**Assessing social behavior phenotypes in adult zebrafish:**

**shoaling, social preference and mirror biting tests**

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**Abstract**

Zebrafish are a popular model organism in neuroscience research, recently emerging as an excellent species to study complex social phenotypes. For example, zebrafish actively form shoals, which can be used to quantify their shoaling behaviors. Zebrafish also display strong social preference when placed in a tank with conspecific fish, a trait that can easily be quantified in the two-compartment preference test. The mirror biting test, based on mirror image stimulation, is another well-established method for studying zebrafish boldness and sociability. This chapter will describe three simple and efficient paradigms - shoaling, social preference and mirror biting tests - for quantifying social behaviors in adult zebrafish. Reflecting different aspects of zebrafish social phenotypes, these models can be used individually, or within a test battery.

**Key words:** adult zebrafish, social behaviors**,** shoaling, social preference, conspecific, mirror biting test

## 1. Introduction

Social interactions are an important domain of animal and human behavior [1-5]. In humans, social deficits trigger several serious brain illnesses, such as autism [6], personality disorders [7], affective disorders [8] and schizophrenia [9]. In animals, numerous rodent and primate paradigms have long been used in pre-clinical research of social behavior [2, 10-15]. There is also a strong genetic component in social phenotypes, with a growing number of genetic mutations linked to both animal [16-18] and clinical [19, 20] social deficits.

Although zebrafish are a relatively novel model species for neurobehavioral research [21-24], their utility in studying social behaviors is rapidly developing. The main reason for this is that zebrafish are highly social animals (Table 1) and exhibit robust social behaviors, such as shoaling [25, 26], boldness [27, 28], aggression [25, 29] and social preference [24, 30, 31].

Shoaling behavior (Fig. 1) is very common in fish models [32-34], representing the complex interaction of animals moving together in coordinated movements [21, 25, 32, 34-36]. In fish, this adaptive, evolutionarily conserved behavior has long been investigated in terms of ontogenesis [37], effects of environmental stressors [38], behavioral organization [25], genetics [26, 27, 39] and pharmacological modulation [22, 24, 30, 35, 40]. In zebrafish, shoaling is maintained at a relatively stable high level throughout their lifespan [41] although specific preferences for shoaling conspecifics appear to be learned [31]. Shoaling tests are based on easily quantifiable endpoints (Table 1) collected from video-captured static images (during manual analysis) or using more sophisticated video-tracking (calculated by software programs, as discussed in a separate chapter by Miller and Gerlai in this book).

Social preference is another useful model to study fish social phenotypes [23, 24]. Based on a similar rodent paradigm [13, 42, 43], it assesses zebrafish sociability by observing the interactions between several fish, and assessing zebrafish preference for conspecifics. One commonly used modification of this test uses two fish – the target fish (placed in a ‘conspecific’ compartment) and the experimental fish (placed in the central arena and then given a choice between conspecific or empty arm; Fig. 2, Table 1). Albeit not discussed here, other studies (e.g., [31]) have combined shoaling and social preference tests, assessing preference of an individual fish for shoals of zebrafish in the social preference assays.

The mirror image stimulation is also a well-established fish paradigm, traditionally used for studying their social/aggressive behavior [29, 31, 44, 45]. Similar to other fish species, zebrafish display boldness by butting or biting the mirror when placed in a tank with it (Fig. 3), also "tracing" their reflection as they swim quickly back and forth [28, 46, 47]. Besides aggression, mirror biting also reflects the interaction with a conspecific, and therefore is highly relevant to social behavior [46]. Given their utility in biobehavioral research, this chapter will describe shoaling, social preference and mirror biting tests as useful and time-efficient models for studying adult zebrafish social behaviors.

## 2. Methods and Materials

### 2.1. Animals

Adult fish (e.g., *short-fin* wild type or AB zebrafish) can be obtained from a local commercial distributor or raised in house. Animals (e.g., ~5-8 months old) can be housed in groups of 20–25 fish per 40-L tank, filled with filtered system water maintained at 25–27 °C. Illumination can be provided by ceiling-mounted fluorescent light tubes on a 14:10-h cycle (e.g., on: 6.00 h; off: 20.00 h) according to the standards of zebrafish care. All fish used in these studies must be experimentally naïve. Zebrafish are typically fed twice daily (e.g., Tetramin Tropical Flakes, Petco Inc., San Diego, CA). Animal experiments should be approved by IACUC and adhere to National and Institutional guidelines and regulations.

### 2.2 Equipment

- Adult zebrafish (as in Section 2.1);

- Observation tanks (e.g., 1.5-L trapezoidal tank 15 height × 28 top × 23 bottom × 7 cm width; Aquatic Habitats, Apopka, FL);
- Treatments, e.g., drugs (if used in the experimental design);
- Mirror (sized to fit the side of the tank used e.g., 15 x 7 cm) for the mirror biting test;
- Pre-treatment beaker (e.g., a 3-L plastic container);
- Video cameras (webcams) connected to a computer through USB port.

## 3. Methods

### 3.1 Shoaling Test

***Apparatus and Procedures***

- Expose groups of 8 zebrafish (e.g., using 2 groups, total 16 fish per treatment) from the same holding tank to a drug or drug-free water (control) for a specific period of time (e.g., 5-20 min) in the pre-treatment beaker.
- Place the group in the novel tank and leave to acclimate to the experimental conditions (in our experience, 3 min may be sufficient for fish to pass the initial 'transfer' anxiety and re-establish their natural shoaling behaviors).
- Video-record fish behavior for the next 3 min and remove fish from the tank when finished. In the narrow tank, such as the novel tank test, zebrafish shoaling behavior can be video-recorded by a side-view camera for a 6-min test duration. Make 8 screenshots within a fixed time interval every 20 s during the last 3 min of the observation period.
- Each screenshot should be carefully calibrated and analyzed by trained observers (inter- and intra-rater reliability >0.85), measuring the distances between each fish in the group per screenshot. This can be performed either manually (from a calibrated print-out with a ruler, although this is less accurate) or from a computer-screen (using the more precise software, such as ImageTool; UTHSCSA, San Antonio, TX). To measure the inter-fish

distance in ImageTool, open one frame at a time. Using the virtual ruler tool, measure the distance between every fish and record it in an Excel document.

- In both cases, measure the distance from the center of one fish to the center of another fish. The top/bottom preference can also be assessed by counting the number of fish in top and bottom areas of the tank, per screenshot. Final shoaling data for control and experimental cohorts should represent averaged results for all fish for 8 screenshots per each group. More cohorts and/or more screenshots per cohort can be used in some experiments, if necessary, in order to obtain less variable data. Likewise, larger observation tanks can be used for zebrafish shoaling tests (especially for larger shoals), if necessary.

***Typical results***

The shoaling test is used to assess overall social behaviors in a group of zebrafish. Usually, fish that are within 4 average fish lengths of each other are considered a part of the same shoal. Shoal cohesion is usually stable and maintains a relatively high baseline level in adult zebrafish [41]. Two other endpoints that can be measured in a shoal are nearest neighbor distance and farthest neighbor distance (Table 1). Nearest neighbor distance is measured independently of shoal size, which allows researchers to study shoal cohesion without the number of fish known. Furthermore, the shoaling test may also reflect zebrafish stress or anxiety. For example, stressed fish tend to swim closer together, in tighter shoals with a smaller inter-fish distance [23] (also see [40]). In contrast, when fish are less stressed, the inter-fish distance is significantly larger [23].

Typical shoaling results are shown in Fig. 1 and published shoaling data is summarized in Table 2. For example, ethanol-treated fish exhibit tight shoaling at a low dose, most likely due to the disinhibitory effect of ethanol, allowing conspecifics to approach closer than controls [35]. In contrast, high doses of ethanol evoke a sedative response in zebrafish, manifested in increased nearest neighbor distance and shoal area [35]. Zebrafish treated with the hallucinogenic drug lysergic acid diethylamide

(LSD) swim in a calm and slower fashion, with disrupted shoaling and increased average inter-fish distance [30]. Altered shoaling responses in these fish may reflect hallucinogenic and/or anxiolytic-like effects of LSD, giving important insights into pharmacological modulation of zebrafish social behaviors [30]. In line with this notion, another hallucinogenic drug, ketamine, also evokes anxiolytic responses in zebrafish, as well as inducing looser shoals with increased inter-fish distance [24] (Table 2).

### 3.2. Social Preference Test

***Apparatus and Procedures***

- A typical social preference test can consist of a 50-cm Plexiglas corridor divided into five 10 x 10 cm cells (Fig. 2). The target (conspecific) fish is introduced to an exposure compartment (conspecific box), separated by a transparent divider from the rest of the apparatus (Fig. 2) [36].
- To avoid lateral bias in zebrafish cohorts, the left/right location of target (conspecific) fish must be alternated between the trials. Experimental fish are pre-exposed to a drug or drug-free water (control) for 20 min.
- Control or drug-exposed zebrafish (n=12 in each group) are introduced individually to the central zone of the apparatus, temporarily separated (by transparent sliding dividing doors) from the two arms of the corridor.
- Following the initial 30-s acclimation interval (necessary to reduce transfer/handling stress), the two sliding dividers should be gently lifted, and the zebrafish released to explore the apparatus for 6 min. Fish behavior can be scored manually (by trained observers) or using video-tracking software, assessing the number of entries and time spent in center, conspecific arms or empty arms [36]. The ratios of conspecific:empty arm entries and the respective duration ratios can also be calculated based on this data (Table 1).

**Typical results**

In a social preference test, the experimental fish will generally prefer to spend more time close to a target/conspecific fish (Fig. 2, Table 3), spending over 65-70% of time there. Social preference can also be modulated by environmental factors, such as rearing with fish of own or different strains [31]. Finally, some drugs may affect fish activity levels without altering their social preference. For example, LSD has no overt effect on zebrafish social preference, as the time spent or ratio of entries between conspecific:total and conspecific:empty arms remained unaltered [30]. Likewise, ketamine-treated fish also did not show altered social preference phenotypes, but instead demonstrated more total entries to each arm, consistent with hyperactivity responses typically evoked by this drug [24].

**3.3 Mirror Biting Test**

***Apparatus and Procedures***

This section describes two different modifications of the mirror biting test. Modification 1 uses mirror introduced to the tank with fish already placed in it. Therefore, this procedure is based on higher mirror novelty as well as stronger territoriality of fish behavior. Modification 2 is based on introducing fish to the tank with the mirror – the situation based on stronger novelty of both mirror and tank environment for the fish. While this modification may be less appropriate for anxious fish with high baseline freezing, it can be suitable for more active and less anxious strains (i.e., whose behavior is less confounded by the initial novelty stress).

**Modification 1: Introducing mirror to the tank with fish**

- Place the fish in a small tank (e.g., 21 L) and leave undisturbed for a long period of time (e.g., 18 h) [28].
- Quickly place a mirror into the tank with the fish, trying not to cause excess disturbance [28].
- Manually record zebrafish behaviors (see Table 1 for selected endpoints) during the testing time (e.g., 5 min) or use video recording, which enables data analysis at a later time [28].

**Modification 2: Introducing fish to the tank with mirror**

- Set up the novel tank apparatus with the mirror inside, attached to the inner side wall of the tank. Draw a light line on the tank with a marker 0.5 cm from the mirror, to represent the zone of "contacting the mirror" (Table 1). Draw another line 2.5 cm from the first line (based on an average adult fish length) to represent the zone of "approach to the mirror" (Fig. 3). If using video-recording software, these two lines can be drawn virtually.
- Place one fish in the novel tank and immediately start recording. As specified in Table 1, manually recorded endpoints include the number of mirror contact, approach, latency to first mirror contact, and latency to first mirror approach. With a video-camera and software program, the duration of mirror contacts and approaches can also be recorded.

**Typical Results**

While the two mirror biting test modifications may have some contextual differences (as mentioned earlier), they both seem to be efficient in assessing zebrafish responses (Fig. 3). In both models, zebrafish baseline behavior in the mirror biting test is usually characterized by freezing bouts during the first minute in the testing tank. Then zebrafish gradually start to explore the tank, getting in closer proximity to the mirror (Fig. 3). Depending on the size of the tank, the majority of the biting occurs between minutes 3-4 of the standard 6-min test. With an extended testing period, habituation to the mirror can be seen after minutes 5-6, with a gradual reduction in mirror biting activity as the novelty of the stimulus declines (data not shown). Using Modification 1 of this test and assessing the number of mirror bites, some studies reported interesting strain and sex differences in zebrafish behavior [46]. Other studies found that zebrafish raised in mix-strain groups bit more than those raised in pure-strain groups [28]. Collectively, these findings demonstrate that such types of aggression-related behaviors may have a learned component, and can be easily quantified using the mirror biting test (see Table 4 for details).

### 3.4. Statistical analysis

In all tests described here, the non-parametric Wilcoxon-Mann-Whitney U-test can be used for comparing two groups (parametric Student's t-test may be used for data distributed normally). For more than two groups, analysis of variance (ANOVA), followed by an appropriate post hoc test (e.g., Tukey, Dunn, Newman-Keuls or Dunnet test), must be used. In general, n-way ANOVA can be applied for zebrafish social behavior tests, with typical factors being treatment, dose, sex, strain, time, trial or age. For analyses of inter- or intra-trial habituation (see chapter by Raymond et al. on zebrafish habituation in this book), ANOVA with repeated measures (test time or trials, respectively) can be used.

## 4. Troubleshooting/Notes

- Overall, there are well-documented sex differences in zebrafish behaviors and their sensitivity to various drugs. For example, female zebrafish have altered sensitivity to ethanol exposure [48] and cocaine withdrawal [49]. There are also reported sex differences in zebrafish behavioral models, such as aggression [50], shoaling [51, 52] and feeding in the presence of alarming stimuli [53]. Therefore, it is important to consider different sex subjects and conspecifics in social behavior testing. For this, experimenters may choose to either examine each sex in separate experiments, or use a ~50:50 ratio of female:male zebrafish in their studies.
- Animal locomotor activity commonly affects their performance in various behavioral paradigms, and the same applies to zebrafish neurophenotyping studies. For example, zebrafish hyperactivity may non-specifically increase the number of entries into all of the arms of the social or more mirror approaches in the mirror biting test. If fish display abnormally high locomotion after the acclimation period, experimenters should consider extending the acclimation time (to reduce arousal) or choose another (less active) zebrafish strain.

Abnormally low activity (e.g., due to high freezing) may also be common in zebrafish social paradigms. Accordingly, various automated programs, such as Noldus Ethovision XT7, can be used to measure overall locomotion in zebrafish, and the experimenters can control for it in their studies. If zebrafish activity level is quantified, it can also be used as a covariate in subsequent statistical analyses.

- During a shoaling test, some fish may occasionally swim apart from the group. For example, this may occur if these fish are more active or less anxious than the rest of the shoal. To reduce data variability in this model, experimenters should reduce all pre-experiment stress. This can be achieved, for example, by ensuring that the environment is similar between housing and testing containers; by reducing net handling stress; and/or by allowing sufficient acclimation (e.g., 3 min or longer) before taking screenshots of the video.
- Auditory/chemosensory cues are critical cues in the social tests. In the social preference test, the transparent divider that separates the compartments should be as tightly secured as possible, to prevent any cue transmission from the conspecific fish to the subject fish. Likewise, social preference test dividers should be lifted at precisely the same time. If this not done correctly, the experimental zebrafish may dart into whichever corridor is exposed first, therefore confounding social preference data.
- In all social paradigms described here, if the fish seem to erratically dart unexpectedly or suddenly, it is probably caused by a startling stimulus in the room. To avoid startling the fish (see details of zebrafish startle in chapter by Chanin et al. in this book), sounds produced by the investigators in the experimental room should be kept to an absolute minimum during the testing. Also, avoid sudden or abrupt movements during testing, or any other disturbances of the tank. Blinds that block visual stimuli from the tank may also be useful.

- When using Modification 1 of the mirror biting test, if the fish are frozen the entire testing time, find a way to introduce the mirror that creates the least amount of disturbance. Excess stress created by the mirror's introduction to the tank will lead to longer freezing bouts and increased anxiety related behaviors. One alternative is to present the mirror on the outside of the tank wall.
- Overall, zebrafish may display initial preference at the beginning of the test, and this may confound their subsequent behavioral results. Therefore, the placement of target conspecific fish in the social preference test, as well the mirror in the mirror biting test, should be alternated or randomized to avoid spatial bias.
- In Modification 2 of the mirror biting test, the fish may notice and start biting the mirror in the very beginning of the test. To avoid this situation, introduce fish in the opposite side of the tank from the mirror so that the fish can calm down from the net stress before noticing the mirror. Ensure that all fish in the experiment are introduced to the tank in the same manner (e.g., by placing the net in the bottom of the tank, with fish facing away from the mirror). Using high-quality video-recording and slow motion with frame-by-frame analyses may also help better quantify mirror biting behavior (for example, distinguishing it from "chasing" or "butting" responses, also commonly observed in this test).

**5. Summary**

Social phenotypes are a key part of zebrafish natural behavior, and are equally important in the laboratory environment. As outlined here, examining shoaling phenotypes, social preference and mirror biting responses provides a better understanding of social behaviors as well as stress and anxiety in adult zebrafish. All these three behavioral models reflect different domains - shoaling tendency, social choice or social aggression/boldness, and can be used separately, depending on research goals. However, they may also be used complementarily – for example, combined in a test battery with a sufficient (e.g., several days) inter-test interval. The use of these tests in neurobehavioral research will foster the development of translatable models, thereby contributing to our understanding of human social disorders, such as autism, social phobia and schizophrenia.

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**Figure 1. Zebrafish shoaling test using the novel tank apparatus.** The primary endpoints in this test are detailed in Table 1. The horizontal line in the middle of the tank divides the top of the tank from the bottom of the tank. Inter-fish distances in this test can be either examined manually (using a ruler) or calculated by a computer program (e.g., ImageTool UTHSCSA, San Antonio, TX). The number (or percent) of fish in top (e.g., 5 in this diagram vs. bottom, 3 in this diagram) reflects place preference of the shoal, which is likely to be near the bottom for more anxious fish. Photographs in this diagram (by Kalueff et al.) represent different shoaling patterns in zebrafish, including (left to right) tight, medium and loose shoals.

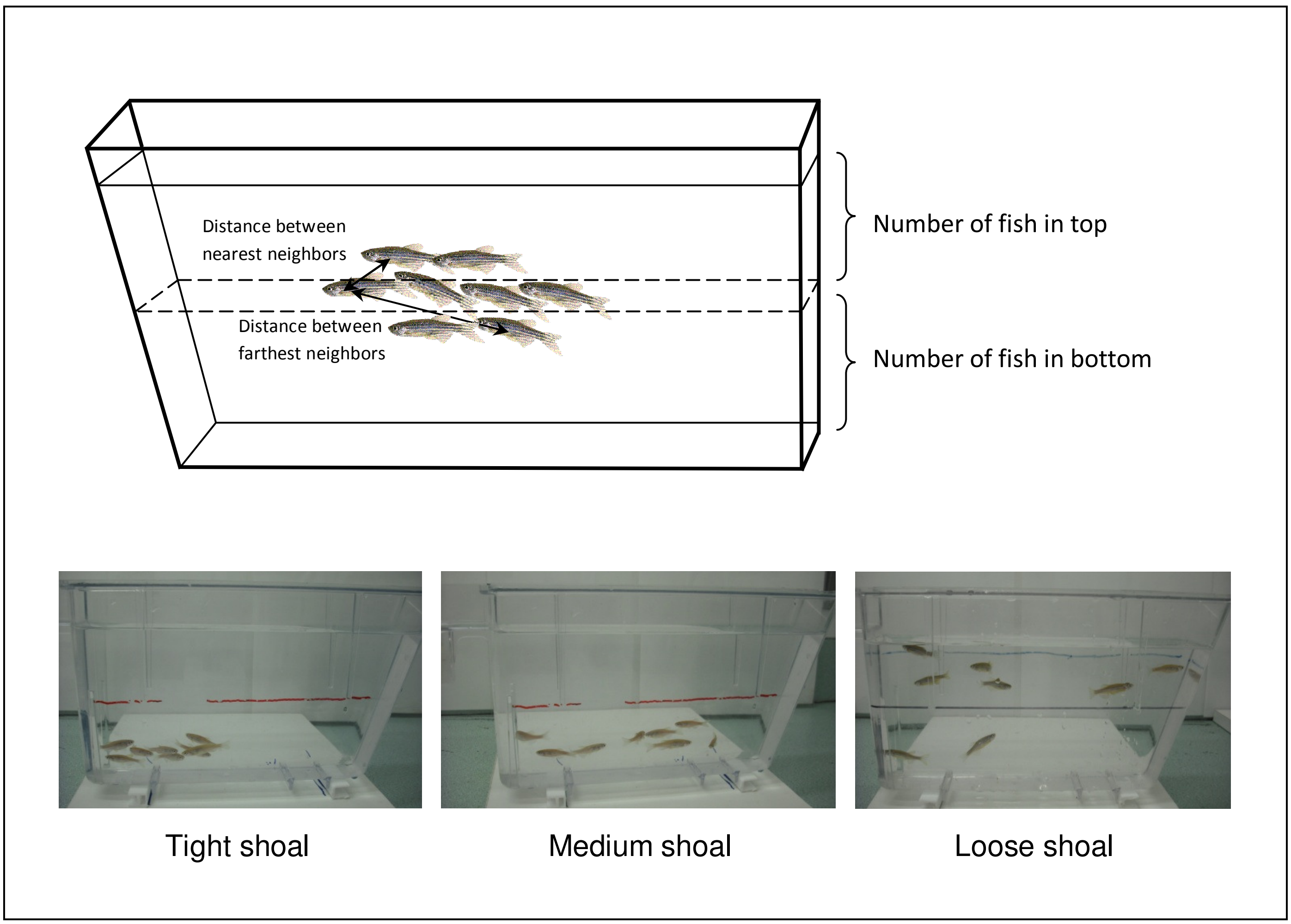


**Figure 2. Zebrafish social preference test.** The experimental fish is placed into the center. There are dividers that separate the center and arms from the boxes on each end. Another zebrafish of the same size is placed in the conspecific box, and the experimenters manually score the entries to arms and time spent in each section (if available, video-tracking software can also be used to quantify zebrafish responses). Bottom panel shows typical behavior observed in naïve adult zebrafish (5-8 month old; $n = 15$) exposed to the social preference test for 6 min (bar diagrams: ** $P<0.01$, ***$P<0.005$ vs. conspecific arm, paired U-test). In line diagrams, note time-course of behavioral responses, as conspecific arm entries and time change (habituate) over time during the test (** $P<0.01$, #$P = 0.5$-$0.1$ (trend), min 6 vs. min 1, paired U-test).

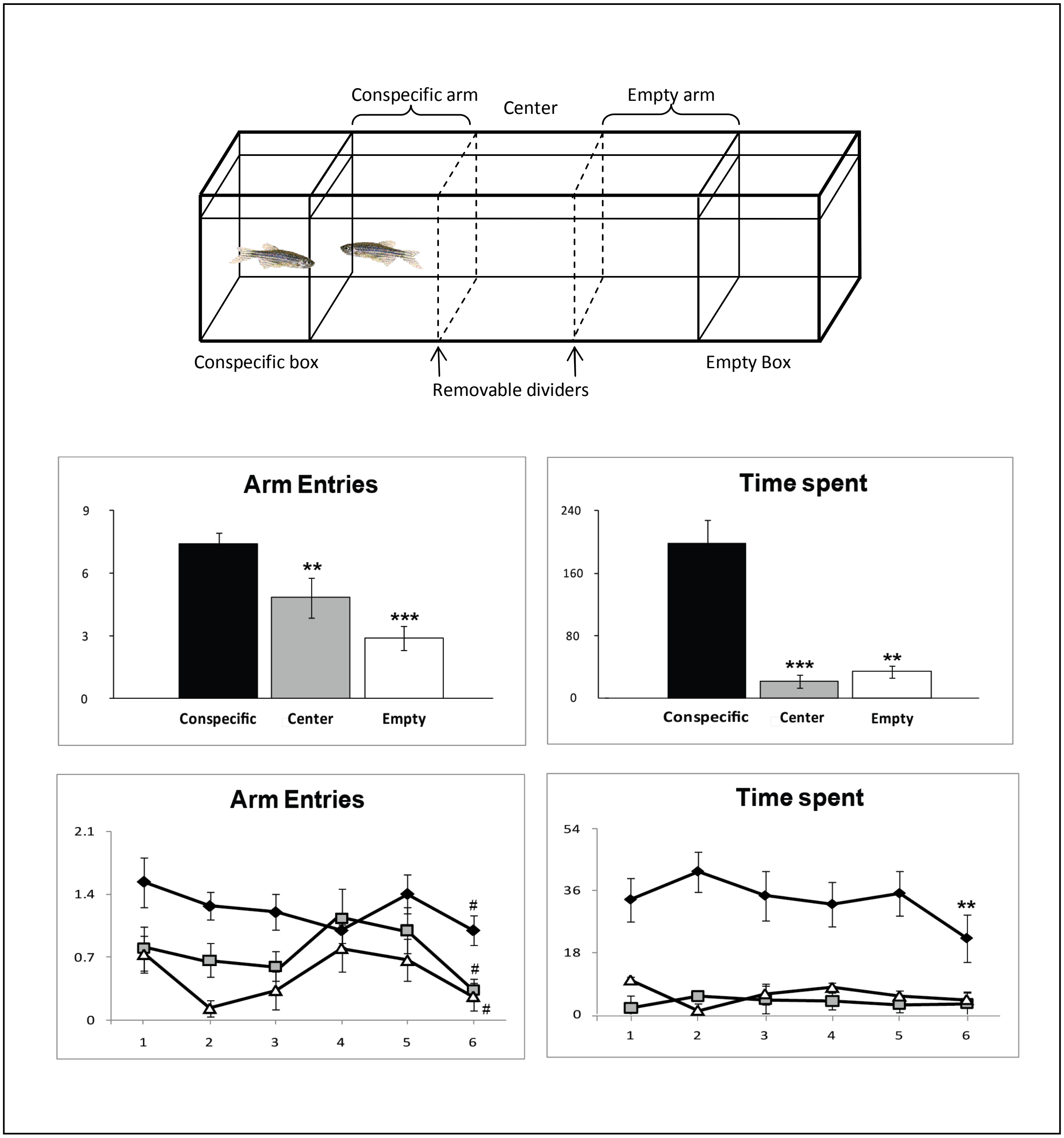
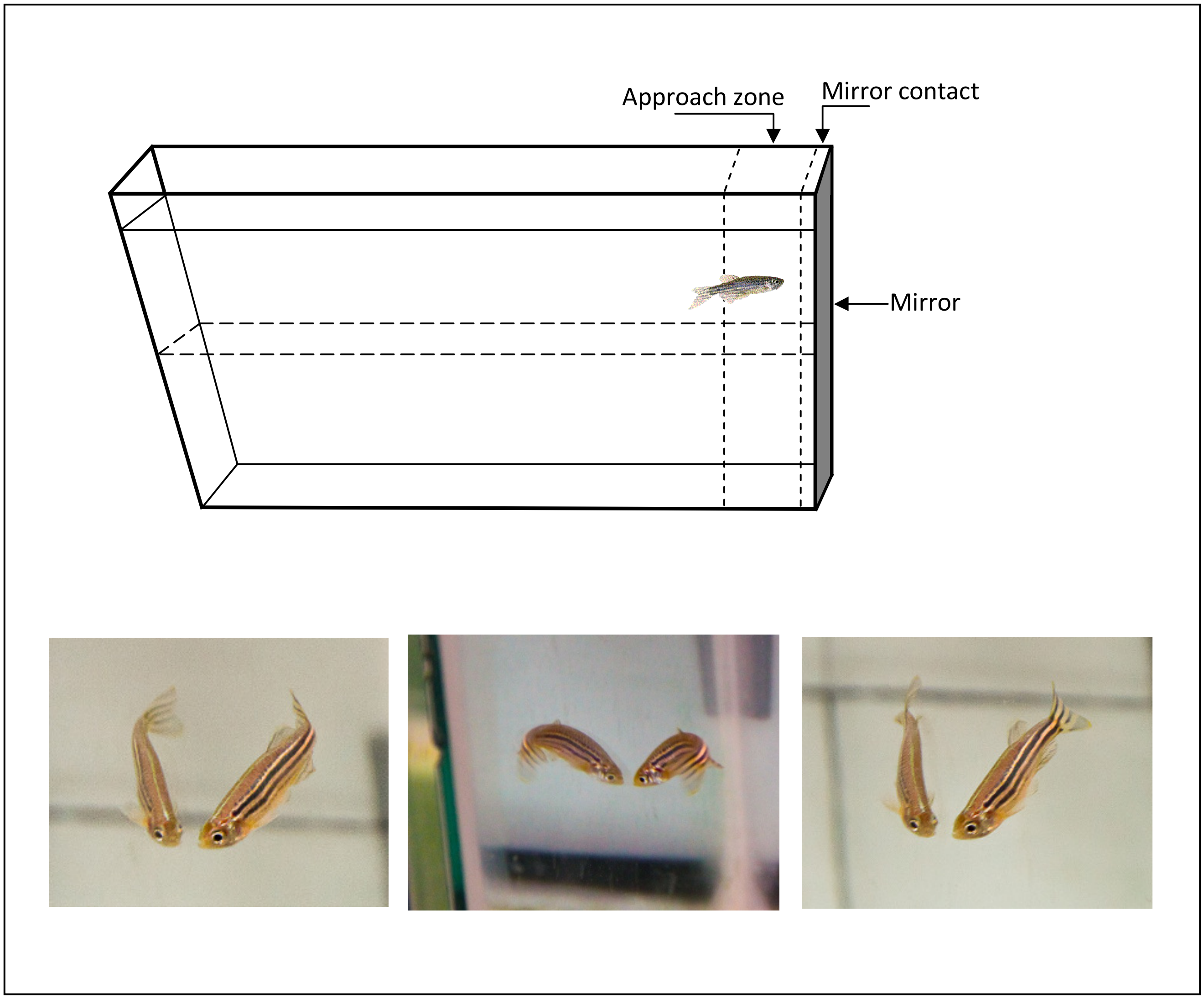

Conspecific arm
Center
Empty arm
Conspecific box
Removable dividers
Empty Box
Arm Entries
9
6
3
0
**
***
Conspecific
Center
Empty
Time spent
240
160
80
0
***
**
Conspecific
Center
Empty
Arm Entries
2.1
1.4
0.7
0
#
#
#
1
2
3
4
5
6
Time spent
54
36
18
0
**
1
2
3
4
5
6

**Figure 3. Mirror biting test apparatus (can be used for both Modifications 1 and 2 described in this protocol) and typical results obtained in this test.** Top panel shows the apparatus and typical behaviors demonstrated by zebrafish in this test (photos by B. Robinson and M. Singer). Bottom panel compares typical behavioral responses observed in naïve adult zebrafish (5-8 month old; n = 12 per group) exposed to two different modifications of the mirror biting test. In Modification 1 (white bars on bottom diagrams), zebrafish were placed in the novel tank apparatus for a 5-min acclimation prior to introducing mirror and recording fish behavior for 5 min. In Modification 2 (black bars on bottom diagrams), zebrafish were exposed for 5 min to the novel tank test apparatus containing a mirror attached to one of its side walls. Note time-course of behavioral responses in both modifications of this test, as the mirror biting behavior, but not approaches, changes with time during the test (* $P<0.05$, ** $P<0.01$ min 5 vs. min 1, paired U-test).


Approach zone
Mirror contact
Mirror

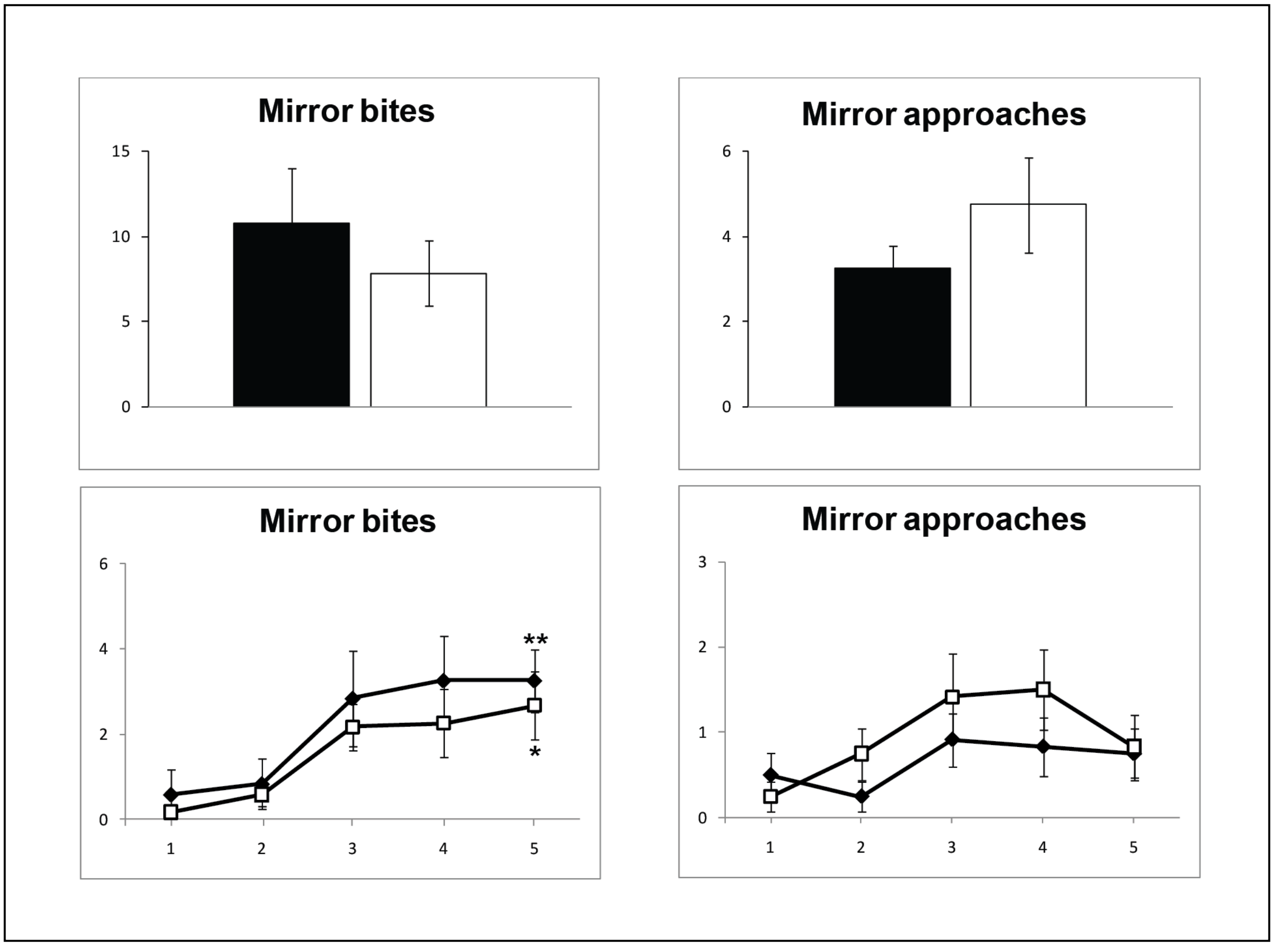

Mirror bites
15
10
5
0
Mirror approaches
6
4
2
0
Mirror bites
6
4
2
0
1
2
3
4
5
**
*
Mirror approaches
3
2
1
0
1
2
3
4
5

**Table 1. Definition of selected endpoints typically assessed in three popular tests of zebrafish social phenotypes**

| Endpoints | Definition | References |
|---|---|---|
| **Shoaling test** | | |
| Average inter-fish distance | Distance between the body center of every member of the shoal | [21-24, 40] |
| Average neighbor distance | Distance for the body center of each fish to the closest neighboring fish | [22, 35, 41, 54] |
| Median distance between any two fish | Middle distance of all the inter-fish distances | [41] |
| Average farthest neighbor | Distance for the body center of each fish to the farthest neighboring fish | |
| Top dwelling | Percent of fish in the top (upper half) of the tank | [24] |
| Thigmotaxis | The average distance of the group from the center of the tank | [41] |
| Variance of inter-fish distance | An index reflecting how homogeneously the fish are distributed in the shoal | [22] |
| Shoal area | The size of the shoal (width and length) | [35] |
| Excursions from shoal | Number of excursions of individual fish away from the shoal | [46]* |
| Duration of excursions | Duration of excursions of individual fish away from the shoal | * |
| Shoal polarization | Absolute size of the summed vector of all fish in the shoal | * |
| Spread of the group | Smallest wedge (extending to edges) that captures within it all the fish | [41] |
| **Social preference** | | |
| Time in target arm | Time spent in the target (conspecific) arm | [30] |
| Time in empty arm | Time spent in the empty arm | [30] |
| Time in center | Time spent in the center of the social preference test apparatus | [30] |
| Target:empty arm time ratio | The ratio between time spent in the target arm to time spent in the empty arm | [30] |
| Target:total time spent ratio | The proportion of time spent in the target arm (relative to the total testing time) | [30] |
| Target arm entries | The number of entries to the target (conspecific) arm | [30] |
| Center entries | The number of entries to the center of the social preference apparatus | [30] |
| Empty entries | The number of entries to the empty arm | [30] |

| | | |
|---|---|---|
| Total arm entries | Sum of target, empty and center entries | [30] |
| Target:empty arm entries ratio | The ratio between entries to the target arm and entries to the empty arm | [30] |
| Target:total arm entries ratio | The ratio between entries to the target arm and total arm entries | [30] |
| **Mirror biting test** | | |
| Mirror biting frequency | Number of times the fish bite the mirror | [28] |
| Mirror biting duration | Time spent biting mirror | [55] |
| Approaches to the mirror | The number of crossing the line denoting the mirror approach zone, but without mirror contact (e.g., 3-0.5 cm from the mirror, and 2.5 cm away from the contact zone) | ** |
| Mirror contacts | The number of crossing the line denoting the mirror contact zone (e.g., 0.5 cm from the mirror) | ** |
| Latency to approach | Time to the first approach to the mirror | ** |
| Aggressive tail beats | The number of aggressive tail beats against the mirror | ** |
| Latency to contact | Time to the first contact with the mirror | ** |

* See chapter by Miller and Gerlai in this book for details. ** These endpoints and zones are based on current protocol, and may be modified by other laboratories, if necessary.

**Table 2. Selected published zebrafish shoaling studies (see Table 1 for definitions of behaviors)**

| Model | Endpoints | Effects | References |
|---|---|---|---|
| **Larval/juvenile zebrafish** | | | |
| 7 vs. 26 days post fertilization | Average inter-fish distance | Reduced | [21] |
| 26 vs. 42 days post fertilization | Average inter-fish distance | Reduced | [21] |
| 59 vs. 76 days post fertilization | Average inter-fish distance | Reduced | [21] |
| **Adult zebrafish** | | | |
| Acute ketamine exposure (20 and 40 mg/L) | Average inter-fish distance, top dwelling | Reduced | [24] |
| Embryonic ethanol exposure (0.25 and 0.5%) | Nearest neighbor distance, average inter-fish distance and its variance | Reduced distances, increased variance | [22] |
| Acute ethanol exposure (0.125, 0.25 and 1.0%) | Nearest neighbor distance, shoal area | Reduced at low doses, increased at a higher dose | [35] |
| Acute lysergic acid diethylamide (LSD, 250 μg/L) | Average inter-fish distance | Increased | [23] |
| Acute alarm substance exposure | Average inter-fish distance | Reduced | [40] |

**Table 3. Social preference tests performed with various experimental manipulations (adult zebrafish studies; see Table 1 for definitions of behaviors).**

| Treatment | Endpoints | Effects | References |
|---|---|---|---|
| Acute ketamine (20 and 40mg/L) | Entries and time spent in center, conspecific arm and empty arms | Increased entries to center, conspecific and empty arms, more time in empty arm | [24] |
| Acute lysergic acid diethylamide (LSD, 250 μg/L) | Entries and time spent in center, conspecific arm and empty arms | Reduced number of total arm entries | [23] |
| Wild type raised with wild type fish | Time spent with wild type or nacre fish | More time spent with wild type | [31] |
| Nacre raised with other nacre fish | Time spent with wild type or nacre fish | More time spend with nacre | [31] |
| Wild type raised with nacre fish | Time spent with wild type or nacre fish | More time spend with nacre | [31] |
| Nacre raised with wild type fish | Time spent with wild type or nacre fish | More time spent with wild type | [31] |

**Table 4. Mirror biting test (Modification 1) performed with various experimental manipulations in adult zebrafish (see Table 1 for definitions of behaviors). If video-recording is used, camera can be positioned with the side- or top-view, and video files re-played in slow motion and annotated manually.**

| **Treatment** | **Endpoints** | **Effects** | **References** |
|---|---|---|---|
| Strain admixture | Mirror biting frequency | Increased in a normally non-aggressive strain (Nadia) when raised with an aggressive strain (TM1) | [28] |
| Comparison of several zebrafish strains | Mirror biting frequency | Higher in TM1 male and female fish compared to Nadia or SH strains | [46] |
| Developmental hypoxia exposure | Mirror biting duration | Reduced (vs. normoxia-reared controls) | [55] |
| Ethanol exposure | Mirror biting duration (minutes 1 and 10 of the test) | Increased at mild doses (0.2-0.5%) and inhibited at a high sedative dose (1%) | [47] |

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