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Short communication

Associative learning in the multichamber tank: A new learning paradigm for zebrafish

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HIGHLIGHTS

- A simple, easy to manufacture learning apparatus was designed.
- Visual discrimination based associative learning was studied.
- Zebrafish were found to exhibit procedural learning and memory.
- Zebrafish were found to exhibit acquisition of CS-US association.
- Maze is argued to be simple enough for high throughput applications.

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ABSTRACT

The zebrafish has been gaining prominence in the field of behavioural brain research as this species offers a good balance between system complexity and practical simplicity. While the number of studies examining the behaviour of zebrafish has exponentially increased over the past decade, the need is still substantial for paradigms capable of assessing cognitive and mnemonic characteristics of this species. Here we describe and utilize a novel visual discrimination task with which we evaluated acquisition of CS (colour)-US (sight of conspecifics) association in adult zebrafish. We report significant acquisition of CS-US association indicated by the increased time the fish spent in and the increased frequency of visits of the target chamber during a probe trial in the absence of reward. Given the simplicity of the apparatus and procedure, we conclude that the new task may be employed to assay learning and memory in adult zebrafish in an efficient manner.

1. Introduction

Despite concerted efforts by a large number of laboratories, the mechanisms of learning and memory are still not clearly understood. For example, the number of genes and gene product known to be involved in neuronal plasticity ([1]) is a small fraction of the number of genes found expressed in the vertebrate brain including the zebrafish brain (e.g [2]). Notably, a large proportion of these expressed genes are not yet functionally annotated, but are suspected to be involved in mnemonic or cognitive processes. For these reasons, laboratory species that would aid gene identification via large-scale mutagenesis screens have been proposed [3]. The zebrafish is one such species. Over the past few decades substantial amount of genetic information has been collected and numerous recombinant DNA methods have been developed for this species [4].

The bottleneck in zebrafish behaviour genetics research has been the behavioural characterization of this species and the development of appropriate behavioural testing procedures and apparati [3,5]. Although these questions are still relatively underexplored, the number of studies attempting to address them has been rapidly increasing over the past few years. For example, studies of learning and memory in zebrafish now show that zebrafish are capable of active avoidance [6], simple CS-US associative learning as well as complex latent spatial learning [7]. [also reviewed in 8]. Furthermore various apparati including the plus-maze (+) [9,10], the T-maze [11], and the shuttle-box [12] have been developed. In addition, different stimuli including animated images presented on a computer screen have been explored for zebrafish learning paradigms [13,14]. Despite these recent developments, the number

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of learning paradigms available for zebrafish is significantly smaller compared to those developed for traditional vertebrate laboratory organisms including the rat or the mouse [3]. Proper characterization of changes in mnemonic and/or cognitive processes induced by mutations or drugs may require multiple behavioural tasks that tap into similar central nervous system functions while having different performance demands [15]. Given the complex nature of learning and memory and the relative paucity of test paradigms that can measure these processes in the zebrafish, there is a substantial need for the development of efficient and robust learning and memory tasks as well as for the characterization of these processes in zebrafish.

In the current study, we developed and tested a new apparatus that we employed to analyze visual discrimination, an associative learning task performed in a cheap aquarium retrofitted with acrylic inserts. Although not novel in its conceptual features, the task has some advantages and/or complementary features to currently existing paradigms. For example, compared to the T-maze it allows the investigator to study the subjects' choices between more than two options. Compared to the plus or radial mazes, the test apparatus has a smaller physical footprint, and it is also easier to construct. Last, although simple in terms of experimental set up and easy to conduct, the shuttle box paradigm developed for zebrafish was found to lead to less than robust acquisition of memory [12].

In summary, the purpose of the current study is to determine whether this simple maze-design in which zebrafish are required to associate a visual cue (CS) with a reward (also a visual stimulus, the sight of conspecifics) may be employed efficiently to induce and quantify associative learning in zebrafish.

2. Methods

2.1. Animals and housing

Sixteen zebrafish (Danio rerio) of the AB strain were used in this experiment. All fish tested were sexually mature, 6–10 months old, young adults (males and females 50–50%). The fish were bred and raised at the University of Toronto Mississauga (UTM) vivarium. They were housed in high-density racks (Aquaneering Inc., San Diego, CA, USA) equipped with multistage filtration that included a mechanical filter, fluidized glass bed biological filter, an activated carbon filter, as well as a fluorescent UV light sterilizing unit. Ten percent of the water was replaced daily with deionized water supplemented with 60 mg/l Instant Ocean Sea Salt (Big Al's Pet Store, Mississauga, ON).

During the habituation phase, fish were housed in groups of 8–10, in 2.8 l rearing tanks (Aquaneering Inc.) placed in a high-density rack system. After habituation fish were individually housed for identification purposes, in 1 l tanks that were part of a high-density rack system (Aquaneering Inc.). The water temperature was maintained at 26 ± 2 ◦ C. Illumination was provided by fluorescent light tubes from the ceiling with lights turned on at 07:00 h and off at 19:00 h. Fish were fed a mixture of ground flake food (4 parts, Tetramin Tropical Flakes, Tetra, USA) and powdered spirulina (1 part, Jehmco Inc., Lambertville, NJ, USA).

In the current paradigm we allowed the experimental fish to interact with a group of conspecific called the stimulus fish. We used five zebrafish as stimulus. In nature zebrafish have been found to form shoals ranging in size between only a few to hundreds of members. In the laboratory, varying the number of members of animated shoals between 3–8 was also found not to have a significant effect on the strength of the shoaling response [16], but in numerous past studies we employed 5 stimulus fish [13,14,17,18], and thus we decided to use the same number of fish here as well. To distinguish "stimulus fish" from the experimental subject, we injected the former with 0.05 μl of blue marking tissue dye (Sigma Aldrich) near the caudal fin as described before [19].

All behavioural experiments were video-recorded from an overhead camera (JVC Everio GZ-MG500, Yokohama, Japan), and later replayed for observation-based quantification using Observer Colour Pro XT (Noldus Info Tech., Wageningen, The Netherlands).

2.2. Experimental maze

The new maze (Fig. 1) was made of Plexiglas, rectangularly shaped, measuring 60 cm × 47 cm × 25 cm (length × width × height). While designing the maze, we kept simplicity in mind. For example, the interior construction of the maze utilizes acrylic inserts that are rectangular and do not have to be glued in complicated shape or angles. Furthermore, these inserts may be lowered into a standard 40 l pet store variety tank made of glass and thus unlike in Y or T-mazes do not require precision water-tight manufacturing. Along the length of the maze, on either side were four chambers (15 cm × 10.5 cm × 25 cm), which were separated by an open compartment (60 cm × 25 cm × 25 cm). One side contained the four start chambers, labeled one through four. Directly opposite the four start chambers were the four target chambers. Each target chamber had one coloured (yellow, blue, red, or green) removable cue card. The choice of cue colour was based on previous research [20]. The colour cue marking the chamber where the stimulus fish were placed we designated as CS_+, while the other colour cues that mark target chambers without the US are referred to as CS_0. The target chambers were accessible from the open area through a 9 cm tube that connected each target chamber to the open compartment. The opening between the tube and the target chamber was closed by a transparent door, which allowed the experimental subject to see the stimulus fish (the unconditioned stimulus, US) and the cue while preventing the stimulus fish from leaving the target chamber. At the tube entrance, which connected to the open compartment was a slot for a second door. The second door was placed at the entrance, once the experimental subject entered the tube.

2.3. Procedure

The experimental procedure had three phases: habituation, training, and the probe. During the habituation phase the experimental fish were placed in the maze in groups, and the size of these groups gradually decreased from 16 (first day, one 20 min long exploration session), to 8 (second day, two separate 10 min exploration sessions), to 4 (third day, two separate 10 min exploration sessions), to 2 (fourth day, four exploration session of 5 min each) to 1 (fifth day, four 5 min long exploration sessions). For each habituation trial, the fish were/was placed in a different start chamber. During the habituation/exploration sessions, only the start chamber from which the fish were released remained open, while the other three were closed. All reward chambers were open.

Following the habituation, experimental fish entered the training phase. Experimental subjects were housed in the behavioural testing room. A black metal divider (120 cm × 180 cm, width × height) blocked the view of the maze from the holding tanks. Fish were netted from their holding tank, the net with the fish was placed into a 500 ml beaker, and the fish were transported while immersed into the beaker with the net to the experimental tank. The experimental fish was placed singly into the start chamber of the test apparatus and was released by raising the guillotine door. All target chambers were open and accessible to the experimental fish. One of the target chambers contained a stimulus shoal, i.e. the reward (US). A transparent door prevented these fish from leaving the target chamber. The particular colour cue (CS_+) marking the location of the target chamber containing the stimulus fish

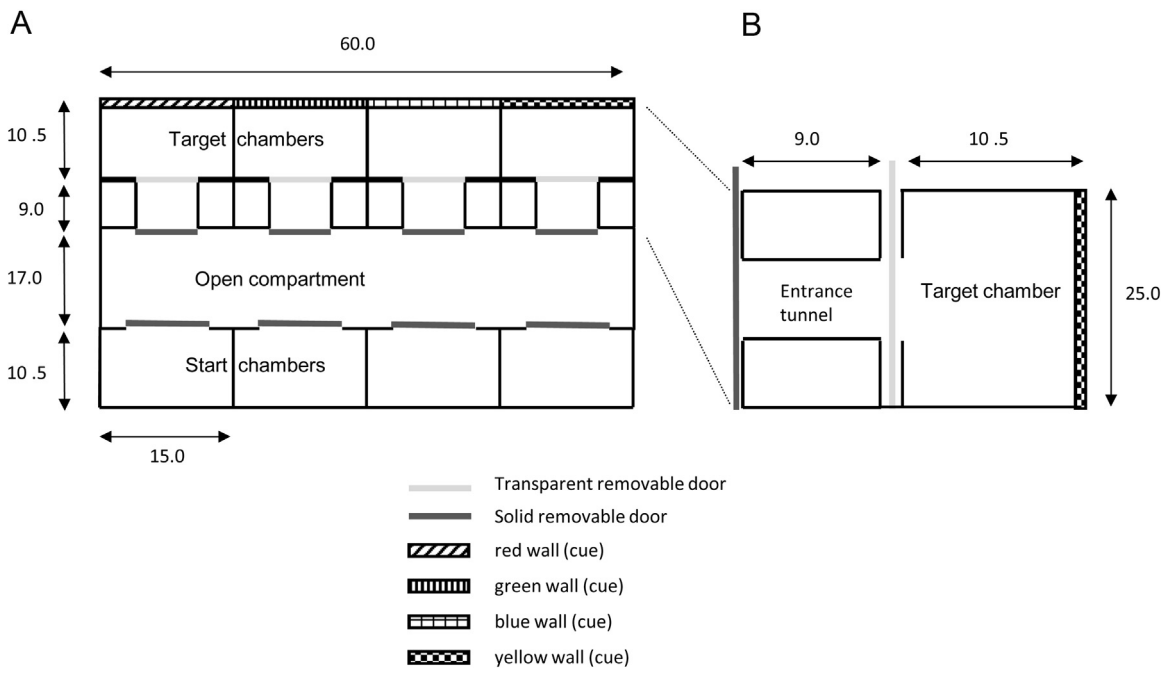

Fig. 1. The blue-print of the experimental tank showing the different compartments (start chambers and colour cue marked target chambers) from above (A) and from the side (B). The dimensions of the maze are indicated in cm. The colour designations represent an example. The colour cues may be moved at will. The tunnels are short tubes through which the experimental subjects could swim into the colour cue marked target chambers.

remained constant for each experimental fish across all 20 trials but which colour cue was used as CS_+ varied across the experimental fish. The other three (non-rewarded) chambers were also marked by a colour cue (CS_0) but the colours differed from CS_+. There were four starting locations. During the course of a training day each experimental fish received four training sessions, starting once from every starting location. The trial ended 5 min after the start chamber was opened. Each experimental fish was given 20 training trials in total, i.e. four, 5 min trials per day over the course of 5 consecutive days.

Experimental fish were rewarded by being able to stay in close proximity of the shoal. The amount of time the experimental subject spent shoaling was dependent upon how fast they located the shoal. If the experimental subject did not find the shoal during the trial they did not receive any reward. To enter the reward chamber experimental subjects had to swim from the open compartment through the 9 cm tube into the reward chamber. Once the experimental subject entered the 9 cm tube, a door was first placed at the tube's entrance, and subsequently the door to the reward chamber was opened.

During the probe trial, all target chambers with their colour cue cards were open. No reward fish (stimulus shoal) were present. The probe trial began with raising the door of the start chamber, and ended 5 min later. Each experimental fish received a single probe trial. An overhead digital camera (JVC Everio GZ-MG500, Yokohama, Japan) recorded the trials for behavioural analysis. To minimize the possibility of straight-line direct swimming into the previously rewarded target chamber, during the probe the start chamber and the previously rewarded target chamber were always two chambers away from each other for all experimental fish.

2.4. Quantification of behaviour and statistical analysis

The digitally recorded video files were analyzed by a trained individual using an event recording software application, The Observer XT 9.0 (Noldus, Info Tech., Wageningen, The Netherlands). During training we measured the latency to locate the reward chamber, and during the probe we measured the percentage of time spent in the reward chamber as well as in the other three chambers of the experimental tank. The time spent in these later chambers were averaged and compared to the time spent in the target chamber.

Data were analyzed using SPSS (version 21) for the PC. We employed a repeated measure analysis of variance (ANOVA) to examine the effect of Training Day (repeated measure with 5 levels) on the latency to enter the target chamber, a measure we argued may be used to conclude about procedural learning during training as well as about motivation and motor ability of the experimental subjects. In addition, we also investigated whether the latency was reduced as training progressed comparing performance on each possible pairs of days using one tailed repeated measures ANOVA (two levels) with Bonferroni correction for multiple comparisons.

To determine whether fish formed an association between the colour cue and the stimulus (reward) shoal we measured the performance of the fish in the probe trial during which the US (conspecific stimulus fish) was absent and compared the percent of time experimental fish spent in the target (previously rewarded) chamber and the average of the percent of time spent in the three other (non-rewarded) chambers using a paired two-tailed *t*-test with significant difference accepted when $p < 0.05$. We performed a similar analysis for the frequency of visits to the target (previously rewarded) versus visits to the other three (non-rewarded) chambers. In addition, we also compared how effective a CS the different colours were, i.e. we compared the target chamber preference (both the percent of time in and the frequency of entry to the target chamber) across fish trained with the four different colour cues using univariate ANOVA followed by Tukey HSD Posthoc tests when warranted.

3. Results

Fig. 2 shows the latency to reach the reward chamber across the five training days. Training day was found to have a significant effect on the latency to reach the reward chamber ($F(4, 60) = 4.234$,

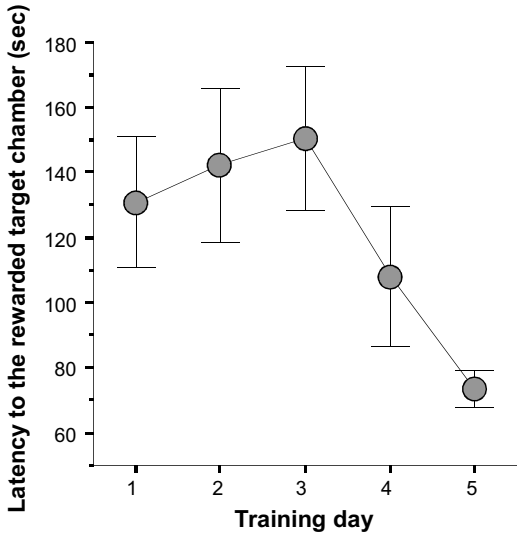

Fig. 2. The latency to enter the chamber containing the stimulus shoal (rewarded target chamber) significantly diminishes by the fifth day of training. Mean ± S.E.M are shown. n = 16.

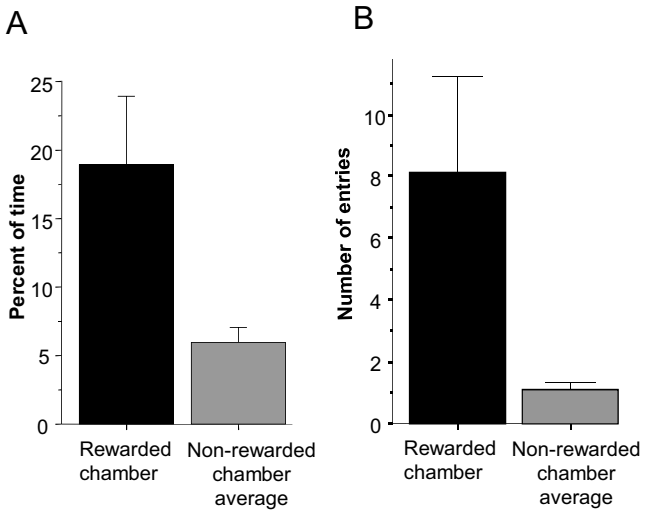

Fig. 3. The percent of time experimental fish spent in the chamber marked by the correct colour cue (CS_+) (Panel A) and the number of entries (frequency) to this chamber (Panel B) are significantly higher compared to the average percent of time in and the average number of entries to the cambers marked by the other colour cues (CS_0) during the probe trial. Mean ± S.E.M. are shown. The black bars represent the values obtained in the rewarded target chamber, whereas the average of the performance measured in the other three chambers are shown by the grey bars. n = 16. Note that during the probe trial, the US (the stimulus shoal) was not present in the experimental apparatus.

p = 0.004). Post hoc two-level repeated measure one tailed ANOVA with Bonferroni correction revealed that the latency to reach the target chamber was significantly smaller on day 5 compared to all previous training days ($F(1, 15) = 8.955$, Bonferroni corrected $p < 0.05$) except compared to day 4.

Fig. 3, panel A, shows the percentage of time the experimental fish spent in the chamber marked by the correct associative cue (CS_+) versus the average of the percent of time they spent in the other three chambers marked by the other colour cues (CS_0) during the probe trial. Note that during this trial, the US (the conspecific stimulus fish) was absent. The percent of time fish spent in the target chamber in the vicinity of the CS_+, versus the average percent of time they spent in the other three (non-rewarded) chambers in the vicinity of the incorrect cues (CS_0) was found significantly different ($t = 2.610$, $df = 15$, $p = 0.02$). Fig. 3, panel B shows the number (frequency) of visits to the target chamber marked by CS_+ versus the average of the entries to the three other, previously non-rewarded, chambers marked by CS_0. The difference between the number of entries to these two parts of the maze was found significant by a paired two-tailed *t*-test ($t = 2.249$, $df = 15$, $p = 0.04$). Also notably, comparison of performance of fish trained with the four different colour cues as CS_+ found no significant colour cue effect for the percent of time fish spent in the previously rewarded target chamber ($F(3, 12) = 2.587$, $p > 0.10$) or for the frequency of visits to this target chamber ($F(3, 12) = 2.697$, $p > 0.05$) during the probe trial.

4. Discussion

The small number of behavioural assays available for zebrafish is considered a bottleneck for using this species in the field of behavioural neuroscience [5,8], a problem especially noticeable in the light of the vast number of genetic tools available for this species [4]. Furthermore, analysis of learning and memory often requires multiple test paradigms. Arguably, a single learning test is never sufficient for proper interpretation of potential mutation or drug effects on cognitive and/or mnemonic processes because numerous performance features idiosyncratic to the employed task may be affected by the genetic or the pharmacological manipulation. Given that these effects are not known to the experimenter, the use of multiple learning tasks with different performance features have been recommended [3,15]. Here, we presented and employed a simple apparatus that can be used to measure learning and memory in adult zebrafish. The paradigm is not novel conceptually as it is based upon the principles of colour discrimination learning, a type of associative learning that requires the animal to distinguish between reinforced (CS_+) and unreinforced visual cues (CS_0). However, we argue that the relative ease with which the apparatus can be manufactured and used may make it a useful addition to already existing learning tasks.

Using the newly developed apparatus, we found that by the fourth training day, experimental subjects started to reduce their latency to enter the target chamber, and by the fifth training day this latency reduction became significant. Notably, during training the conspecific stimulus fish (the rewarding stimulus) were always present. Their sight and potentially their smell as well as auditory cues were all accessible to the experimental fish. Thus, the reduction of latency to enter the target chamber containing the stimulus fish during training cannot be interpreted as a sign of acquisition of the association between the colour cue (CS_+) and the presence of the stimulus fish (US). Although acquisition of the CS-US association might have contributed to the decrease of latency to enter the rewarded chamber, the latency reduction is likely better explained by procedural learning, i.e. acquisition of information required for the fish to perform well in the maze in general. This information may include getting habituated to human handling, the general layout of the maze, the movements the subject had to perform when swimming out from the start chamber through a short tunnel to the stimulus fish, etc. In addition, the decreasing entrance latency also signifies that the experimental fish continued to be motivated to actively move in the maze and seek out the rewarded target chamber. This is an important point as it suggests the motivation to find and to move to close physical proximity to shoal mates (the stimulus fish) did not diminish throughout the training. This motivational feature of the task is somewhat novel compared to Y, T, + or radial arm mazes in which food is utilized as reward for zebrafish [21]. We decided to use live stimulus fish, instead of more traditional reinforcers (e.g. food) for several reasons. Previously, Al-Imari & Gerlai [22] showed that the sight of conspecifics is rewarding. Subsequent studies also revealed that

behind this reward lies the functioning of the dopaminergic system [17,23,24]. The strong motivation of a single experimental fish to seek out and join a shoal in a novel environment is not surprising given that zebrafish form shoals in nature and in the laboratory [25]. Thus, here we emphasize that the use of a stimulus shoal has been found to be an excellent motivator in zebrafish learning studies as this stimulus represents a non-satiating reinforcer that requires no prior deprivation (e.g. restricted feeding) that may have deleterious health consequences [12]. The problem with live stimulus fish, however, is that such a stimulus is inherently variable. We used stimulus fish that had been well habituated to the experimental procedure, and for this reason we expected these stimulus fish to behave in fairly stable and consistent manner. Nevertheless, we could not precisely control the behaviour of the stimulus fish, and thus likely could not fully eliminate trial to trial variation in their behaviour. Such control would only be possible with the use of artificial stimuli, e.g. computer animated images of zebrafish. Computer animated images have been successfully utilized for inducing strong shoaling responses [23,26,27] as well as for acquisition of short and long term memory in zebrafish [13,14]. Whether this artificial stimulus is appropriate for the current paradigm will be empirically answered in the future.

Memory of the acquisition of CS-US association may be evaluated from the probe trial results. During the probe trial, the rewarding stimulus (US) was absent, yet the experimental fish spent disproportionately high percentage of time in the chamber that contained the CS_+. Zebrafish is a fast swimming fish; in nature and in the laboratory they do not have stable territories. Thus, we expected zebrafish to actively explore the maze. The increased time they spent in the target chamber marked by CS_+ is not because they remained in this chamber once they found it, but rather it is due to the significantly increased number of visits to this chamber as compared to the visits to the other chambers. This latter finding demonstrates that throughout the probe trial, zebrafish showed a consistent preference for the chamber associated with the correct colour cue. Thus, we conclude that experimental fish successfully acquired the association between the CS_+ and US.

What the basis of this association is, we do not know. It is notable that intrinsic (prior-training or innate) colour bias is unlikely to explain the post-traning CS_+ preference, because the colours designated as the CS_+ varied across the different subjects. Our analysis also indicated a lack of significant difference in the performance of fish as to which colour served as CS_+. Nevertheless, because the colour cues remained in a fixed position throughout training and the probe, the experimental subject could locate the reward (target) chamber in three principally different ways: one, colour cue − reward association; two, place −reward association; and three, ego-centric cue − reward association. Previously, Karnik & Gerlai [28] showed that zebrafish can learn two distinct types of associations during training demonstrating that, similarly to mammals with intact hippocampal function, this fish species is capable of simultaneous acquisition of non-conflicting spatial cues as well as a separate single associative cue. The current paradigm will allow the investigator to explore these questions and dissociate the effect of such cues. For example, one could train the fish to find a particular location with or without the separate single associative cue marking the location, and/or one could conflict spatial and single associative cues during probe trial, and one could perform such studies for ego-centric cues vs. other cues.

Although other mazes and associative learning tasks have been successfully employed, we argue that the current test apparatus and paradigm will have a place in the tool box of zebrafish learning and memory methods. There are two principally different reasons for this argument. One is practical, and the other is more fundamental. First, the practical reason why we hope this new maze will be useful is that its design is simple and thus the maze can be manufactured easily and cheaply. The chambers, tubes, and doors require minimal engineering and expertise to make, and all these pieces may be lowered into a standard glass aquarium. Importantly, unlike complex Y, T, or multiple arm mazes (+ or radial arm mazes), the manufacturing of our apparatus does not involve having to cut multi-angled complex shapes, and also does not require watertight construction (the structure is inside a regular glass tank). The physical barriers force the experimental fish to make clear choices that are easy to quantify using observation-based or video-tracking methods both from a side and from an overhead view. The ability to clearly see the fish from the side is an added bonus because this view enables the investigator to observe and quantify numerous subtle behavioural responses including fin erection displays (aggression), erratic movement (fear), and other motor patterns described for the zebrafish [29], which would aid interpretation of results. Last, the apparatus is small, and conducting behavioural tests using multiple tanks is simple. Parallel running of multiple learning apparati facilitates higher throughput, and thus the paradigm may be useful for drug or mutation screens. The second, more fundamental, reason why we hope this simple task may be used in the analysis of learning and memory in zebrafish is that it differs from most other learning tasks in its procedural demands. It is an appetitive task, but it uses conspecifics as the reward instead of the more traditional food reward. Performance in the task requires active swimming similarly to most other mazes utilizing appetitive conditioning, but unlike in T, Y, + or multi-arm mazes, the distances zebrafish need to traverse in the current apparatus are small, and the fish are required to navigate through tight spaces, making egocentric cue based navigation somewhat difficult.

We acknowledge that many of the above arguments are speculative at this point, and that their validity will have to be ascertained by empirical studies comparing different learning paradigms in a systematic manner. Nevertheless, given the simplicity and versatility of the current paradigm, we hope that its utility will be recognized, and it will be successfully used in mutation and drug screens leading to better understanding of the mechanisms underlying the complex processes of learning and memory, the ultimate goal of the current study.

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