The behavior response in Zrbrafish *Danio rerio* to mirror images

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**Abstract.** Behavioral response experiment of fish to mirror images is one of the main ways to test the social and aggressive behavior. The behavior changes of Zrbrfih *Danio rerio* in tanks contain different number of mirrors was tested in this study. The result indicated that the latency of zebrafish from entry tank which contains mirrors to start moving (\(P < 0.05\)) and their frequency of midline crossing (\(P < 0.001\)) were all lower than control group (contains no mirror), and decreased with increasing number of mirrors. The total swimming time of zebrafish in tank contains mirrors increased with increasing numbers of mirrors and significantly higher than control group (\(P < 0.001\)). Additionally, the time spent of zebrafish in side that contains mirrors significantly higher than the reverse side (\(P < 0.05\)), and this difference degree increased with increasing numbers of mirrors.

**1. Introduction**

Fish is an ancient vertebrate, they have a high diversity of species, and their behavior patterns are also complex and diverse such as recognize and avoid predators, Aggressive behavior[1], social classification[2], schooling[3,4], exploring[5], learning and cognition[6], thigmotaxis[7,8], color preference [9], dark/light preference[10], response to mirror images[11,12]. Among them, behavioral response experiment of fish to mirror images was getting more attention as an important way to test social and aggressive behavior[11].

Behavioral response to mirror images is commonly used to test the self-cognition of mammals[13]. Current research has suggested that fish are incapable of recognizing themselves in the mirror and tend to treat the mirror image as their companies [14]. Because of its advantages of high controllability and repeatability, the image in the mirror has become an effective method to evaluate the behavioral responds of fish to their companies such as aggressive and social behaviors[11,14].

Zebrafish *Danio rerio* is a small freshwater social fish and emerging as a new model organism for the study of neuropharmacology and behavior. However, there are few studies on their response to mirror images. In this study, different numbers of mirrors were used to test the behavioral response to mirror image as well as the preference for the mirror area in zebrafish. The goal of this work was to enrich the behavioral data of zebrafish, and to understand better the behavior of respond to mirror image.
2. Materials and methods

2.1. Animals and housing
Zebrafish (2.019 ± 0.149 cm, 0.06 ± 0.02 g) used in present study is from Institute of Hydrobiology, Chinese Academy of Sciences. The age of all fish was 2 months old. They were kept in 20L transparent polycarbonate tanks with recirculating water. The photoperiod was artificially controlled at 12L: 12D from 08:00 to 20:00. Water temperature, pH, and dissolved oxygen were maintained at 25–27°C, 7.32–8.75 and 7.22–8.54 mg/L, respectively. Fish were fed to satiation with ozone-disinfected frozen red worms (*Chironomidae flaviplumus* larva) (Yuerle, Tianjin, China) twice daily.

2.2. Procedures and apparatus
The experimental apparatus is shown in the Figure1. A single test fish was introduced into the center of the test tank contains aerated tap water at 25-27°C; filming began 5 min after adaptation. Test fish behavior was observed for 30 min by a video tracking system composed of CCD camera and analysis software (TTQ, Shenzhen, China). Latency of test fish from experiment began to start moving, frequency of boundary line crossing, time spent in mirror area, and total swimming time were recorded.

![Figure 1](image.png)

Figure 1. Experiment apparatus. It was a transparent seamless glass test tank (30 × 15cm and 20cm high). One (a), two (b) and three (c) mirrors (15cm × 20cm) are placed close to tank wall respectively.

2.3. Data analysis
Data are given as the mean ± standard deviation (SD) and were checked for assumptions of normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. The difference was analyzed by one-way ANOVA or Kruskal-Wallis nonparametric test between groups. Statistical processes were performed using the SPSS (version 19.0, IBM, Chicago, USA) and Office Excel (version 2010, Microsoft, Redmond, USA). Figures were created using Excel 2010, Originlab (version 8.0, Originlab, Northampton, USA) and Adobe Photoshop (version CS6.0, Adobe, San Jose, USA). Data were presented as mean ± SD.

3. Results
In the tank with mirrors, zebrafish preferred swimming close to the mirror (Fig.2). Compared to control group (no mirror), the latency of test fish from experiment began to start moving significantly decreased in tank contains one (*P* = 0.048) and three mirrors (*P* = 0.001), while is no significant change in tank contains two mirrors (*P* = 0.081). And it was no difference between three experimental groups (*P* = 0.057, Fig.3). The frequency of boundary line crossing decreased with increasing number of mirrors and all was significantly lower than control group (*P* < 0.001), and it was significantly different between three experimental groups (*P* < 0.001, Fig.3). Time spent of test fish in two side of tank was no different (*P* = 0.174) in control group, while they spent clearly more time in mirror area than reverse side in three experimental groups (*P* < 0.05, Fig.4). The total swimming time of test fish increased with increasing number of mirrors and all was significantly higher than control group (*P* < 0.001), and it was significantly different between three experimental groups (*P* = 0.001, Fig.5).
Figure 2. The behavior patterns of zebrafish in the tanks that contain mirrors.

Figure 3. The latency of zebrafish from experiment began to start moving (a) and frequency of boundary line crossing (b) in the tanks that contain different number of mirrors.

Figure 4. Time spent of zebrafish in two side in the tanks that contain different number of mirrors.
4. Discussion
The mirror in the tank has a significant influence on the behavior of zebrafish. They tend to start moving faster, spend more time to swim, and tend to stay in the mirror area when they entry the tank contains mirrors. And these behaviors became more apparent as the number of mirrors increases. The test fish were able to see images from three mirrors simultaneously owing to the mirrors were placed in a "U" shape and close to three walls of tank in this study. Therefore, the increase in the number of mirrors actually means an increase in the number of companions for the test fish. The mirror test is commonly used to test the behavioral response of fish to companions according to the theory that most fish can't recognize themselves in the mirror \[14\]. Fishes will exhibit the same social or aggressive behavior when confronted with mirror images as when confronted with living companions \[11,12\]. Therefore, we suggested that those behavior patterns of zebrafish when they confronted with mirrors such as swimming close to mirror were aggressive or social behavior. Freezing on the bottom was considered a sign of anxiety and stress in many fish \[15,16\], thus both the decreased latency and the increased swimming time indicated that the anxiety and stress in zebrafish were significantly reduced in the presence of the mirrors, and this reduce effect was more severe in tank contains more mirrors. This is consistent with previous research showing that fish prefer larger groups \[17\]. The results of this study indicate that zebrafish is a good model fish for mirror testing and social behavior testing, and prove that mirror testing is a good method to test the social behavior of fish.

5. Conclusions
In conclusion, zebrafish showed significant behavioral response to the image in the mirror. Their total swimming time significantly increased but frequency of boundary line crossing decreased in tank contains mirrors compared to tank contains no mirrors. They preferred to stay at mirror area and swimming close to the mirror. These behaviors became more apparent as the number of mirrors mirror increases.

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References

[1] Carreno Gutierrez, H., Vacca, I., Pons, A.I., Norton, W.H.J. (2017) Automatic quantification of juvenile zebrafish aggression. Journal of neuroscience methods, 296: 23-31.

[2] Teles, M.C., Oliveira, R.F. (2016) Androgen response to social competition in a shoaling fish. Hormones and behavior, 78: 8-12.

[3] Moretz, J.A., Martins, E.P., Robison, B.D. (2006) The effects of early and adult social environment on zebrafish (Danio rerio) behavior. Environmental Biology of Fishes, 80: 91-101.

[4] Romey, W.L., Rossmann, D.S. (1995) Temperature and Hunger Alter Grouping Trade-Offs in Whirligig Beetles. American Midland Naturalist, 134: 51-62.

[5] Brown, C., Braithwaite, V.A. (2004). Size matters: a test of boldness in eight populations of the poeciliid Brachyraphis episcopi. Animal Behaviour, 68: 1325-1329.

[6] Fernandes, Y., Tran, S., Abraham, E., Gerlai, R. (2014) Embryonic alcohol exposure impairs associative learning performance in adult zebrafish. Behavioural brain research, 265: 181-187.

[7] Richendrfer, H., Pelkowski, S.D., Colwill, R.M., Creton, R. (2012) On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behavioural brain research, 228: 99-106.

[8] Schnorr, S.J., Steenbergen, P.J., Richardson, M.K., Champagne, D.L. (2012) Measuring thigmotaxis in larval zebrafish. Behavioural brain research, 228: 367-374.

[9] Luchiari, A.C., do Amaral Duarte, C.R., de Morais Freire, F.A., Nissinen, K. (2006) Hierarchical status and colour preference in Nile tilapia (Oreochromis niloticus). Journal of Ethology, 25: 169-175.

[10] Blaser, R.E., Penalosa, Y.M. (2011). Stimuli affecting zebrafish (Danio rerio) behavior in the light/dark preference test. Physiology & behavior, 104: 831-837.

[11] Cattelan, S., Lucon-Xiccato, T., Pilastro, A., Griggio, M. (2017) Is the mirror test a valid measure of fish sociability? Animal Behaviour, 127: 109-116.

[12] Chen, J., Lei, L., Tian, L., Hou, F., Roper, C., Ge, X., Zhao, Y., Chen, Y., Dong, Q., Tanguay, R.L. (2018) Developmental and behavioral alterations in zebrafish embryonically exposed to valproic acid (VPA): An aquatic model for autism. Neurotoxicology and teratology, 66: 8-16.

[13] Broom, D.M., Sena, H., Moynihan, K.L. (2009). Pigs learn what a mirror image represents and use it to obtain information. Animal Behaviour, 78: 1037-1041.

[14] Rowland, W.J. (1999) Studying visual cues in fish behavior: a review of ethological techniques. Environmental Biology of Fishes, 56: 285-305.

[15] Bass, S.L., Gerlai, R. (2008) Zebrafish (Danio rerio) responds differentially to stimulus fish: the effects of sympatric and allopatric predators and harmless fish. Behavioural brain research, 186: 107-117.

[16] Maximino, C., de Brito, T.M., Colmanetti, R., Pontes, A.A., de Castro, H.M., de Lacerda, R.I., Morato, S., Gouveia, A., Jr. (2010) Parametric analyses of anxiety in zebrafish scototaxis. Behavioural brain research, 210: 1-7.

[17] Ruhl, N., Mcrobert, S. (2010) The effect of sex and shoal size on shoaling behaviour in Danio rerio. Journal of Fish Biology, 67: 1318-1326.