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Research report

Can zebrafish learn spatial tasks? An empirical analysis of place and single CS–US associative learning

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HIGHLIGHTS

- Zebrafish were trained to learn CS–US and location-US association concurrently.
- Acquisition of the CS–US association did not interfere with acquisition of location–US association.
- Similar performance is found in rodents at the context and cue dependent fear conditioning.
- Similarities between zebrafish and rodent performance represent translational relevance.

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ABSTRACT

The zebrafish may be an ideal tool with which genes underlying learning and memory can be identified and functionally investigated. From a translational viewpoint, relational learning and episodic memory are particularly important as their impairment is the hallmark of prevalent human neurodegenerative diseases. Recent reports suggest that zebrafish are capable of solving complex relational-type associative learning tasks, namely spatial learning tasks. However, it is not known whether good performance in these tasks was truly based upon relational learning or upon a single CS–US association. Here we study whether zebrafish can find a rewarding stimulus (sight of conspecifics) based upon a single associative cue or/and upon the location of the reward using a method conceptually similar to 'context and cue dependent fear conditioning' employed with rodents. Our results confirm that zebrafish can form an association between a salient visual cue and the rewarding stimulus and at the same time they can also learn where the reward is presented. Although our results do not prove that zebrafish form a dynamic spatial map of their surroundings and use this map to locate their reward, they do show that these fish perform similarly to rodents whose hippocampal function is unimpaired. These results further strengthen the notion that complex cognitive abilities exist in the zebrafish and thus they may be analyzed using the excellent genetic tool set developed for this simple vertebrate.

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1. Introduction

The zebrafish has been becoming increasingly popular in behavioral brain research. This species appears to strike an optimal compromise between system complexity and practical simplicity. On the one hand, it is a vertebrate with a sophisticated brain whose basic layout [1] and neurochemical properties [2] are similar to those of higher order vertebrates including mammals. On the other hand, it is small (4 cm long), prolific (200 eggs per spawning per female every other day) and cheap and easy to maintain in large numbers in captivity. Also importantly, numerous genetic tools have been developed for the zebrafish and a large amount of genetic information has been accumulated for it making this species one of the most preferred study organisms of geneticists [3]. Not surprisingly, these features have made zebrafish perhaps the best research tool for high throughput screening in a variety of sub-disciplines of biology [4].

Zebrafish have been increasingly utilized in the analysis of vertebrate learning and memory [5–14]. A large amount of information has been obtained about the mechanisms of learning and memory with the use of the primary model organism of biomedical research, the house mouse [15]. However, according to some estimates, the number of molecular targets (genes and their protein products) involved in learning and memory discovered so far is at least an order of magnitude fewer than what may actually underlie these complex brain functions (e.g. [12]). The zebrafish has been suggested as an ideal research tool for tackling this complexity [10,12] primarily because it offers the investigator the ability to conduct

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high throughput screens and identify a large number of mutants and subsequently the genes carrying the mutations [4].

In order for such discoveries to have potential translational relevance, ideally one would like zebrafish to possess behavioral features that resemble those of mammals (face validity). Also, ideally, one would want to find that mechanisms that subserve the chosen behavioral function are similar between humans and the laboratory study species (construct validity). Relational learning and episodic memory have been thoroughly investigated in mammalian species partly because these phenomena have important human clinical implications. In our species, relational learning and episodic memory are known to be dependent upon the normal functioning of the hippocampal formation (e.g. [16]). It has also been repeatedly demonstrated that the hippocampus is particularly vulnerable to neurodegenerative processes associated with, for example, Alzheimer's Disease [17,18]. Indeed, impairment of relational learning and episodic memory is one of the core symptoms of Alzheimer's Disease (e.g. [19,20]). An important form of relational learning is spatial learning [21].

Here we define spatial learning as the process that allows the human or non-human animal to acquire spatial cues and, importantly, the dynamic relationships among these cues [22]. Briefly, according to this definition, spatial learning leads to the establishment of a spatial map, a neural representation of the external environment. Could zebrafish develop such a spatial map?

Recent studies with zebrafish [14] suggested that similarly to another cyprinid, the gold fish [23], zebrafish too are capable of performing well in spatial tasks and that their learning performance is dependent upon the NMDA-receptor similarly to what has been found with mammals [13,24]. The zebrafish studies showed that the subjects could locate a reward and show a preference for a particular location in their environment which previously contained the rewarding stimulus [14]. Similar results are often regarded as sufficient evidence for spatial learning ability in the mammalian literature and indeed this is how the gold fish and zebrafish results have been interpreted too. However, the possibility exists that a subject that shows good spatial learning performance identifies the location of the reward not by being able to develop a dynamic spatial map of the external environment but by associating the reinforcer's location with a single cue. In this latter case, performance in the spatial learning task would appear excellent (the subject finds the location well) but this performance would not reflect true relational (spatial) learning but rather simple CS–US associative learning.

A similar problem surfaced in the rodent neurobehavioral genetics research: mutant mice, mice from inbred strains (e.g. DBA/2) with abnormal hippocampal function, and rats with lesioned hippocampus, which were expected to be impaired in spatial tasks, were occasionally found to perform well in such tasks [25–27]. For example, in the context and cue dependent fear conditioning paradigm in which the subject needs to recognize/identify the context (the place where it was shocked), DBA/2 mice showed a strong response to the context. That is, these mice exhibited a robust amount of freezing when placed in the chamber where they previously received the electric shocks [25]. A similar finding was also obtained with hippocampal lesioned rats [27], which exhibited a good freezing response to the shock chamber despite their anatomically confirmed hippocampal impairment. In both of these examples the experimenters concluded that perhaps the hippocampally impaired rodents turned their spatial task into a non-spatial, simple CS–US associative task by selecting a single cue from the "background" and associating this single cue with the reinforcer. This speculation was supported by empirical data. When the experimenters provided a single salient associative cue (CS) predictive of the delivery of the shock (US) during training, the hippocampally impaired rodent (DBA/2 mouse or lesioned rat) could not respond to the shock chamber alone at a subsequent probe trial. In other words, once a salient cue was experimentally provided and paired with the US, the hippocampally impaired rodent could not select yet another cue from the context and thus was unable to respond to the place where the shock training occurred. It could, however, still learn the association between the US and the experimentally provided salient CS (elemental as opposed to relational learning). Also importantly, mice and rats with intact and unimpaired hippocampal function were able to respond to the place even when the salient cue was used during training. That is, rodents with intact spatial learning abilities could learn both the association between the salient CS and US (elemental learning) and the location of the US (relational or spatial learning).

In the current study we employed the above logic in the analysis of the learning capabilities of zebrafish. Here, we trained our experimental zebrafish by presenting them with a reward (sight of conspecific stimulus fish) whose location was constant in the test tank. In addition to being in the same place, the stimulus was also predicted by a red plastic cue card that was placed behind the tank of the stimulus fish. The question we were asking was whether experimental zebrafish could identify the correct location of the stimulus fish by learning both distinct pieces of information: one, the association between the stimulus fish and the red cue card, and two, the association between the stimulus fish and some other features of the location of the stimulus fish.

2. Methods

2.1. Animals and housing

Forty zebrafish (*Danio rerio*) of the AB strain were used in the experiments. All fish tested were young sexually mature, 6–10 months old adults (males and females 50–50%). The fish were bred and raised at the University of Toronto Mississauga (UTM), and housed in high density racks (Aquaneering Inc., San Diego, CA, USA) with multistage filtration that contained a mechanical filter, a fluidized glass biological filter, an activated carbon filter, and a fluorescent UV light sterilizing unit. 10% 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). Fish were housed in groups of 8 in 40 L acrylic tanks (model# ZFC-1.0, Aquaneering Inc.) prior to testing. The water temperature was maintained at $26 \pm 2\,^{\circ}C$. 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).

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

2.2. Apparatus

The test apparatus (Fig. 1) was a transparent Plexiglas square "open-field" (80 cm × 80 cm × 20 cm, length × width × depth). Four transparent (26 cm × 15 cm × 10 cm, length × width × depth) stimulus tanks were placed in the tank on each side of the field along the edge and equidistant from the corners, such that they were accessible on only three sides. A (10 cm × 10 cm × 10 cm) start box was placed in the center of the tank that served as the release apparatus. The experimental tank was filled with system water to a height of 10 cm such that water levels in the stimulus tanks and the experimental tank were equal. Water was maintained at $27\,^{\circ}C$ by thermostat controlled aquarium heaters (EHEIM JAGER Model #7357890, Deizisau, Germany).

2.3. Procedure

The procedure had three phases: habituation, training and probe. To acclimatize fish to the test apparatus, all experimental subjects received three 1 h long habituation sessions (one session per day on consecutive days) followed by one 5 min long session on the fourth day. For the habituation sessions of the first day, 8 fish were placed in the experimental tank at a time. The second day 4 fish and the third day 2 fish were released in the tank for habituation. On the fourth day a single fish was placed in the experimental tank at a time. During the habituation sessions all stimulus tanks were empty and no additional cues were delivered.

Following habituation, experimental zebrafish were divided randomly into two groups: paired (for which the stimulus fish were presented in a single spatial location which was also marked with a red cue card) and not-paired (for which the stimulus fish were presented at random locations, and for which the red cue card

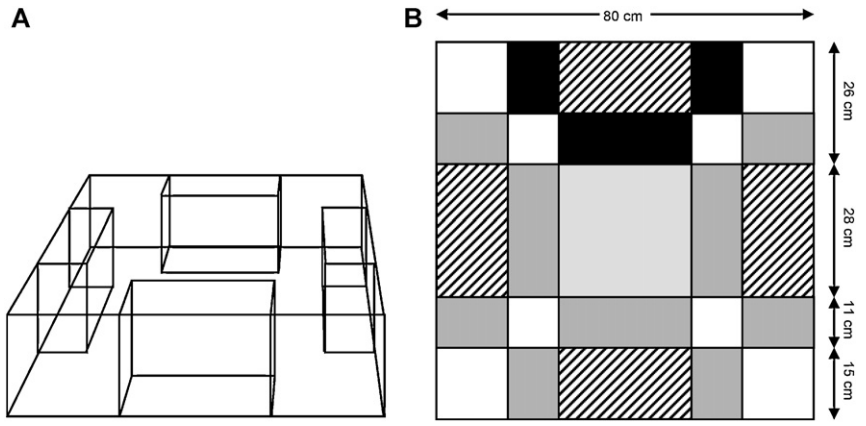

Fig. 1. The physical layout of the large open experimental tank is shown in panel A. This tank contained four stimulus tanks placed adjacent to the walls. All walls of the apparatus were made of glass and were transparent. One of the stimulus tanks contained five conspecific stimulus fish during training. Behind one of the stimulus tanks a red plastic cue card could be placed. The dimensions of the experimental tank along with the dimensions of certain areas of this tank are shown in panel B. The areas shown with different shading served as a template for quantification of the location of the single experimental subject both during training and at a probe trial. The black areas represent the target zone that is proximal to the location of the stimulus. The gray areas represent the proximity zones which correspond to the target zone but are adjacent to stimulus tanks without the stimulus. The striped areas represent the stimulus tanks, and these areas are not accessible to the experimental fish. The white areas represent locations from which the content of the stimulus tanks are not possible to see due to reflection from the glass walls of the stimulus tanks (diffraction coefficient differences between glass and water). These latter two types of areas (striped and white) are excluded from the quantification and analysis of the location of the fish.

was given also at random locations). During the training trials, one of the stimulus tanks contained five conspecific zebrafish of the same strain and size as the experimental fish (the gender ratio within the stimulus shoal was approximately 50–50%). A red cue card (27 cm × 16 cm) was attached to the back of one of stimulus tanks as follows. Fish in the "paired" group received the cue card always behind the tank that contained the conspecifics and both this cue card and the stimulus tank that contained the conspecifics were placed in a constant location relative to extra-tank visual cues. On the other hand, fish in the "not-paired" group received the cue card at random order behind the 4 stimulus tanks and the stimulus tank that contained the stimulus fish was also placed at the four possible locations in a random order. The sight of conspecifics has been shown to be rewarding [9] and thus no other reinforcement was employed. The rationale for presenting the cue card and the stimulus fish at the same location for the paired group was principally the same as in the context and cue dependent fear conditioning where both the contextual (diffuse spatial background) cues as well as a salient associative cue (CS) were presented with the reinforcer (US, electric shock).

Each day, four 5-min long training trials were administered between 12:00 h and 17:00 h for a total of 5 consecutive days, i.e. training consisted of 20 trials in total. For each trial, the experimental fish was removed individually from the holding tank and transferred to the center start box of the experimental tank, where it was allowed to acclimatize for 10 s. The box was then lifted remotely using a metal rod and the trial commenced. The fish was allowed to explore the experimental tank while the experimenter waited outside of the testing room. The room in which the experiment was conducted contained numerous visual cues including large pieces of equipment, shelf units, fluorescent light fixtures and several complex three-dimensional Styrofoam blocks attached to the walls of the room and painted with different colors.

The probe trial took place one day after the last training trial. All procedures and conditions were the same for the probe as in the training trials except that no stimulus fish were presented and only a single probe trial of 5 min was run for each fish. From each of the two training groups, experimental fish were randomly assigned to one of two probe groups: probe with cue card and probe with no cue card. That is, the experimental design was a 2 × 2 between subject design with training as a factor with two levels as described before (paired and not-paired), and probe, the second factor, also with two levels (cue card present, cue card absent). In the 'cue card present' probe trial experimental fish received the cue card at any one of the four locations behind one of the stimulus tanks. The location of presentation across multiple experimental fish followed a random order. This probe trial was expected to test whether experimental fish learned the association between the conditioned stimulus (cue card) and the reward (stimulus fish). The 'no cue card present' probe trial was expected to reveal whether the past spatial location of the stimulus fish was learned.

2.4. Quantification of behavior

The behavior of experimental fish was recorded for the first and last trials of the training session and most importantly also for the probe trial. Behavior was quantified using Observer ColorPro XT (Noldus Info Tech., Wageningen, The Netherlands). The testing arena was divided into imaginary sections consisting of the center, three non-target proximity zones, and the target zone (Fig. 1, panel B). The target and proximity zones were identical in area size and were the rectangular zones whose outer edge was 11 cm away from the nearest wall of the corresponding stimulus tank (Fig. 1, panel B). From these zones, experimental fish could have unimpeded view of the content of the stimulus tanks (stimulus fish from the target zone, indicated by solid black, and no stimulus fish from the proximity zones, indicated by solid gray shading, Fig. 1, panel B). The amount of time spent in the target zone, the three proximity zones and the center was quantified respectively for the 1st and the 20th training trial as well as for the cue present and no cue present probe trials. During training trials, the target zone is defined as the zone in which the stimulus tank with stimulus fish is placed (as shown in Fig. 1, panel B). During the cue present probe trial, the target zone is defined as the zone in which the stimulus tank with the cue card is present. During the no cue probe trial, the target zone is defined for the paired zebrafish group as the zone where the stimulus fish were present during training.

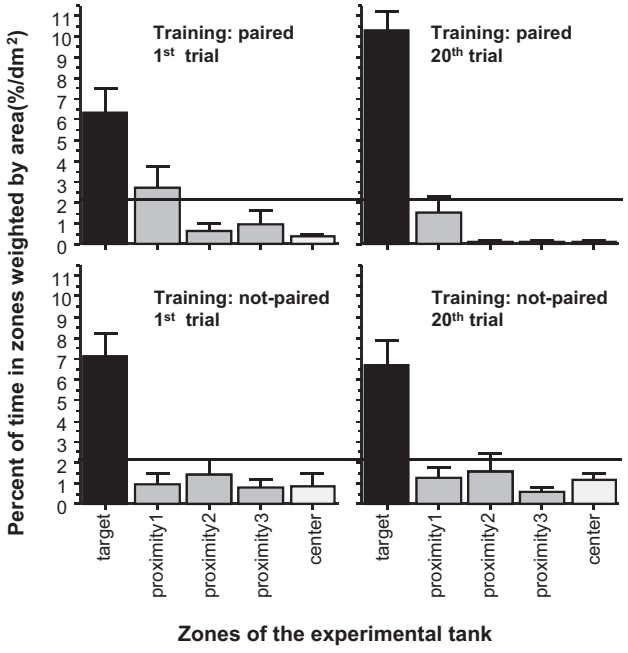

Fig. 2. Zebrafish prefer to stay in close proximity of the stimulus tank that contains stimulus fish (US) throughout training (the 1st [upper and lower bar graphs on the left] and the 20th [upper and lower bar graphs on the right] trials are shown representing the first and last trial of training). The data are expressed as percent of time spent per unit of area of the tank. Mean ± S.E.M. are shown, sample sizes (n) equal 20 for both the paired and the not-paired training group. Random chance is indicated by the straight horizontal line. Note the strong preference shown toward the target zone (black bars) that contained the stimulus tank with the conspecific stimulus fish in both the paired training group (upper two graphs) and in the not-paired training group of fish (lower two graphs).

The target zone for the not-paired group, on the other hand is defined during the no-cue present probe trial as the subjective "North" target zone.

In addition to the location of the fish, we also measured the percent of time the fish were immobile (lack of locomotion, fish remain in the same position, usually occurs near the bottom or the surface of the water) and the percent of time for which the fish moved erratically (a species-specific and stereotypical movement pattern whereby the fish zig-zag moving with a higher than normal swimming speed and changing directions more than one per second) (Fig. 7). Immobility and erratic movement have been found to be associated with fear in zebrafish [28–30], a behavioral state that may interfere with learning in appetitive conditioning tasks.

2.5. Data analysis

For data analysis we first calculated the percent of time fish spent in each of the above described segments of the experimental tank during the 1st and the 20th trials of the training as well as during the probe trial. Subsequently we calculated the percent of time fish spent per unit of area to make the time in the different segments comparable across these segments (the area of the center and of the target and proximity zones differ). We expressed the thus calculated values as %/dm^2 (percent of time per square decimeter) and analyzed these values using one sample *t*-tests and univariate Variance Analysis (ANOVA). Using ANOVA we investigated whether training (two levels: paired, not-paired) and probe (cue card present or cue card absent) had a significant ($p < 0.05$) effect on target zone time. In addition, we also investigated whether performance in any of the four groups of fish was significantly better than chance level using one tailed one sample *t*-tests with Bonferroni correction for multiple comparisons. Chance level performance is calculated as the total percentage of time (i.e. 100%) divided by the total area of the maze (4720 cm^2 = 47.2 dm^2). Using this calculation, random chance comes to 2.1186%/dm^2, and this was the value to which we compared the performance of fish from each group at their 1st and 20th training trials as well as at the probe trial. In addition to the location of the fish, we analyzed the effect of training (paired vs. not-paired), on motor patterns immobility and erratic movement during training, using two sample two tailed *t*-tests. We also investigated the effect of training (paired vs. not-paired) and the type of probe trial (cue card present, cue card absent) on these behavioral measures using univariate two factorial ANOVA using the probe trial data.

3. Results

First we examined performance of our experimental fish during training. Here we report the results for the first and the last (the 20th) trial of the training. Fig. 2 demonstrates that fish both in the paired and not-paired training groups preferred to stay in the target zone, which contained the stimulus fish. The percent of time (weighted by area) they spent in the target zone was above random chance, an observation that is supported by one sample *t*-tests (paired training 1st trial $t = 3.434$, df = 19, $p < 0.01$; paired training 20th trial $t = 8.973$, df = 19, $p < 0.001$; not-paired training 1st trial $t = 4.798$, df = 19, $p < 0.001$; not-paired training 20th trial $t = 3.881$, df = 19, $p < 0.01$). These results suggest that all fish remained motivated to stay in close proximity of the stimulus fish throughout training, a finding that is in line with the strong shoaling tendency of zebrafish reported previously [9,31–33].

Fig. 3 shows the percent of time fish remained immobile during the 1st and the 20th trials of the training. The figure depicts an apparent difference in immobility between the fish of the paired and not-paired training groups, suggesting that perhaps the paired group started out more passively and became more active by the end of the training while the not-paired group did the opposite. However, these apparent differences did not turn out to be significant (trial 1, paired vs. not-paired fish $t = 1.262$, df = 38, $p > 20$; trial 2, paired vs. not-paired fish $t = -1.59$, df = 38, $p > 0.10$).

Fig. 4 depicts the percent of time fish moved erratically during the 1st and the 20th trials of the training. It is notable that this behavior occurred rarely. Statistical analysis found no significant differences between the two training groups at either trial (1st trial $t = -0.454$, df = 38, $p > 0.65$; 20th trial $t = 1.55$, df = 38, $p > 0.10$).

In summary, during training, fish of both the paired and the not-paired training groups showed strong preference for the unconditioned stimulus. Also notably, all experimental fish exhibited only small amount of behavioral responses associated with fear and these responses did not differ between the training groups.

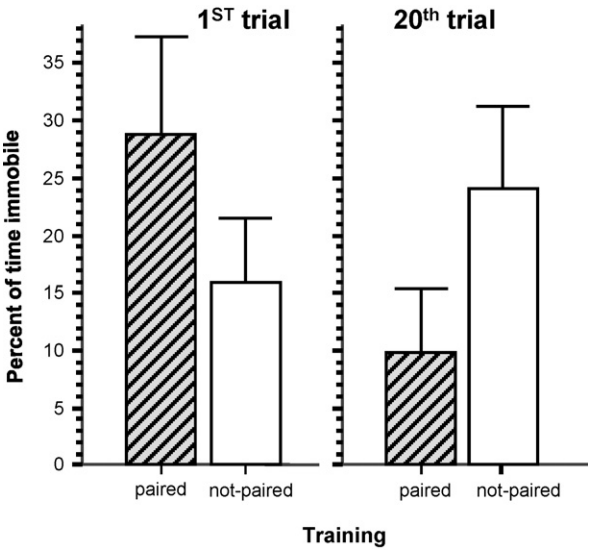

Fig. 3. Percent of time experimental fish remained immobile during the 1st and 20th trials of the training does not significantly differ between fish of the paired training and not-paired training groups. Mean ± S.E.M. are shown, sample sizes (n) equal 20 in each group.

Fig. 5 shows the spatial distribution of experimental fish during the probe trials. Each of the four sets of graphs corresponds to a particular group of fish in this 2 (paired or not-paired training) × 2 (cue card present vs. cue card absent at probe) experimental design. From this figure it appears that fish of the paired group exhibited a strong preference for the cue card. Furthermore, it also appears that fish of the paired group that were given no cue card preferred the location of target zone where the stimulus fish used to be in prior training trials. However, no such preferences were found in fish that received the not-paired training. ANOVA confirmed these observations and for the target zone time found a significant training effect ($F(1, 29) = 13.216$, $p < 0.001$) and a non-significant probe effect ($F(1, 29) = 0.005$, $p > 0.90$) and training × probe interaction ($F(1, 29) = 2.206$, $p > 0.15$). We plotted only the target zone data in Fig. 6 and compared each group to random chance. The results demonstrated that fish of the paired training spent significantly

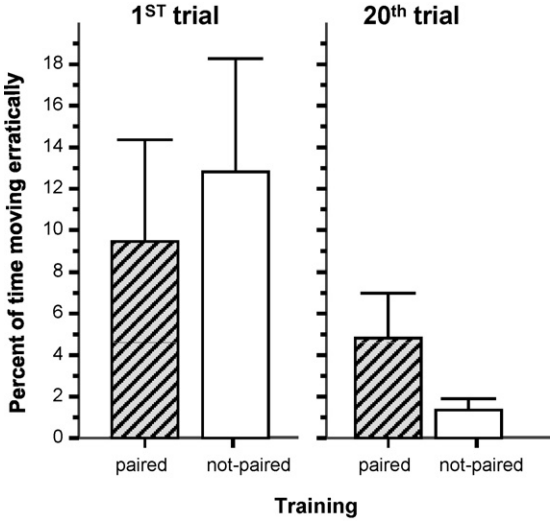

Fig. 4. Percent of time experimental fish moved erratically during the 1st and 20th trials of the training does not significantly differ between fish of the paired training and not-paired training groups. Mean ± S.E.M. are shown, sample sizes (n) equal 20 in each group.

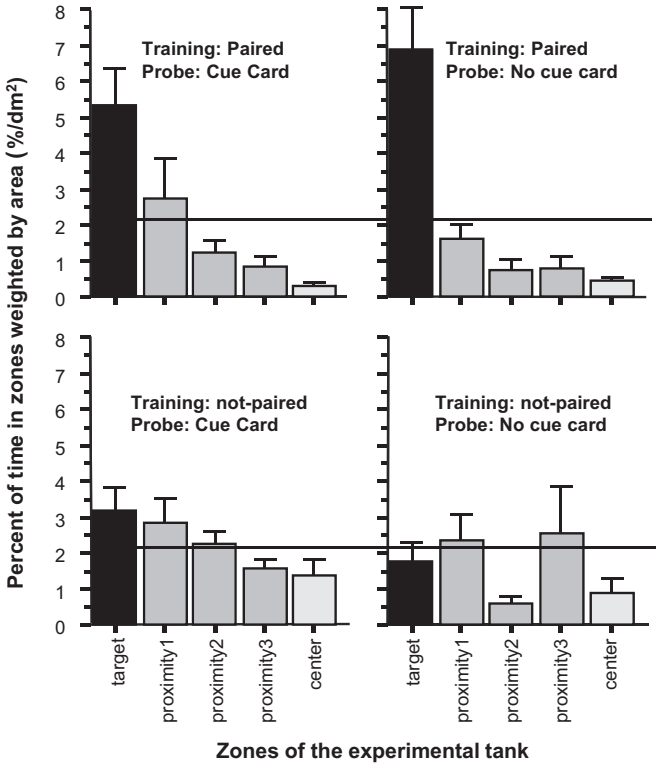

Fig. 5. Preference of experimental zebrafish toward different zones of the experimental tank during probe trial. The data are expressed as percent of time spent per unit of area of the tank. Mean ± S.E.M. are shown, sample sizes (n) equal 10 in each group. Random chance is indicated by the straight horizontal line. Two types of probe trials are administered: one set of fish are given a probe in which the red cue card is presented (cue card) and another set of fish are given a probe in which no cue card is present (during this probe trial the fish may find the location of the previous target based on extra-maze visual cues). Note the strong preference toward the location of the red cue card (target zone, indicated by the black bar) shown by fish that received paired training (upper left graph). Note that the location of the red cue card randomly changed across experimental fish and that there were no stimulus fish present in any stimulus tanks during this probe trial. Also note the strong preference toward the past fixed location of the stimulus fish (target zone indicated by the black bar) shown by fish of the paired training group during this probe trial when no stimulus fish and no red cue card are presented (upper right graph). Last, notice that the target zone induced no preference (no significant difference compared to random chance) in the not-paired training group at either probe trial (lower left and right graphs).

more time near the cue card (that defined the target zone) than chance during the probe ($t = 3.134$, df = 8, $p < 0.05$). Furthermore, the other set of fish of the paired training group that were not given a cue card during the probe trial spent significantly above chance amount of time in the target zone where the stimulus fish used to be even though this location was not marked by the cue card ($t = 3.996$, df = 9, $p < 0.01$). On the contrary, fish of the not-paired training group did not differ from chance in either probe (cue card present $t = 1.692$, df = 6, $p > 0.25$; cue card absent $t = -0.650$, df = 6, $p = 0.90$).

In addition to the location of the fish, we also analyzed the amount of immobility and erratic movement the fish exhibited during their probe trial (Fig. 2). We found no significant training (ANOVA immobility $F(1, 29) = 1.795$, $p > 0.15$; erratic movement $F(1, 29) = 0.181$, $p > 0.65$), and probe effects (ANOVA immobility $F(1, 29) = 0.198$, $p > 0.65$; erratic movement $F(1, 29) = 0.526$, $p > 0.45$) or training × probe interaction (ANOVA immobility $F(1, 29) = 0.766$, $p > 0.35$; erratic movement $F(1, 29) = 0.630$, $p > 0.40$) suggesting that fear related behavioral responses were statistically indistinguishable among the four groups of fish examined.

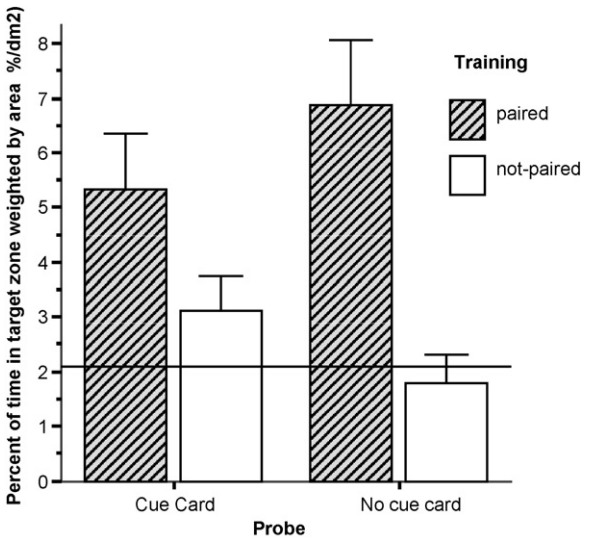

Fig. 6. Zebrafish that received the paired training show significant preference toward the zone marked by the red cue card and also toward the location of the zone that used to contain the stimulus fish. The data are expressed as percent of time spent per unit of area of the tank. Mean ± S.E.M. are shown, sample sizes (n) equal 10 in each group. Random chance is indicated by the straight horizontal line. Two types of probe trials are administered: one set of fish are given a probe in which the red cue card is presented (cue card) and another set of fish are given a probe in which no cue card is present (during this probe trial the fish may find the location of the previous target based on extra-maze visual cues). Note that fish of the paired training group show a significantly stronger preference toward both the red cue card and also toward the location where the stimulus fish used to be presented, as compared to the fish that received the not-paired training. Also note that performance of the paired training group is significantly above chance for both the red cue card as well as the location whereas the performance of the not-paired group is statistically indistinguishable from chance.

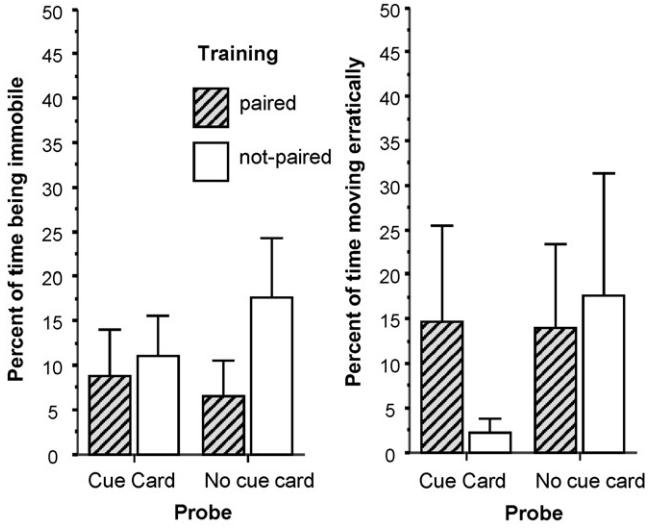

Fig. 7. The percent of time fish remained immobile or performed erratic movement does not differ across the paired and not-paired training groups tested in the cue card present (cue card) and cue card absent (during this probe trial the fish may find the location of the previous target based on extra-maze visual cues) probe trials. Mean ± S.E.M. are shown, sample sizes (n) equal 10 in each group.

4. Discussion

We found our experimental zebrafish to be able to associate the sight of conspecifics (reward) with a visual cue. These zebrafish responded to the cue alone during a probe trial by staying in close proximity of the cue. This result confirms that zebrafish have the ability to perform well in associative learning tasks [14]. Even more

importantly, zebrafish that were trained to make this association were also able to show significant and robust preference for the past location of the reward at a probe trial (during which the reward was absent) even when the associative cue was not presented. This is an important finding as it implies that zebrafish are capable of learning the association between reward and more than just a single experimenter provided associative cue.

Learning and memory have been intensively investigated from many perspectives. From a mechanistic viewpoint our knowledge is exponentially increasing but a lot remains to be discovered [12,15]. The zebrafish holds great promise in this discovery process as it may allow unraveling a large number of genetic and biochemical targets associated with these phenomena [4,12]. This is because the zebrafish is particularly amenable to high throughput screening [3,4]. However, in addition to the practical question of how one may be able to conduct high throughput learning and memory screens [12], from a translational perspective one may also have to ascertain that the behavioral phenomena studied in the model organism have some relevance to human. An important human learning and memory function is relational learning and episodic memory. Because of the clinical relevance of these processes (e.g. [18–20]) it may be important to know whether lower order vertebrates used as model organisms in the laboratory may possess similar cognitive and mnemonic characteristics. The zebrafish and its close relative, the gold fish, have been shown to perform well in a type of relational learning task, spatial learning [14,23]. However, good performance in a spatial learning task is rarely examined as to whether it indeed reflects spatial learning or whether it is due to an alternative, non-spatial (and thus non-relational) solution [25,27].

One could examine this question in multiple ways. One could control every single cue in the external as well as in the intra-maze environment. For example, by systematically rotating and/or conflicting these cues, one could investigate which cues the experimental subject has learned. Consequently, one could address the question whether the learning performance indeed involved the development of a dynamic relational map of several of these cues. This is, however, not yet practically feasible with zebrafish. Although the visual system of this fish is among the most well studied organs from developmental, anatomical, physiological and genetic perspectives [34], we know practically nothing about the behavioral aspect of this system. Briefly, we do not know what complex visual stimuli zebrafish are sensitive to and what cues these fish may ignore. But we do know that certain visual cue constellations and characteristics (color and pattern, movement and size) induce different and robust behavioral responses while certain other visual cue characteristics are mainly ignored by the zebrafish [35–38]. Similarly, we know practically nothing about the behavioral effects of lateral line and auditory cues in zebrafish. Thus, at this point it would be difficult to construct a laboratory environment in which all cues are properly controlled and thus the question of whether zebrafish are capable of building a spatial map could be answered.

In the rodent literature a simple and practical task has been successfully administered to prove or disprove the development of an, at least rudimentary, spatial map. This task is based upon the ability of the rodent (mice and rats) to detect geometry of their environment, i.e. the proportions of the experimental enclosure in which they are placed [39]. For example, a rodent that is placed in a rectangular test cage which is gently rotated 180 degrees around, will chose the corner opposite to the rewarded one after such a rotation is performed proving that the subject attended to and learned the geometry of its environment. However simple such an experiment may seem to be, it is not feasible with zebrafish. These fish are particularly sensitive to human intervention and in response to any sign of movement in their environment will exhibit freezing (immobility), a form of fear, which then would prevent the experimenter from quantifying any appetitively reinforced learned response. It is also notable that at this point we do not have any evidence that zebrafish (or any other fish as far as we know) would be sensitive to such geometric cues and could sufficiently distinguish the longer and shorter walls of the fish tank. For these reasons we decided to use a different approach.

The alternative task, as we described in the introduction and method sections, follows the logic of the 'context and cue dependent fear conditioning' paradigm. This latter paradigm, which is frequently used to study hippocampal function in mice and rats, may be solved by the experimental subject in two different ways [25,27]: one, by learning the constellation of contextual cues (the spatial solution) associated with the location of the reinforcement and two, by picking out a single background cue with which the reinforcer is associated (the non-spatial solution). However, it has been shown that the latter solution is not possible for the experimental rodent that suffers from hippocampal dysfunction if a salient associative CS is experimentally provided. In other words, without the hippocampus, rodents are unable to learn two things at a time and thus can only remember the salient experimentally provided CS but not the spatial information associated with the reinforcer. Fish do not possess a hippocampus, at least not one that has the typical mammalian tri-synaptic circuit, but they do posses a region, the lateral pallium, believed to be the evolutionary precursor of the mammalian hippocampus [40]. It is thought that an intact tri-synaptic circuit is crucial for the hippocampus to perform its function in mammals [15]. Based upon finding no classical hippocampal circuitry in the fish thus one may argue that these simple vertebrates should not be able to learn two pieces of information associated with the reinforcer. However, our results clearly suggest otherwise.

They demonstrate that fish can learn both the associative cue as well as some information that allows them to locate the place of a prior reward in their environment. The nature of this latter piece of information at this point is not known. It is possible that fish did acquire a dynamic spatial map and used a true spatial solution in our task. Alternatively, it is also possible that they used a single "background" cue in addition to the experimentally provided CS and identified the appropriate target location without having to rely on a dynamic spatial map. Our current experiment cannot distinguish between these two possibilities. Nevertheless, by analogy to the context and cue dependent fear conditioning task [25], our findings do show that fish behave similarly to rodents with an intact hippocampus.

Our results thus are compatible with the notion that perhaps even fish have relational learning capabilities. Whether this ability is exclusively dependent upon the lateral pallium or whether other brain regions contribute to it as well is not known at this point. Nevertheless, given the strong translational relevance of the zebrafish, the demonstration of complex forms of learning in zebrafish similar to that of rodents is noteworthy: it will justify the mechanistic analysis of complex cognitive functions using this simple vertebrate model organism.

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