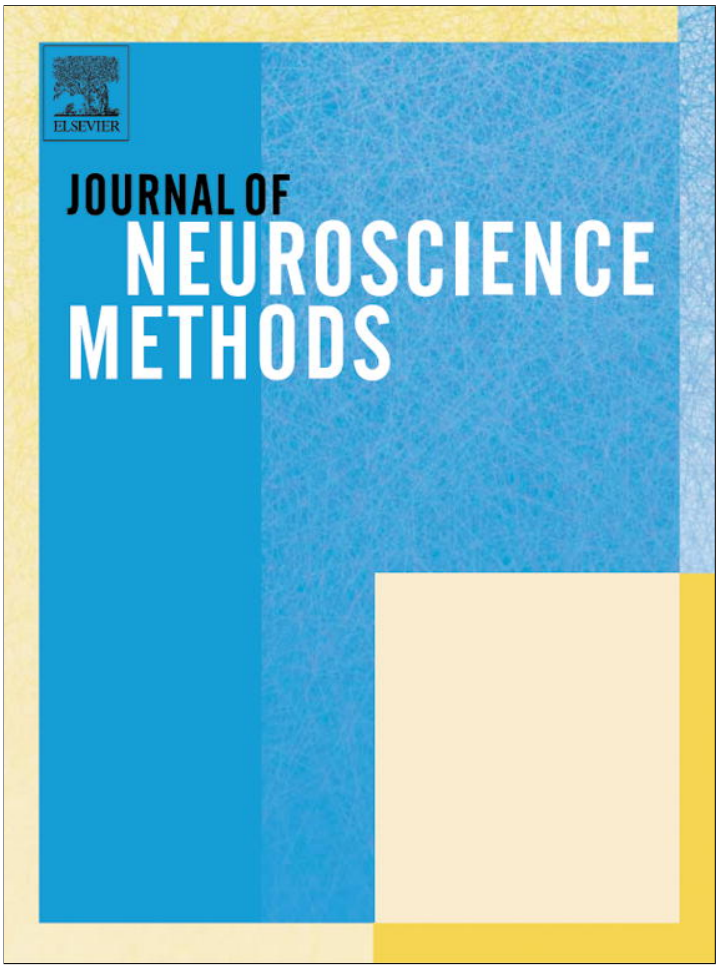

(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

Journal of Neuroscience Methods 210 (2012) 266–271

Contents lists available at SciVerse ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Automated high-throughput neurophenotyping of zebrafish social behavior

Jeremy Green[a], Christopher Collins[a], Evan J. Kyzar[a], Mimi Pham[a], Andrew Roth[a], Siddharth Gaikwad[a], Jonathan Cachat[a], Adam Michael Stewart[a], Samuel Landsman[a], Fabrizio Grieco[b], Ruud Tegelenbosch[b], Lucas P.J.J. Noldus[b], Allan V. Kalueff[a,c,*]

[a] Department of Pharmacology and Neuroscience Program, Tulane University Medical School, 1430 Tulane Avenue, New Orleans, LA 70112, USA
[b] Noldus Information Technology BV, Nieuwe Kanaal 5, Wageningen, The Netherlands
[c] Zebrafish Neuroscience Research Consortium (ZNRC) and ZENEREI Institute, 309 Palmer Court, Slidell, LA 70458, USA

HIGHLIGHTS

- Zebrafish are an excellent model species to study complex social phenotypes.
- We describe a novel methodology for automated video-tracking of zebrafish shoaling.
- Our method is bi-directionally sensitive to various experimental manipulations.
- A significant correlation was found between novel and traditional (manual) analyses of shoaling.

ARTICLE INFO

Article history:
Received 15 June 2012
Received in revised form 12 July 2012
Accepted 23 July 2012

Keywords:
Zebrafish
Social behavior
Shoaling
Automated quantification
Video tracking

ABSTRACT

Zebrafish (*Danio rerio*) are rapidly becoming an important model organism in neuroscience research, representing an excellent species to study complex social phenotypes. Zebrafish actively form shoals, which can be used to quantify their shoaling behaviors, highly sensitive to various experimental manipulations. Recent advances in video-tracking techniques have enabled simultaneous tracking of multiple subjects, previously assessed by manual scoring of animal behavior. Here we examined the effect of group-size in the shoaling paradigm (ranging from 2 to 8 fish), and evaluated the ability of novel video-tracking tools to accurately track an entire shoal, compared to traditional manual analysis of shoaling phenotypes. To further validate our approach, the effects of the psychotropic drugs lysergic acid diethylamide (LSD) and 3,4-methlenedioxymethamphetamine (MDMA), as well as exposure to alarm pheromone, previously shown to affect zebrafish shoaling, were examined. Overall, a significant difference in group size was shown in the 2-fish vs. the 3-, 4-, 5-, 6-, 7- and 8-fish groups. Moreover, both LSD and MDMA treatments reduced shoaling (assessed by increased inter-fish distance) as well as proximity (time spent together) among fish. In contrast, exposure to alarm pheromone yielded an increase in shoaling and in proximity in a time-dependent manner. Importantly, a highly significant correlation for manual vs. automated analyses was revealed across all experiments. Collectively, this study further supports the utility of zebrafish to study social behavior, also demonstrating the capacity of video-tracking technology to assess zebrafish shoaling in a high-throughput and reliable manner.

1. Introduction

The characterization of social interactions in both humans and animals is a key approach for studying social behavior (Miller et al., 1987; Potegal et al., 1993; Price et al., 1994; Sassenrath and Chapman, 1976; Saverino and Gerlai, 2008). However, the biological mechanisms underlying social behavior in vertebrates are complex, and remain poorly understood. Deficits in social interactions represent a common endophenotype of various neurobehavioral disorders, including schizophrenia (Figueira and Brissos, 2011) and autism (Veness et al., 2012).

Due to their fully characterized genome, robust behavioral responses and high-throughput nature, zebrafish (*Danio rerio*) have emerged as a complementary model in biomedical research (Cachat et al., 2011; Kyzar et al., 2012; Stewart et al., 2011). While primates and rodents have traditionally been utilized to study the

Abbreviations: ANOVA, analysis of variance; LSD, lysergic acid diethylamide; MDMA, 3,4-methlenedioxymethamphetamine; SIM, Social Interaction Module.

* Corresponding author at: Department of Pharmacology, SL-83, Tulane University Medical School, 1430 Tulane Ave., New Orleans, LA 70112, USA.
Tel.: +1 504 988 3354.
E-mail address: avkalueff@gmail.com (A.V. Kalueff).

0165-0270/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneumeth.2012.07.017

genetic and neural underpinning of social interactions (Fano et al., 2001; Ribeiro Do Couto et al., 2009; Sassenrath and Chapman, 1976), zebrafish can also be used for examining both normal and aberrant social behavior (Miller and Gerlai, 2007). Shoaling, an important evolutionarily conserved behavior, has long been identified in zebrafish (Mc and Bradner, 1998; Reyhanian et al., 2011; Ward et al., 2008), representing the interaction of a number of animals moving together in coordinated movements (Buske and Gerlai, 2011b; Krause et al., 2000). In zebrafish, shoaling is an innate behavior maintained at a relatively stable and high level throughout the lifespan (Miller and Gerlai, 2007), but is also modulated by social learning (Engeszer et al., 2004). Extensively used in zebrafish research, shoaling assays have been used to study ontogenesis (Fukuda et al., 2010), effects of environmental stressors (Brierley and Cox, 2010), behavioral organization (Krause et al., 2000), genetic factors (Wright and Krause, 2006; Wright et al., 2006, 2003) and pharmacological modulation (Buske and Gerlai, 2011a; Grossman et al., 2011; Kurta and Palestis, 2010; Riehl et al., 2011; Speedie and Gerlai, 2008).

Conventional shoaling tests have long relied upon manual analysis of easily quantifiable endpoints collected from photographs or video-captured static images. Simpler paradigms have included measuring the preference of a single zebrafish placed in a central compartment of a test tank flanked by two adjacent compartments which contain a shoal of conspecifics or are empty (Wright and Krause, 2006). More recent attempts to assess the internal dynamics of association among fish in free-swimming shoals have focused predominantly on measuring inter- and intra-fish distances over the course of a series of video frames (see Pham et al., 2012; Miller and Gerlai, 2007 for review).

While computationally based programs have been developed to quantify several parameters of group cohesion in zebrafish, locations of the animals must still be coded manually (Miller and Gerlai, 2007). Thus, the previous applications of video-tracking technology in shoaling tests have focused on increasing the efficiency and speed of manual coding, and not full-scale automation. In particular, no methodology was available that enables an automatic identification of freely swimming fish location within a shoal from a video source.

Here we apply the Social Interaction Module (SIM; Noldus, 2011) of EthoVision XT8.5 software (Noldus Information Technology, Wageningen, Netherlands) for detecting multiple unmarked animals in a social context, capable of assessing zebrafish shoaling behavior by simultaneously tracking all fish and recording dynamic changes in social behavior between the subjects. Overall, this approach offers a novel, high-throughput method of measuring zebrafish shoaling with its temporal dynamics on par with traditional manual analyses, as well as the ability to track alterations in shoaling behavior evoked by various experimental manipulations.

2. Methods

2.1. Animals and housing

A total of 171 adult (5–8-month-old) 'wild type' short-fin zebrafish (~50:50 male:female ratio) were obtained from a commercial distributor (50 Fathoms, Metairie, LA). All fish were given at least 14 days to acclimate to the laboratory environment and housed in groups of 20–30 fish per 40-L tank. Tanks were filled with filtered system water and maintained at 25–27 °C. Illumination (1000–1100 lx) was provided by ceiling-mounted fluorescent lights on a 12-h cycle (on: 6:00 h, off: 18:00 h) according to the standards of zebrafish care (Westerfield, 2000). All fish used in this study were experimentally naïve and fed Tetramin Tropical Flakes (Tetra USA, Blacksburg, VA) twice a day. Following behavioral testing, the animals were euthanized in 500 mg/L Tricaine (Sigma–Aldrich, St. Louis, MO).

2.2. Behavioral testing and manual analyses of shoaling data

Behavioral testing was performed between 11:00 and 15:00 h using tanks with water adjusted to the holding room temperature. Prior to testing, fish were pre-exposed in a 1-L plastic beaker for 20 min to either drug-treated or drug-free vehicle solution. During testing, zebrafish behavior was recorded by 2–3 highly trained observers, manually scoring different behavioral endpoints (inter-rater and intra-rater reliability >0.85) with subsequent automated analysis of recordings by EthoVision XT8.5 software paired with the SIM add-on package.

The shoaling test, assessing social/group behavior in zebrafish, was chosen based on the sensitivity of zebrafish shoaling to various psychotropic drugs and experimental manipulations (Grossman et al., 2010; Saverino and Gerlai, 2008; Speedie and Gerlai, 2008). Experiment 1 examined the effect of group size (number of subjects) in the shoaling paradigm. In this experiment, 7 groups of zebrafish, differing in sample size (from 2 to 8) were placed for 20 min in 1-L plastic beaker (with drug-free water) for acclimation, and group-tested in the novel tank observation apparatus (a 1.5-L trapezoidal Plexiglas tank 15 cm height × 28 cm top × 23 cm bottom × 7 cm width; Aquatic Habitats, Apopka, FL). This experiment allowed us to establish optimal shoal size (4 fish) for generating robust behavioral data without slowing computing time or high degree of subject overlap, representing a common problem with bigger shoals.

In Experiment 2, groups of 4 zebrafish (n = 8–12 per drug) were pre-exposed in a 1-L plastic beaker for 20 min to either drug-treated water (100 μg/L LSD or 80 mg/L MDMA) or drug-free water, and group-tested (three 4-fish cohorts per trial) in the novel tank. This experiment was used to confirm the sensitivity of our method to pharmacological modulation of zebrafish behavior by compounds that can *disrupt* shoaling.

Finally, in Experiment 3, groups of 4 fish (n = 8 in each cohort) were allowed to acclimate in the novel tank for 3 min, prior to being treated with 7 mL of drug-free control water or freshly extracted alarm pheromone, which were added directly to the novel tank (see Cachat et al., 2010; Egan et al., 2009 for details of alarm pheromone extraction) prior to 6-min video-recording. This experiment was used to confirm the sensitivity of our method to experimental modulation of zebrafish behavior by stressors that *increase* shoaling cohesion.

All zebrafish shoaling behavior was video-recorded for 6 min, and manually analyzed using 24 screenshots made every 15 s over the entire observation period. A total of 72 screenshots (24 per each shoal) per drug were used for analyses in this study, similar to Grossman et al. (2010) and Pham et al. (2012). Each screenshot was calibrated to the size of the tank and analyzed by trained observers, measuring the distances (cm) between each fish in the group using ImageTool software (University of Texas Health Sciences Center, San Antonio, TX), and averaging this data to obtain an average inter-fish distance per screenshot, as described in Grossman et al. (2010).

2.3. Video-tracking and track analysis

Recorded videos were also analyzed with EthoVision XT8.5 software (Grieco et al., 2011), as described previously (Cachat et al., 2011), with the addition of the SIM. All arenas were calibrated across the bottom wall of the tanks, and the calibration axes were placed to designate the origin (0,0) at the tank center. Behavioral data were exported to Excel to generate total and per-minute plots for each endpoint. The SIM (Noldus, 2011) is an add-on to

the EthoVision XT program that enables tracking multiple subjects across an entire trial, using either color marking tracker or center-point detection. Since marking zebrafish with unique colors is methodologically difficult, center-point detection of unmarked animals (using the same algorithms as the marker-assisted tracking) was chosen as the default setting for all experiments, followed by averaging data for each group. EthoVision XT software analyses each frame and distinguishes the object(s) from the background on the basis of their greyscale/brightness values, extracting the coordinates of the geometric center and surface area for each object per frame (see Noldus, 2011; Grieco et al., 2011 for methodological details). Average inter-fish distance was calculated by averaging inter-fish distances between all members of the shoal. Inter-fish distance was defined as the distance between two subjects as measured from the center point of each fish. Proximity duration (s) was defined here as the average amount of time a subject spent close (within 0.5 cm) to another subject. In all experiments, the subject loss due to misdetection by video-tracking software was <2%.

2.4. Statistical analysis

The experimental data was analyzed using one-way ANOVA (factor: treatment or shoal size), one-way ANOVA with repeated measures (time) followed by post hoc Tukey testing for significant ANOVA data, or by non-paired Mann–Whitney *U*-test, where appropriate. Comparison of manual and SIM-generated data was determined by Spearman correlation. Data were expressed as mean ± SEM; significance was set at $p < 0.05$ in all experiments of this study.

3. Results

In Experiment 1, there were no significant differences in inter-fish distance between groups of 3, 4, 5, 6, 7 and 8 fish for manual or automated recordings (Fig. 1). The 2-fish shoal displayed a smaller inter-fish distance vs. the other groups in both manual ($F_{(6,167)} = 12.8$, $p < 0.0001$) and automated data ($F_{(6,41)} = 13.9$, $p < 0.0001$; Fig. 1). Comparing the manual results to the SIM-generated data, we found a similar pattern, with significant correlation between manual and automated data for each of the groups ($R = 0.7$, $p < 0.0001$). There was also a strong negative correlation between inter-fish distance and proximity for the 2-fish shoals ($R = -0.77$ to -0.90, $p < 0.01$–0.05).

Experiment 2 (Fig. 2) assessed the effect of LSD and MDMA on zebrafish shoaling. Both hallucinogenic drugs significantly increased inter-fish distance relative to controls in both manual ($F_{(2,53)} = 21.7$, $p < 0.0001$) and SIM-generated recordings ($F_{(2,53)} = 68.7$, $p < 0.0001$). In addition to inter-fish distance, we also assessed proximity duration, which was significantly decreased vs. control by both drugs ($F_{(2,53)} = 12.8$, $p < 0.05$; Fig. 3). As in Experiment 1, there was significant correlation ($R = 0.80$, $p < 0.0001$) between manually recorded and SIM-generated shoaling data. The LSD-treated group also showed significant correlation ($R = -0.40$, $p < 0.05$) between inter-fish distance and proximity (albeit the control and MDMA-treated groups did not reach significance).

Having demonstrated the ability of SIM to accurately track a decrease in shoaling by LSD and MDMA treatments, we designed Experiment 3 to examine the efficacy of our approach in tracking a tightening of the shoal evoked by alarm pheromone, a procedure generally well-known to evoke stress responses in zebrafish, including a marked tightening of the shoals (Jesuthasan and Mathuru, 2008; Parra et al., 2009; Speedie and Gerlai, 2008). Automated data shows that alarm pheromone significantly decreased inter-fish distance (tighter shoals) as compared to the controls in both manual ($p < 0.0001$) and SIM-generated data ($p < 0.001$), which

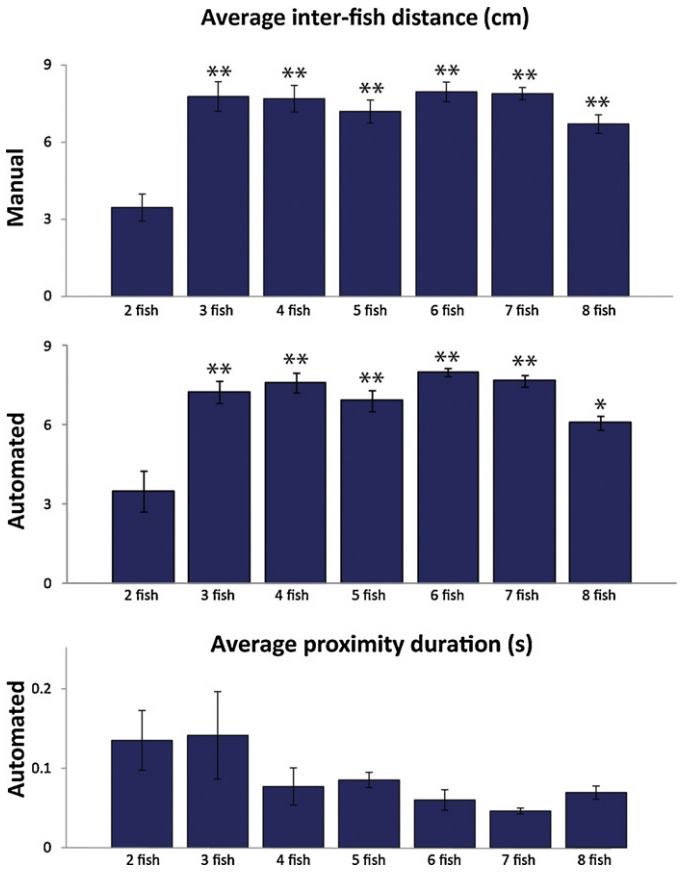

Fig. 1. Behavioral effects of different shoal sizes (2–8 fish) on zebrafish in the novel tank (Experiment 1). Shoaling endpoints (average inter-fish distance and average proximity duration) were obtained in the standard 6-min shoaling paradigm in the novel tank ($n = 6$–8 per group). Data was generated by Noldus EthoVision XT 8.5 software using side-view video-recording. Significant correlation was demonstrated between manual and automated data for each of the groups (Spearman correlation; $R = 0.7$–0.9, $p < 0.0001$). $^{*}p < 0.05$, $^{**}p < 0.001$ vs. 2-fish shoal cohort by post hoc Tukey test for significant ANOVA data.

was especially pronounced within the first 90 s of treatment (Fig. 2). Since alarm pheromone is a fast-acting stressor inducing rapid, short-lasted behavioral effects in zebrafish, we further examined the time-course of its effects on zebrafish shoaling using 15-s time bins. Although showing no significance vs. control for cumulative proximity ($p < 0.1$), the time course of alarm pheromone actions shows some immediate responses to alarm pheromone, as detected by SIM within 90 s of the exposure (Figs. 2 and 3).

A significant correlation ($R = 0.15$, $p < 0.001$) was found between manual and SIM-generated inter-fish distance data (albeit statistically significant, this correlation is rather weak in this experiment, due to the complex, dynamic nature of alarm pheromone action on zebrafish (Fig. 2) that increases the overall variance of shoaling data). Finally, a significant negative correlation of the SIM-generated inter-fish distance with proximity ($R = -0.45$, $p < 0.0001$) was also observed for the experimental group in this study.

4. Discussion

This study introduces a novel automated video-tracking method which is capable of assessing zebrafish shoaling behavior (without the need to mark individual fish), and is bi-directionally sensitive to experimental manipulations that affect zebrafish shoaling responses. Complementing currently used zebrafish shoaling assays (Buske and Gerlai, 2011a; Grossman et al., 2011; Kurta and Palestis, 2010; Riehl et al., 2011; Speedie and Gerlai, 2008), this

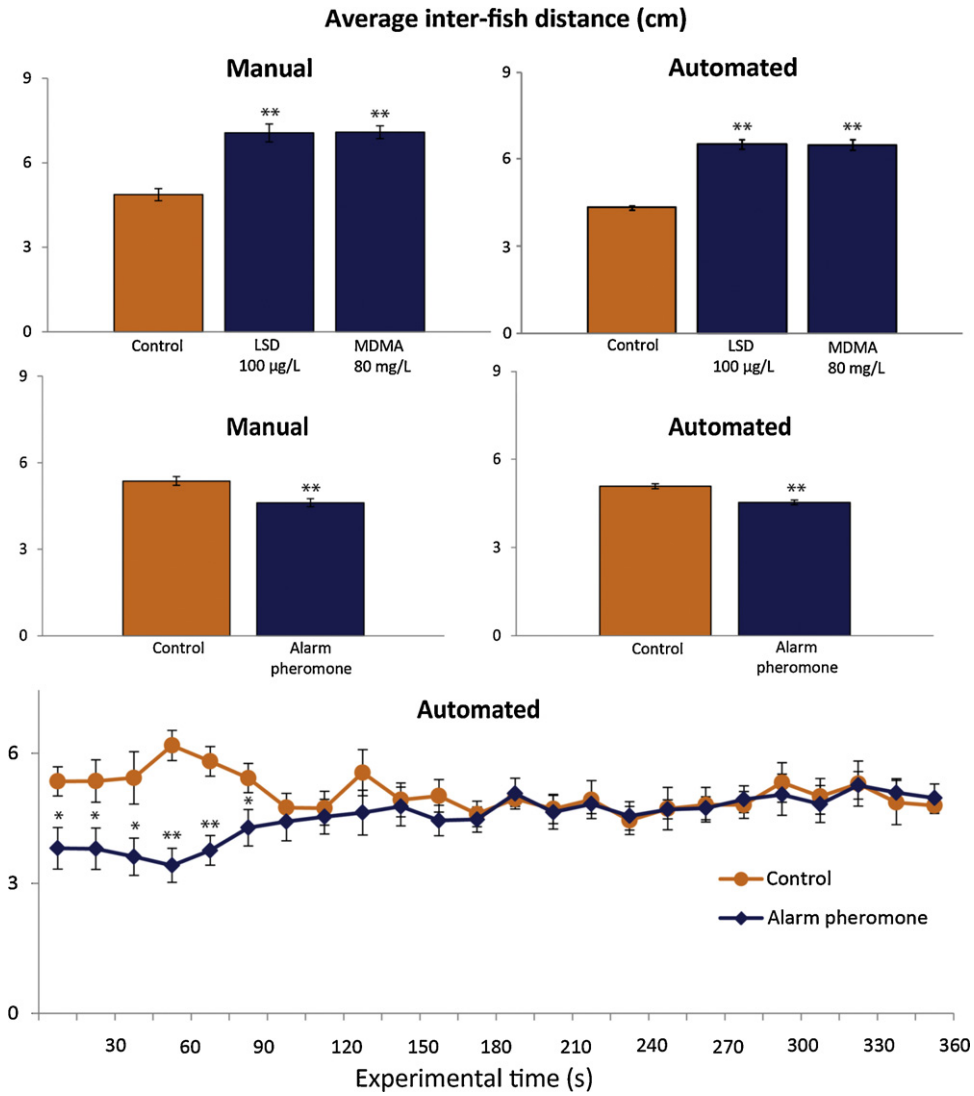

Fig. 2. Behavioral effects of acute 20-min exposure to the hallucinogenic drugs lysergic acid diethylamide (LSD), 3,4-methlenedioxymethamphetamine (MDMA; Experiment 2) and acute 3-min exposure to alarm pheromone (Experiment 3) on zebrafish tested in the novel tank. Shoaling endpoints (average inter-fish distance and proximity duration) were obtained in the standard 6-min shoaling paradigm for 100 µg/L LSD, 80 mg/L MDMA and 7 mL of alarm pheromone extract. Data was generated by Noldus EthoVision XT 8.5 software using the side-view video-recording; $^{*}p < 0.05$, $^{**}p < 0.001$ vs. respective control by post hoc Tukey test (for significant ANOVA data) or by *U*-test, where appropriate.

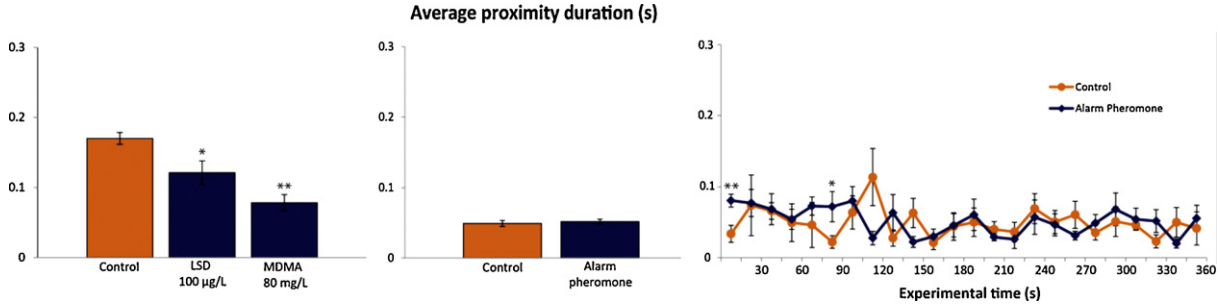

Fig. 3. Behavioral effects on proximity duration (s) following acute 20-min acute exposure to the hallucinogenic drugs lysergic acid diethylamide (LSD), 3,4-methlenedioxymethamphetamine (MDMA; Experiment 2) and acute 3-min exposure to alarm pheromone (Experiment 3) on zebrafish tested in the novel tank. Shoaling data (average proximity duration) were obtained in the standard 6-min shoaling paradigm for 100 µg/L LSD, 80 mg/L MDMA and 7 mL of alarm pheromone extract. Data was generated by Noldus EthoVision XT 8.5 software using the side-view video-recording; $^{*}p < 0.05$, $^{**}p < 0.001$ vs. respective control by post hoc Tukey test for significant ANOVA data.

protocol allows for a fast and efficient analysis of an already robust behavioral paradigm. Our results have shown a high correlation between manual and automated results, validating this new, high-throughput approach to phenotyping zebrafish shoaling responses. Previously used manual methods of shoaling analysis have relied on relatively large shoals (e.g., including groups of 8 fish) to effectively quantify shoaling behavior (Grossman et al., 2010). The ability of zebrafish to swim in groups presents some limitations to computer-based shoaling analysis, mainly due to overlapping of objects if tested in groups of 6, 7, or 8 zebrafish. For example, as fish travel behind each other, the computer can lose track of subjects if too many are present, ultimately resulting in higher percentage of subject loss. However, in our study, this percentage was rather low (<2% subject loss), confirming the optimal detection settings used here. Groups of 5 fish and under proved to be easier to track in SIM, as results from Experiment 1 (showing no difference in inter-fish distance between groups of 3, 4, 5, 6, 7 and 8 fish) support the notion that smaller groups do, in fact, display similar shoaling tendencies to the larger (e.g., 8-fish) shoals. Comparing the manual results with the software-generated data shows high correlation among all groups, especially the 4-fish group, indicating that automated recordings of zebrafish group behavior can be as effective as manual recording, and that 4-fish shoals can be optimal for such studies.

In addition to the ability to track zebrafish shoals of differing size, SIM also demonstrated the ability to track and differentiate the effects of various experimental modulations. For example, SIM was able to properly track and analyze decreased shoaling behavior evoked by LSD and MDMA administration as well as accurately identify increased shoaling (decreased inter-fish distance) in fish treated with alarm pheromone. As expected with an increased inter-fish distance, SIM detected a decrease in proximity for both LSD and MDMA, with an increase in proximity under the influence of alarm pheromone at the initial phase of alarm pheromone action. These observations enable us to introduce the proximity duration as an additional useful index for testing social phenotypes in zebrafish (Fig. 3), complementing the traditional measures used in previous shoaling studies (see Pham et al., 2012; Miller and Gerlai, 2007 for details). Importantly, the proximity parameters can be adjusted using the SIM. In the present study, a stringent 0.5-cm criterion was applied to characterize close proximity of zebrafish in the observed shoals. While this yielded robust behavioral effects (Fig. 3) here, our earlier pilot analyses utilizing a different (2.5 cm; ~one body length) setting showed no significant difference between any of the groups (data not shown), thereby emphasizing the importance of correct selection of proximity parameters for obtaining reliable data.

While not without limitations, the application of video-tracking software to zebrafish has been shown to be as effective as manual recording. First, the SIM program can track multiple subjects based on different colors, either applied by marker or sticker, which can be useful in future studies using zebrafish. However, the software is still able to track multiple subjects at once without the use of individual colors, as is the case with our zebrafish experiments. To optimize detection and prevent subject loss, a smaller number of subjects is required. Based on our extensive testing and comparison of manual to automated data, the software applied here will most effectively track 3 to 6 fish (subject lost rate <2%). To further optimize the analysis, we found an ideal video resolution to record our test videos, which balances the amount of strain put on the computer while also giving enough picture clarity to ensure subject detection. We found that recording in 640 × 480 pixels (30 fps) gave us the best results, and while a 720-pixel resolution was clearer, the improved clarity did not result in a overt benefit to justify the increased computational time for analyzing the videos.

Overall, this study presents a novel protocol for analyzing zebrafish social behavior – a paradigm previously limited to time-intensive manual analyses. The method described here demonstrates the capacity of video-tracking technology to assess zebrafish shoals of various sizes, as well as bi-directional modulation of shoaling behavior by various treatments. This protocol can be useful in future high-throughput studies focusing on the biological mechanisms underlying social behavior (including screening drugs or genetic mutations affecting social interaction) in zebrafish. Finally, while currently using a selected software package (SIM), this study can serve as a proof-of-concept, to foster the development of alternative tools and their use by the research community for neurophenotyping of social behaviors in zebrafish and other aquatic models.

Acknowledgements

The study was supported by Tulane University Intramural and Pilot funds, and by ZNRC collaborative initiative. Funders had no involvement in the study design, data collection and analysis, or the preparation of this MS. Noldus Information Technology (Wageningen, Netherlands) has provided necessary software, intellectual contribution, IT-related expertise and support for this collaborative project. Authors not affiliated with this company have no conflict of interest regarding this study. The authors thank Ari Davis and Mohamed El-Ounsi for their help with this study.

References

Brierley AS, Cox MJ. Shapes of krill swarms and fish schools emerge as aggregation members avoid predators and access oxygen. Curr Biol 2010;20:1758–62.
Buske C, Gerlai R. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotoninergic systems in adult zebrafish. Neurotoxicol Teratol 2011a;33:698–707.
Buske C, Gerlai R. Shoaling develops with age in Zebrafish (*Danio rerio*). Prog Neuropsychopharmacol Biol Psychiatry 2011b;35:1409–15.
Cachat J, Stewart A, Grossman L, Gaikwad S, Kadri F, Chung KM, et al. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. Nat Protoc 2010;5:1786–99.
Cachat J, Stewart A, Utterback E, Hart P, Gaikwad S, Wong K, et al. Three-dimensional neurophenotyping of adult zebrafish behavior. PLoS One 2011;6:e17597.
Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 2009;205:38–44.
Engeszer RE, Ryan MJ, Parichy DM. Learned social preference in zebrafish. Curr Biol 2004;14:881–4.
Fano E, Sanchez-Martin JR, Arregi A, Castro B, Alonso A, Brain P, et al. Social stress paradigms in male mice: variations in behavior, stress and immunology. Physiol Behav 2001;73:165–73.
Figueira ML, Brissos S. Measuring psychosocial outcomes in schizophrenia patients. Curr Opin Psychiatry 2011;24:91–9.
Fukuda H, Torisawa S, Sawada Y, Takagi T. Ontogenetic changes in schooling behaviour during larval and early juvenile stages of *Pacific bluefin* tuna *Thunnus orientalis*. J Fish Biol 2010;76:1841–7.
Grieco F, Loijens L, Krips O, Smit G, Spink A, Zimmerman P. EthoVision XT Reference Manual. Version 8.5. Wageningen: Noldus Information Technology; 2011.
Grossman L, Stewart A, Gaikwad S, Utterback E, Wu N, Dileo J, et al. Effects of piracetam on behavior and memory in adult zebrafish. Brain Res Bull 2011;85:58–63.
Grossman L, Utterback E, Stewart A, Gaikwad S, Chung KM, Suciu C, et al. Characterization of behavioral and endocrine effects of LSD on zebrafish. Behav Brain Res 2010;214:277–84.
Jesuthasan SJ, Mathuru AS. The alarm response in zebrafish: innate fear in a vertebrate genetic model. J Neurogenet 2008;22:211–28.
Krause J, Butlin RK, Peuhkuri N, Pritchard VL. The social organization of fish shoals: a test of the predictive power of laboratory experiments for the field. Biol Rev Camb Philos Soc 2000;75:477–501.
Kurta A, Palestis BG. Effects of ethanol on the shoaling behavior of zebrafish (*danio rerio*). Dose-Response 2010;8:527–33.
Kyzar E, Zapolsky I, Green J, Gaikwad S, Pham M, Collins C, et al. The Zebrafish Neurophenome Database (ZND): a dynamic open-access resource for zebrafish neurophenotypic data. In: Zebrafish; 2012.
Mc RS, Bradner J. The influence of body coloration on shoaling preferences in fish. Anim Behav 1998;56:611–5.
Miller LG, Thompson ML, Greenblatt DJ, Deutsch SI, Shader RI, Paul SM. Rapid increase in brain benzodiazepine receptor binding following defeat stress in mice. Brain Res 1987;414:395–400.
Miller N, Gerlai R. Quantification of shoaling behaviour in zebrafish (*Danio rerio*). Behav Brain Res 2007;184:157–66.
Noldus IT. Using EthoVision XT 8.5 with the Social Interaction Test. Quick start guide. Wageningen: Noldus Information Technology; 2011.

Parra KV, Adrian Jr JC, Gerlai R. The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). Behav Brain Res 2009;205:336–41.
Pham M, Raymond J, Hester J, Kyzar E, Gaikwad S, Bruce I, et al. Assessing social behavior phenotypes in adult zebrafish: shoaling, social preference, and mirror biting tests. In: Zebrafish protocols for neurobehavioral research; 2012. p. 231–46.
Potegal M, Huhman K, Moore T, Meyerhoff J. Conditioned defeat in the Syrian golden hamster (*Mesocricetus auratus*). Behav Neural Biol 1993;60:93–102.
Price J, Sloman L, Gardner Jr R, Gilbert P, Rohde P. The social competition hypothesis of depression. Br J Psychiatry 1994;164:309–15.
Reyhanian N, Volkova K, Hallgren S, Bollner T, Olsson PE, Olsen H, et al. 17alpha-ethinyl estradiol affects anxiety and shoaling behavior in adult male zebra fish (*Danio rerio*). Aquat Toxicol 2011;105:41–8.
Ribeiro Do Couto B, Aguilar MA, Lluch J, Rodriguez-Arias M, Minarro J. Social experiences affect reinstatement of cocaine-induced place preference in mice. Psychopharmacology (Berl) 2009;207:485–98.
Riehl R, Kyzar E, Allain A, Green J, Hook M, Monnig L, et al. Behavioral and physiological effects of acute ketamine exposure in adult zebrafish. Neurotoxicol Teratol 2011;33:658–67.
Sassenrath EN, Chapman LF. Primate social behavior as a method of analysis of drug action: studies with THC in monkeys. Fed Proc 1976;35:2238–44.
Saverino C, Gerlai R. The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. Behav Brain Res 2008;191:77–87.
Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). Behav Brain Res 2008;188:168–77.
Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, et al. Zebrafish models to study drug abuse-related phenotypes. Rev Neurosci 2011;22:95–105.
Veness C, Prior M, Bavin E, Eadie P, Cini E, Reilly S. Early indicators of autism spectrum disorders at 12 and 24 months of age: a prospective, longitudinal comparative study. Autism 2012;16:163–77.
Ward AJ, Duff AJ, Horsfall JS, Currie S. Scents and scents-ability: pollution disrupts chemical social recognition and shoaling in fish. Proc Biol Sci 2008;275:101–5.
Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed. Univ. of Oregon Press; 2000.
Wright D, Krause J. Repeated measures of shoaling tendency in zebrafish (*Danio rerio*) and other small teleost fishes. Nat Protoc 2006;1:1828–31.
Wright D, Nakamichi R, Krause J, Butlin RK. QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). Behav Genet 2006;36:271–84.
Wright D, Rimmer LB, Pritchard VL, Krause J, Butlin RK. Inter and intra-population variation in shoaling and boldness in the zebrafish (*Danio rerio*). Die Naturwissenschaften 2003;90:374–7.