on either side of the experimental tank (Fig. 1). Each experimental animal underwent exposure to one combination of the three different stimuli: live shoal-motorized mobile, live shoal-video, and motorized lure-video. For each stimulus pairing, the position of each stimulus type was counterbalanced such that an equal number of trials was run with each stimulus on the left side as the number of trials with each stimulus on the right side. Between the tanks prior to and during the habituation period, opaque dividers were inserted to obstruct the view of the fish in the experimental tank during the habituation period. The rearmost wall and the floor of the experimental tank were covered with a white opaque layer throughout the trial in order to reduce visual distractions from the surrounding area and reduce shadows in the tank. Experimental fish were placed into the center tank and given a three-minute acclimation period before the tank dividers were removed to reveal the two conspecific groups. Subjects were then allowed to explore the observation tank for a 10 min free swim period in which locomotor behavior was recorded and analyzed.

2.3. Data collection

A Panasonic WV-CP504 SD5 camera on a Manfrotto adjustable tripod was placed one meter away from the experimental tank. Video was captured from the camera using a video digitizer board (Euryesys H264) on a Dell Precision T3610 computer running Windows 7 Pro for the operating system. Ethovision XT10 (Version 10, Noldus Information Technology) recorded and analyzed subjects' behavior in real time. The program's arena was adjusted to contain distinct upper left, upper right, lower left, and lower right quadrants (each quadrant constituted 25% of the total arena). The detection settings were adjusted so that stimuli were analyzed at 29 frames per second. After the removal of the dividers, Ethovision XT10 tracked and analyzed subjects' swimming behavior for the 10 min free swim period. To characterize zebrafish preferences, the program was directed to quantify time spent in each zone and number of zone transitions. In addition, swim velocity, standard deviation and variance in velocity, and number of moving and freezing occurrences was recorded to distinguish preference from exploration and/or anxiety behaviors, as zebrafish exhibit different swimming profiles when faced with a novel or anxiogenic stimulus than a socially appetitive stimulus (see Stewart et al., 2012).

Heatmaps were generated with Ethovision XT10 to visually depict the difference in subject zone localization between stimuli configurations. The software generated a heatmap for each trial based on the time spent in each area of the tank and then aggregated all heatmaps within each condition. Warmer colors denote greater time spent in those locations. Heatmaps were extracted with a resolution of 4462 × 2356 and a smoothing of 25 to most accurately portray the size of experimental zebrafish.

2.4. Statistical analysis

All statistical analyses were performed using IBM SPSS (V.24) for Windows. Data were analyzed with a Linear Mixed Model (LMM) using Type III Sums of Squares at $\alpha = 0.05$. The model was set with diagonal covariance structure for heterogeneous variance, and degrees of freedom for the denominator of mixed-model F ratios were adjusted according to the Maximum Likelihood estimator for LMM. When significant main effects or interactions were revealed, unplanned comparisons were made using the Bonferroni correction for familywise error. Because all stimulus pairings were counterbalanced to control for any potential position effects, the Side of a stimulus (left or right) was initially entered as a 2-level factor in the LMM. No substantial statistical interactions of Side were revealed. Given the advantages of the LMM, including flexibility in selecting the covariance structure, reduced sensitivity to unequal sample size, and its ability to handle missing data (Wang and Goonewardene, 2004), and the relatively stronger statistical power of LMM over mixed-factor ANOVA (Bolker et al., 2009), subsequent analyses were not collapsed across side position. Rather, the side for one particular member of a stimulus pair was randomly selected for the left and subsequent 3(Stimulus Pairing between) by 4(Quadrant Zone within) LMM analyses were conducted and results compiled for the following stimulus pairings: Mobile Model: Left – Video: Right (n = 19), Live Shoal: Left - Mobile Model: Right (n = 38), and Live Shoal: Left - Video: Right (n = 40). Descriptive statistics are reported as mean +/- s.e.m.

3. Results

3.1. Heatmap

Ethovision generates heatmaps using a proprietary algorithm. Aggregating individual traces for all subjects within a given condition created heatmaps for all conditions. Based on the heatmaps, subjects spent more time on the side of the live shoal when given a choice between live conspecifics and mobile or video stimuli, suggesting a preference for the live shoal (see Fig. 2). Further, subjects spent more time on the side of the mobile shoal (notably at the height of the mobile) versus the video shoal, and also spent more time in the middle of the tank in this condition. These results suggest a range of fidelity for the different conspecific models presented, with each model eliciting a unique response from subjects, and subjects demonstrating a clear preference for models of higher fidelity. Multiple statistical analyses were conducted in order to quantify the results observed from the heatmaps. These statistical results are reported with the higher fidelity model on the left.

3.2. Percent of cumulative duration within quadrant

When analyzing the percent of cumulative duration within quadrants, there was no significant main effect of Stimulus Pairing, F (2,336.54) = 0.10, p = .905; however, there was both a significant main effect of Quadrant, F (3,181.82) = 64.20, $p < .001$, as well as a significant interaction of Stimulus Pairing and Quadrant, F (6,181.65) = 7.14, $p < .001$. Across all stimulus pairings, subjects spent the greatest percentage of session time in the lower quadrant closer to the higher fidelity stimulus (M = 43.29%, SEM = 1.82) than

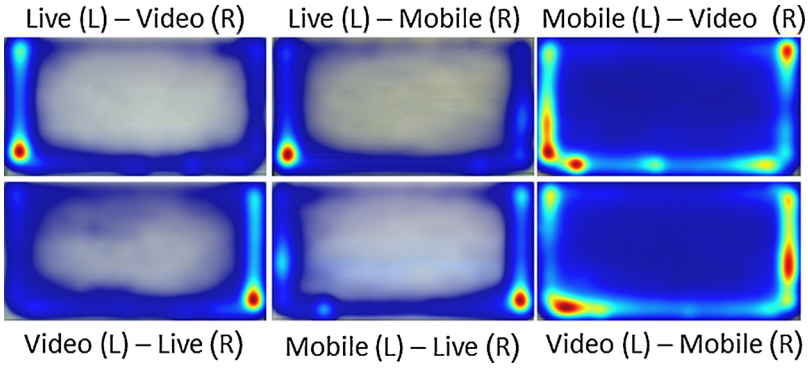

Fig. 2. Heatmap visualizations of subjects' position in the test arena. Each heatmap is labelled with the position of each stimulus (live, mobile, or video) noted in parentheses (R: right, L: left). Color intensity from cool to hot indicates an increasing number of visual samplings of the subject in the position such that cool colors (gray to blue) indicate relatively fewer samplings which include subjects in that particular location while warm to hot colors (yellow/orange to red) indicate relatively more samplings which include subjects in that particular location.

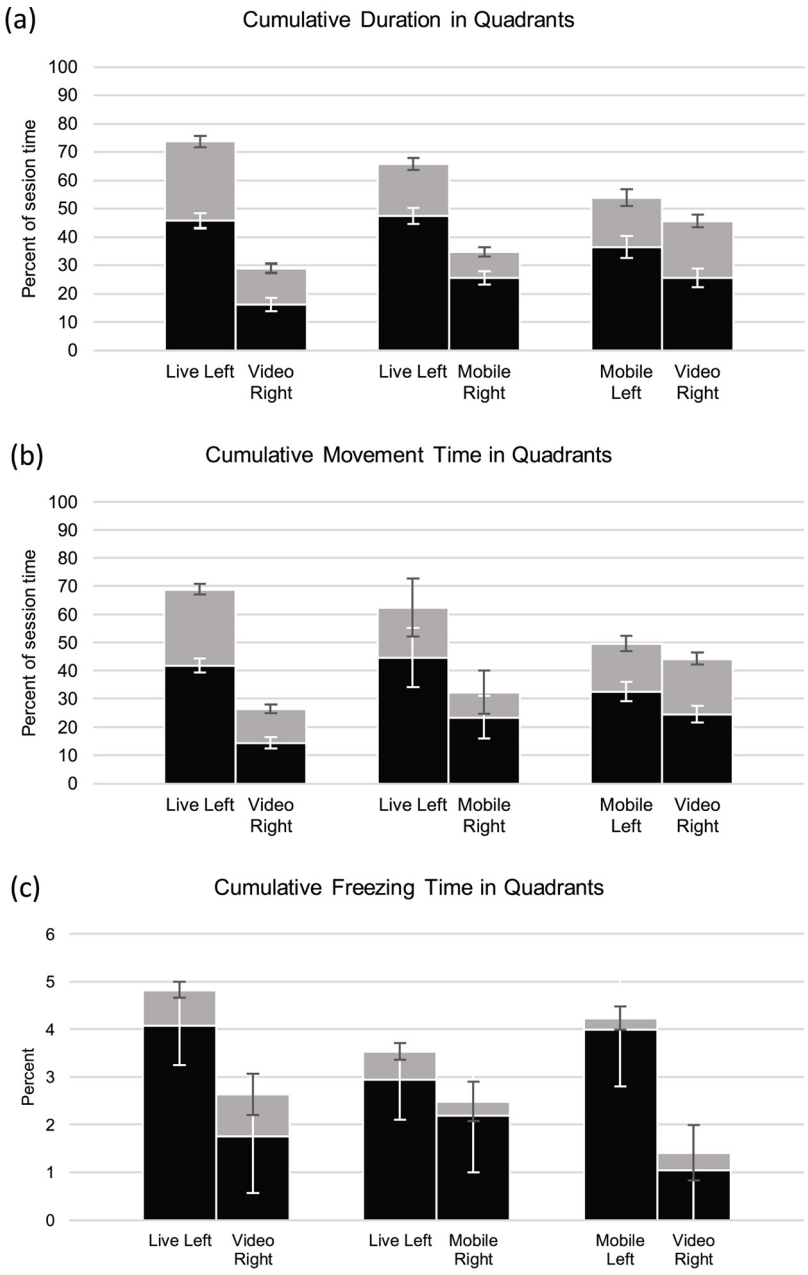

Fig. 3. Effects of stimulus condition on session time per quadrants. (a) total duration in quadrant (b) cumulative movement time in each quadrant (c) cumulative freezing time in each quadrant. Black tone represents lower zones. Gray tone represents upper zones. Error bars are +/- 1 *SEM*.

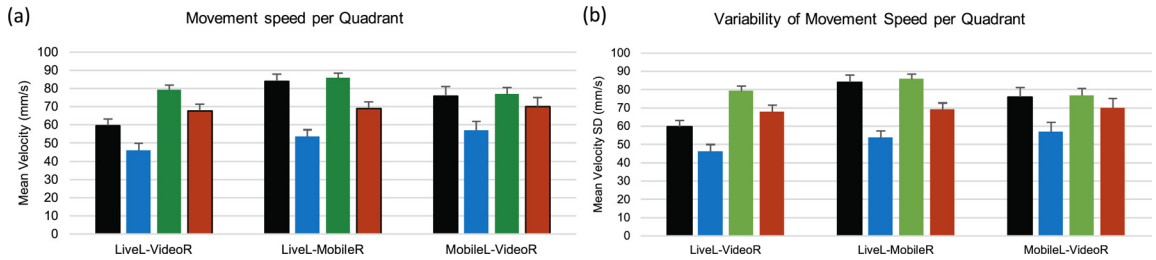

Fig. 4. Effects of stimulus condition on movement parameters per quadrant. (a) average subject velocity in mm/s (b) average *SD* in subject velocity in mm/s. Black tone represents the lower left quadrants. Blue tone represents the upper left quadrants. Green tone represents the lower right quadrants. Red tone represents the upper right quadrants. Error bars are +/- 1 *SEM*.

any other quadrant (see Fig. 3a). Furthermore, subjects spent the lowest percentage of session time in the upper quadrant closer to the lower fidelity stimulus (M = 14.08%, *SEM* = 1.06) than in any other quadrant. Across all stimulus pairings, the difference between the Upper-Left (M = 21.21%, *SEM* = 1.36) and Lower-Right (M = 22.43%, *SEM* = 1.56) quadrants was not significant. For the pairwise comparisons in the Live-Video stimulus pairing, subjects spent a significantly greater percentage of time in both the upper (M = 27.90%, *SEM* = 1.99) and lower (M = 45.82%, *SEM* = 2.68) quadrants on the Live stimulus side than in the upper (M = 12.90%, *SEM* = 1.65) and lower (M = 16.12%, *SEM* = 2.36) quadrants on the Video stimulus side. A similar pattern was found for the pairwise comparisons in the Live-Mobile stimulus pairing; subjects spent a significantly greater percentage of time in both the upper (M = 18.37%, *SEM* = 2.09) and lower (M = 47.49%, *SEM* = 2.75) quadrants on the Live stimulus side than in the upper (M = 9.20%, *SEM* = 1.58) and lower (M = 25.62%, *SEM* = 2.33) quadrants on the Mobile stimulus side. A slightly different pattern was revealed for the pairwise comparisons in the Mobile-Video stimulus pairing; subjects spent a significantly smaller percentage of time in the upper (M = 17.67%, *SEM* = 2.89) quadrant than the lower (M = 36.55%, *SEM* = 3.89) quadrant on the Mobile stimulus side, while there was no significant difference in the percentage of time they spent in either the upper (M = 20.13%, *SEM* = 2.20) or lower (M = 25.55%, *SEM* = 3.29) quadrants on the Video stimulus side.

3.3. Percent of session time moving

Analysis of the percent of session time subjects spent moving indicated no significant main effect for Stimulus Pairing, F (2,342.364) = 0.018, p = .982. However, there was a significant main effect for Quadrant, F (3,187.30) = 61.88, p < .001, as well as a significant interaction of Stimulus Pairing and Quadrant, F (6,187.113) = 8.51, p < .001. Across all stimulus pairings, subjects spent the greatest percentage of session time moving in the lower quadrant closer to the higher fidelity stimulus (M = 39.61%, *SEM* = 1.64) than any other quadrant (see Fig. 3b). Furthermore, subjects spent a lower percentage of session time moving in the upper quadrant closer to the lower fidelity stimulus (Mobile or Video; M = 13.56%, *SEM* = 0.984) than in any other quadrant. The difference between the Upper-Left (M = 20.66%, *SEM* = 1.30) and Lower-Right (M = 20.75%, *SEM* = 1.36) quadrants was not significant. For the pairwise comparisons in the Live-Video stimulus pairing, subjects spent a significantly greater percentage of session time moving in both the upper (M = 27.11%, *SEM* = 1.89) and lower (M = 41.74%, *SEM* = 2.41) quadrants on the Live stimulus side than in the upper (M = 12.02%, *SEM* = 1.53) and lower (M = 14.35%, *SEM* = 2.07) quadrants on the Video stimulus side. A similar pattern was found for the pairwise comparisons in the Live-Mobile stimulus pairing; subjects spent a significantly greater percentage of session time moving in both the upper (M = 17.75%, *SEM* = 2.00) and lower (M = 44.53%, *SEM* = 2.48) quadrants on the Live stimulus side than in the upper (M = 8.89%, *SEM* = 1.47) and lower (M = 23.39%, *SEM* = 2.04) quadrants on the Mobile stimulus side. A slightly different pattern was revealed for the pairwise comparisons in the Mobile-Video stimulus pairing; subjects spent a significantly smaller percentage of session time moving in the upper (M = 17.12%, *SEM* = 2.75) quadrant than the lower (M = 32.55%, *SEM* = 3.50) quadrant on the Mobile stimulus side, while there was no significant difference in the percentage of time they spent moving in either the upper (M = 19.76%, *SEM* = 2.05) or lower (M = 24.50%, *SEM* = 2.88) quadrants on the Video stimulus side.

3.4. Percent of session time freezing

Analysis of the percent of session time subjects spent freezing revealed a significant main effect for Quadrant, F (3,128.25) = 10.79, p < .001, but there was neither a significant main effect of Stimulus Pairing, F (2,201.54) = 0.324, p = .724, nor a significant interaction of Stimulus Pairing and Quadrant, F (6,128.37) = 0.29, p = .941. Across all stimulus pairings, subjects spent a significantly greater percentage of session time freezing in the lower quadrant closest to the higher fidelity stimulus (M = 3.67%, *SEM* = 0.56) than either the upper quadrant closest to the higher fidelity stimulus (M = 0.52%, *SEM* = 0.12) or the upper quadrant closest to the lower fidelity stimulus (M = 0.51%, *SEM* = 0.28) while there was no significant difference in the percentage of session time freezing between either lower quadrants (M = 1.67%, *SEM* = 0.79) (see Fig. 3c). Because there was no significant interaction of Stimulus Pairing and Quadrant, *post hoc* comparisons were not explored beyond the main effect of Quadrant.

3.5. Average velocity

Analysis of subject velocity showed main effects for both Stimulus Pairing, F (2,353.02) = 8.42, p < .001, and Quadrant, F (3,185.79) = 35.08, p < .001, as well as a significant interaction of Stimulus Pairing and Quadrant, F (6,185.90) = 2.43, p = .028. Across all quadrants, subjects in the Live-Mobile condition (M = 73.11 mm/s, *SEM* = 1.73) swam faster than the subjects in the Live-Video condition (M = 63.19 mm/s, *SEM* = 1.73); there was no significant difference in the average velocity between the subjects in Live-Video condition and the Mobile-Video (M = 69.87 mm/s *SEM* = 2.42) (see Fig. 4a). There was also no significant difference in average velocity between the subjects in the Mobile-Video and the Live-Mobile conditions. Across all Stimulus Pairings, subjects swam at the slowest average velocity in the lower quadrant closest to the higher fidelity stimulus (M = 52.17 mm/s, *SEM* = 1.78) and at the fastest average velocity in the upper quadrant closest to the lower fidelity stimulus (M = 80.59 mm/s, *SEM* = 2.48). There was no significant difference in the average velocity of subjects in the Upper-Left (M = 73.20 mm/s, *SEM* = 2.45) and Lower-Right quadrants (M = 68.93 mm/s, *SEM* = 2.40).

The significant interaction of Stimulus Pairing by Quadrant was explored by comparing cell means using unplanned comparisons. All the following pairwise comparisons in the Live-Video stimulus pairing were significant; subjects had the slowest average velocity in the lower quadrant on the same side as the Live Stimulus (M = 46.06 mm/s, *SEM*

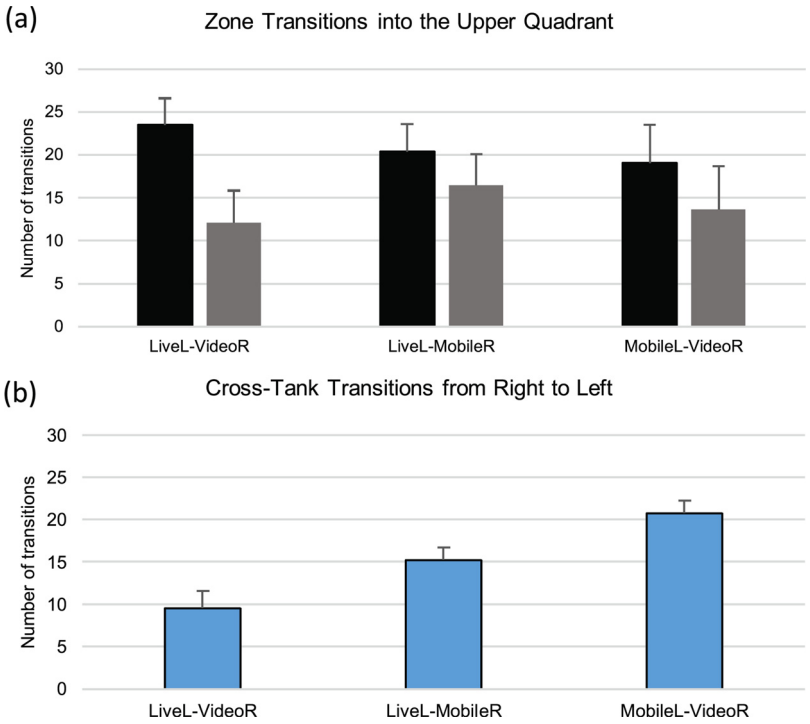

Fig. 5. Effects of stimulus condition on zone transitions. (a) transitions from lower quadrants to upper quadrants (b) transitions from right to left. Black tone represents left zones. Gray tone represents right zones. Blue tone represents both zones on one side. Error bars are +/- 1 *SEM*.

= 2.61), followed by the upper quadrant on the same side as the Live Stimulus (M = 59.63 mm/s, SEM = 3.59) then by the lower quadrant on the same side as the Video Stimulus (M = 67.78 mm/s, SEM = 3.64), Subjects in the Live-Video stimulus pairing had the fastest average velocity in the upper quadrant on the same side as the Video stimulus (M = 79.27 mm/s, SEM = 3.89).

A different pattern was found for the significant pairwise comparisons in the Live-Mobile stimulus pairing. Subjects had the slowest average velocity in the lower quadrant on the same side as the Live Stimulus (M = 53.63 mm/s, SEM = 2.68), followed by the lower quadrant on the same side as the Mobile Stimulus (M = 69.04 mm/s, SEM = 3.60) then by the upper quadrant on the same side as the Live Stimulus (M = 84.03 mm/s, SEM = 3.78). Subjects in the Live-Mobile stimulus pairing had the fastest average velocity in the upper quadrant on the same side as the Mobile stimulus (M = 85.75, SEM = 9.69). The identical pattern was revealed for the significant pairwise comparisons in the Mobile-Video stimulus pairing. Subjects had the slowest average velocity in the lower quadrant on the same side as the Mobile Stimulus (M = 56.83 mm/s, SEM = 3.79), followed by the lower quadrant on the same side as the Video Stimulus (M = 69.98 mm/s, SEM = 5.08) then by the upper quadrant on the same side as the Mobile Stimulus (M = 75.931 mm/s, SEM = 5.20). Subjects in the Mobile-Video stimulus pairing had the fastest average velocity in the upper quadrant on the same side as the Video stimulus (M = 76.75, SEM = 5.17).

3.6. Variability in velocity

One measure of variability in velocity is the standard deviation of each subject's swim speed during the session. Performing the LMM on each subject's SD score revealed a significant main effect of Stimulus Pairing, F (2, 262.80) = 3.52, p = .03, as well as Quadrant, F (3,159.36) = 6.52, p < .001, but there was no significant interaction of Stimulus Pairing and Quadrant, F (6,159.6) = 1.96, p = .07). Across all Quadrants, the only significant pairwise comparison between Stimulus Pairing groups indicated that subjects in the Live-Video group (M = 58.07 mm/s, SEM = 6.20) had lower variability in their swim speeds than subjects in the Live-Mobile Group (M = 81.42 mm/s, SEM = 6.28), but there was no difference in variability between either the Live-Video or Live-Mobile groups and the subjects in the Mobile-Video group (M = 67.99 mm/s, SEM = 8.76) (see Fig. 4b). Across all Stimulus Pairings, the only significant pairwise comparison between the Quadrants indicated that subjects in the Lower Left quadrant (M = 49.52 mm/s, SEM = 3.16) had lower variability in their swim speeds than subjects in the Upper Left quadrant, (M = 87.27 mm/s, SEM = 10.75) but there were no other differences between these quadrants and the Upper Right quadrant (M = 70.45 mm/s, SEM = 6.27) and the Lower Right quadrant (M = 69.40 mm/s, SEM = 10.49).

The LMM on the variance of each subject's swim speed during the session was performed using SPSS, but convergence was not achieved and the validity of the model fit was uncertain as a result. The results of the LMM are reported here but are not explored further. There was a significant main effect of Quadrant on subjects' variance in velocity during the session, F (3, 139.48) = 3.58, p = .016, but there was neither a significant main effect of Stimulus Pairing, F (2, 199.30) = 2.33, p = .10, nor a significant interaction of Stimulus Pairing by Quadrant, F (6, 139.43) = 1.52, p = .177.

3.7. Zone transitions

Because the number of transitions from a lower quadrant into an upper quadrant is almost always equal to the number of transitions from an upper quadrant to a lower quadrant, only transitions into the upper quadrant were analyzed by a 2 (Position: Left or Right) by 3 (Stimulus Pairing) LMM. There was a significant main effect for Position, F (1,180.56) = 4.66, p = 0.032. There was neither a significant main effect for Stimulus Pairing, F (2,180.31) = 0.125, p = .883, nor a significant interaction of Position by Quadrant, F (2, 180.31) = 0.642, p = .527. Across Stimulus Pairings, there were more zone transitions from the lower quadrant into the upper quadrant on the side of the higher fidelity stimulus (M = 20.97, SEM = 2.11) than from the lower quadrant into the upper quadrant on the side of the lower fidelity stimulus (M = 14.06, SEM = 2.41) (see Fig. 5a). While

the Position by Stimulus interaction was not significant, pairwise comparisons indicates that the source of the significant main effect of Position is in the Live-Video stimulus pairing. Subjects in the Live-Video condition had significantly greater transitions from the lower to the upper zone on the Live stimulus side (M = 23.5, SEM = 3.09) than from the lower into the upper zone on the Video stimulus side (M = 12.09, SEM = 3.75). No pairwise comparisons in the other Stimulus Pairing groups were significant.

3.8. Cross-tank transitions

A one-way ANOVA was performed on Right to Left transitions across all Stimulus Pairings. Stimulus Pairing had a significant main effect on transitions, F (2,94) = 11.06, $p <$ .001. Again, using the Bonferroni adjustment for unplanned comparisons, subjects in the Live-Video Stimulus Pairing had significantly fewer transitions (M = 9.55, SEM = 6.78) than either subjects in the Live-Mobile Stimulus Pairing (M = 15.24, SEM = 1.43) or the Mobile-Video Stimulus Pairing (M = 20.79, SEM 2.03) (see Fig. 5b). The difference in side transitions between the Live-Mobile Stimulus Pairing group and the Mobile-Video Stimulus Pairing group was not significantly different.

4. Discussion

Consistent with our hypotheses, individual zebrafish preferred to interact with a live shoal over a video or motorized shoal. Fish spent more time in the zone adjacent to the live shoal than the other conspecific models, suggesting zebrafish can discriminate between models of varying fidelity and prefer models of higher realism. Notably, the live shoal elicited a more distinct approach response than the video stimulus, characterized by more time spent near the live shoal. These findings suggest using lower fidelity conspecifics in zebrafish research could lead to specious conclusions regarding their innate social behavior; videos may not be as effective as live conspecifics. While video shoals do elicit approach from zebrafish, subjects can discriminate between this stimulus and other more realistic options.

Our findings contrast with other findings that indicate that live conspecifics offer no advantage over animated or recorded conspecifics (Savarino and Gerlai, 2008; Qin et al., 2014). However, these studies looked for the presence of an approach response in general, which we observe, but not the extent to which a stimulus invites approach. Unlike other binary choice studies (e.g. aquatic T-maze), the current methodology allows researchers to measure the relative degree of subjects' preference between two different test stimuli. We observed a greater preference for live conspecifics than a video playback. Preference for the live shoal may be partially explained by interactions between the stimulus fish and experimental subjects; although the fish are separated, they can still see each other and therefore can respond to visual cues such as reciprocal approaches and swim patterns. To examine the role of visual interactions in zebrafish social preference, these interactions could be blocked by removing the ability of the stimulus shoal to see the subject by installing a one-way window, as has been done by Polverino et al. (2012). Alternatively, if interactions do indeed contribute to the fidelity of a stimulus, researchers may be able to augment the realism of a recorded shoal. For instance, a two-way CCTV feed could be established between a live shoal and a subject, enabling the "video" to interact with the subject. These juxtapositions would indicate whether live conspecifics are advantageous due to innate social recognition rather than an attraction to interactive response.

In addition to an overall preference for a live shoal, subjects also spent more time near the motorized model over the video shoal, suggesting that the robotic model is of higher fidelity than the video. This finding is inconsistent with previous research that demonstrates the intrinsic rewarding power of animated conspecifics (Al-Imari and Gerlai, 2008), the greater fidelity of animated predators than robotic predators (Ladu et al., 2015), and our own perceptions of the differences between the two conspecific models. However, because the motorized replicas do not interact with the subject, have fixed swimming paths, and are morphologically the most dissimilar to live fish (Ruberto et al., 2017), it is important to differentiate between a high-fidelity model and a novel object, as both novelty and realism are uniquely capable of evoking an approach response in zebrafish (Lucon-Xiccato and Dadda, 2014). When faced with an appetitive stimulus, zebrafish tend to localize themselves near the object and remain there. Meanwhile, when presented with a novel object, zebrafish tend to inspect it, expressing an affinity to novelty similar to that of rodents (Lucon-Xiccato and Dadda, 2014). This inspection is characterized by different swimming patterns, including a continuous cycle of approach and retreat, more distance traveled near the object, and occasional anxiety-like responses (Hamilton et al., 2017; Stewart et al., 2012). In the present experiment, fish swam with a higher velocity near the motorized shoal than the video shoal when the live shoal was presented on the other side, despite spending the same amount of time near both stimuli. Together, these observations indicate subjects traveled greater amounts within zones adjacent to the motor, reflecting exploratory behavior rather than static social preference as subjects traveled greater amounts within the zones adjacent to the motorized shoal. Further, in the Motor - Video conditions, a much greater number of left-right zone transitions were observed, reflecting continual approaches and retreats (Hamilton et al., 2017). In addition, subjects spent more time in the lower-left quadrant of the tank in both Motor - Video conditions indicating that there was no definitive preference to either stimuli, and the zebrafish introduced to these conditions responded in a stressed manner, staying in close proximity to the bottom of the tank. Future projects might consider a home base from which subjects could choose to explore a novel object or interact with a recorded shoal (e.g. Stewart et al., 2010). Quadrant localization in our results suggest that zebrafish prefer the motor shoal over the video shoal; however, further analysis is needed to determine the fidelity of the robotic stimulus.

To further investigate which stimuli are preferred, it is necessary to consider the well-catalogued anxiety behaviors exhibited by zebrafish during exploration (Champagne et al., 2010). More diving occurred only on the side closer to the higher fidelity stimulus; however, the lack of interactions for diving and thrashing indicate no stimuli in this study were aversive (Hamilton et al., 2017; Maximino et al., 2018). In the Motor - Video condition, subjects spent more time freezing near the motorized shoal, suggesting that the motorized model is a novel object rather than socially appetitive stimulus, because inspection is sometimes characterized by fear-responses (Stewart et al., 2010). At the same time, more freezing was observed adjacent to the live shoal when the video was presented on the opposite side. In this case, subject location is largely influenced by interactions with the live shoal (Polverino et al., 2012); if the live shoal was unmoving at the bottom of the stimulus tank and interacting with the subject, the subject would remain at the bottom as well.

To advance the understanding of social behavior in zebrafish, future studies might investigate how phenotype influences shoaling. Previous studies have noted sex differences in zebrafish shoaling choice and group preference (Snekser et al., 2010). Zebrafish were previously thought to utilize simple broadcast spawning during reproduction, but their reproductive strategy is now seen as more complex; males compete for a sexually operational female (Pyron, 2003). Another innate zebrafish behavior worth examination is the impact of social cues on the strength of a social reaction. Zebrafish use cues from their external environment to ascertain dominance hierarchies, a process known as social eavesdropping (Abril-de-abreu et al., 2015). Future projects may artificially establish dominance hierarchies in shoals by varying gender and size in order to examine zebrafish preference, delineating the extent to which eavesdropping occurs and how much it affects social decision-making (Jordan et al., 2010). In addition, social behavior is influenced by genetic, developmental, and pharmacological factors. Medical models have been established in zebrafish and used to

investigate the nature of many diseases. A zebrafish model of autism spectrum disorder (ASD; Maaswinkel et al., 2013) and fetal alcohol spectrum disorder (FASD; Fernandes and Gerlai, 2009), of which social impairment is a hallmark, have recently been developed, and our four-quadrant paradigm would allow experimenters to examine whether these animals have a deficit in recognizing social settings or experience an aversion to social situations. Previous research has also investigated toxicology models on the behavioral response of zebrafish with psychoactive substances such as nicotine, ethanol (Miller et al., 2013), anxiolytics (Hamilton et al., 2017), and natural alarm signals (Speedie and Gerlai, 2008). Using a social organism such as zebrafish to understand the effects of these agents could provide useful insight into how these substances affect social disorders. Similarly, researchers might examine how pollutants affect zebrafish shoaling, because pollutants may present real environmental challenges on an ethologically relevant behavior.

The results of the current study illustrate the discriminative capacity of zebrafish in social settings. We observed that zebrafish socially preferred a live shoal over a motorized or video shoal, and also preferred the motorized shoal over video playback of conspecifics. However, depending on the particular objective for a given experiment, alternative models can still provide experimental efficacy. When investigating shoaling behavior in zebrafish, experimenters should consider the type of visual stimuli to employ, because not all models are equally advantageous. Presented individually, the three conspecific modalities appear to invoke similar social behavior, although a robotic model evokes some behaviors representative of anxiety or exploration (Qin et al., 2014; Ruberto et al., 2017). However, these alternative models forgo certain visual components, such as the visual interaction and/or familiarity between stimulus and subject, that may affect how zebrafish models of psychiatric disorder respond to a social stimulus (Maaswinkel et al., 2013). Thus, care must be taken to determine which facets of a visual social stimulus are negligible for the model at hand when using an alternative conspecific model. Further, given the complexity of social behavior at both group and individual levels, multiple analyses should be incorporated in order to delineate the many behavioral responses that can occur. Understanding the fundamental social behavior of zebrafish can in turn help us develop animal models for numerous psychiatric disorders marked by social dysfunction, including ASD, anxiety, FASD, and depression.

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Declarations of interest

The authors have no competing interests.

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