Short communication

# Sight of conspecifics as reward in associative learning in zebrafish (*Danio rerio*)

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## Abstract

Large amount of genetic information has been accumulated for zebrafish. Learning and memory have also been investigated with zebrafish but the number of learning paradigms is limited. Here we present a simple associative task and investigate a novel reinforcer. We show that visual access to a group of conspecifics has rewarding properties and that this reinforcer can support associative learning in zebrafish. We discuss the advantages of this rewarding stimulus and make recommendations for its use in high throughput behavioral screens.
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Considerable amount of genetic information has been accumulated and numerous genetic tools have been developed for zebrafish [10]. Given the prolific nature of zebrafish, its small size, and highly social behavior (shoaling), a large number of animals may be maintained effectively. Thus zebrafish has been proposed as an excellent screening tool, perhaps the lowest order vertebrate with which complex human biological functions and diseases may be modeled and studied [10].

Due to the above, zebrafish is gaining popularity in a number of fields, one of which is behavior genetics [6]. However, despite the exponential increase of the number of publications on the behavior of zebrafish [22], this species is still incomparably less frequently used in behavioral studies than classical vertebrate laboratory organisms such as the rat or the mouse. Briefly, there is considerable need for the characterization of its behavior and for the development of reliable behavioral test methods.

A challenging problem of neuroscience has been the unraveling of the mechanisms of learning and memory. Compared to the number of genes suspected to be involved, we understand only a fraction of the complex mechanisms of these processes. It is therefore suggested that model organisms in which high throughput mutation (and drug) screening may be performed

could be useful [7,22]. Zebrafish is an ideal candidate for this purpose [1]. For such applications to be successful, one needs to develop high throughput and simple test paradigms. In order for such paradigms to work, one has to properly investigate the behavioral phenomena, i.e. zebrafish behavior must be thoroughly characterized.

Characterization of learning and memory of zebrafish is rapidly advancing. Zebrafish has been shown to be capable of active avoidance learning [26], appetitive reinforcement-based learning in a spatial alternation task [24], and visual discrimination learning [3]. It has been shown to acquire social preference during early development, an imprinting-like phenomenon [5]. Nicotine has been shown to significantly affect learning in zebrafish in a manner also found in mammalian species (ref. [13] and references therein). L1, a cell adhesion molecule, has been shown to play crucial roles in memory consolidation in zebrafish, similarly to its known roles in mammals [19,20]. Reinforcing properties of drugs of abuse have been analyzed [17] and the genetic aspects of rewarding properties of food and opiates have been investigated in this species [12]. Last, the first automated computerized analysis of learning application has been developed for zebrafish [11].

In the current paper we propose that a new unconditioned stimulus may be utilized in reward-based conditioning applications, visual access to conspecifics. Zebrafish is a social species known to aggregate, i.e. form groups, a behavior termed shoaling

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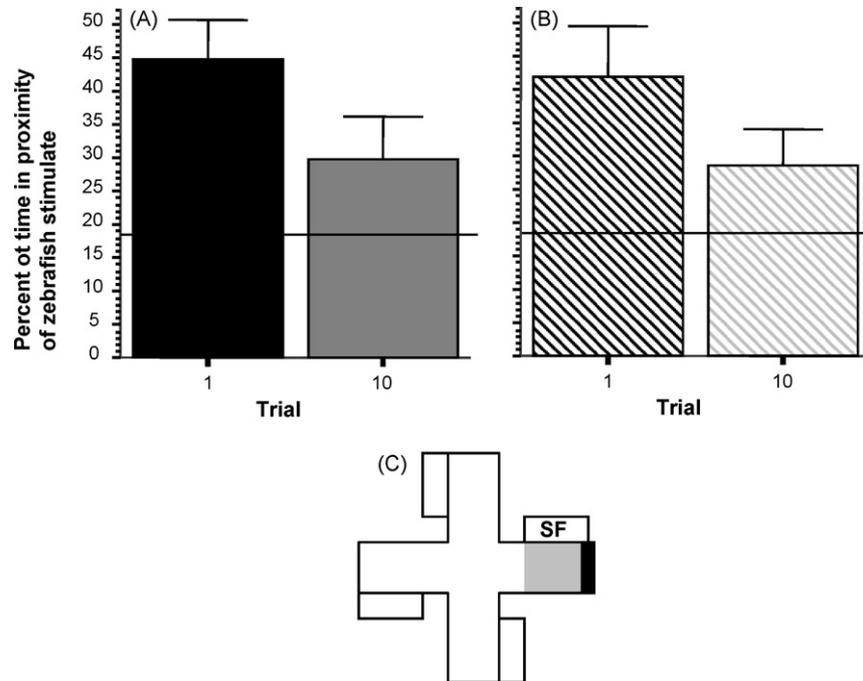*E-mail address:* robert_gerlai@yahoo.com (R. Gerlai).

Fig. 1. Percent of time zebrafish spent in the proximity of the stimulus fish (unconditioned stimulus) during training. Panel A: paired group (in which the stimulus fish and the red cue card were co-presented at the same location in the maze); Panel B: unpaired group (in which the stimulus fish and the red cue card were presented at random locations in the maze). Means ± S.E.M. are shown. Random chance (18.8%, refers to the "proximity" area as a percent of the total area of the maze) is indicated by the horizontal line. Panel C shows the basic experimental set up, the plus maze with the proximity area (grey) near the stimulus fish tank (SF) and the red cue card (indicated by the black bar at the end of one of the arms of the maze). Note that zebrafish spent significantly more time near the stimulus fish compared to chance both at the 1st and at the 10th trial in both the paired and the unpaired group. But also note that this preference decreased from the 1st to the 10th trial.

[18]. This behavior is observed in the laboratory and in nature [15,4]. The adaptive significance of this behavior includes predator avoidance [14], increased reproductive success [9], and more effective foraging [16]. The practical aspect of this behavior is that it may be utilized in learning paradigms. Previously, visual access to a conspecific has been shown to have rewarding properties in an instrumental conditioning paradigm in another fish species, paradise fish (*Macropodus opercularis*) [8]. One may propose that the sight of conspecifics will be reinforcing for zebrafish too and may support associative, or other types of, learning.

In the current paper, we investigate this question. First we study if experimental zebrafish prefers staying close to a group of conspecifics, the stimulus fish. Second, we investigate whether co-presentation of the stimulus fish (unconditioned stimulus) with a visual cue (conditioned stimulus) can be associated by zebrafish and whether zebrafish can learn that the visual cue is predictive of the presence of the stimulus fish.

Experimental zebrafish were bred using a genetically heterogeneous stock obtained from a local pet store (Big Al's Aquarium Inc., Mississauga). The rationale for the choice of this outbred population is explained in detail elsewhere [2]. The first filial generation of these fish bred and raised in-house under controlled conditions was used for testing (50–50% males – females, age = 6–7 months at the time of tests). Fish were housed singly in 3 l transparent acrylic tanks that were part of a high-density rack system designed for zebrafish (Aquaneering Inc., San Diego, USA). The operation of this system and detailed housing conditions are described elsewhere [2]. Notably, sin-

gle housing allowed us to identify the fish without having to employ invasive marking methods, but the tanks were transparent and they used a central filtration unit, thus single housing was not associated with visual or olfactory isolation. In addition to the experimental zebrafish, we used stimulus fish which were from the same cohort (identical in size, color, and pattern) as the experimental zebrafish.

The apparatus was a plus-shaped maze with four arms (length of each arm = 35 cm, width of each arm = 11.25 cm, center of the plus maze = 11.25 cm × 11.25 cm square, depth of the maze = 20 cm). One small tank (30 cm × 15 cm × 20 cm, length × width × height) was placed next to each arm of the maze as shown in Fig. 1. Each tank contained water but only one of them, the stimulus tank, had seven stimulus fish, the other tanks had no fish. White plastic sheets placed on the sides of each small tank (except for the side facing the maze) prevented experimental zebrafish to see into the tanks until they swam to the proximity area (Fig. 1).

Fish were first habituated to the maze for a minimum of 4 days: they were placed in the maze in progressively decreasing shoal sizes. Following habituation, the fish were divided randomly into two groups. In the "paired" group a red plastic cue card (10 cm × 7.5 cm, width × height) was placed at the end of the stimulus arm next to the stimulus tank. The red cue card and the stimulus fish were always presented together as shown in Fig. 1, but the location of their co-presentation changed from trial to trial. In the "unpaired" group the red cue card and the stimulus fish were placed in different arms and their locations changed from trial to trial too. All fish were tested singly in the

maze receiving 10 training sessions each lasting for 5 min (two trials per day, for 5 days, with inter-trial interval of 2.5 h each day between the hours 10:00 and 16:00). A digital video-camera (Sony DCR-HC20, Sony Corporation, Japan) was positioned above the maze and the 1st and the last (10th) training sessions were video-recorded. The recordings were subsequently replayed and analyzed using the Observer Color Pro software (Noldus, Wageningen, The Netherlands). The percent of time zebrafish spent in the proximity of the stimulus fish relative to session length was quantified. Proximity is defined as the area of the maze from where the stimulus fish were visible to the experimental fish (see Fig. 1). Following training, a probe trial was conducted: the red cue card was presented alone, i.e. no stimulus fish were present, and the amount of time the zebra fish spent in the proximity area marked by the cue card was quantified. In case of acquisition of association between the cue card and the stimulus fish, higher than chance (18.8%) time spent in the proximity area marked by the cue card is expected. Chance level is determined based on the size of the proximity area relative to the total area of the maze.

Statistical analyses were conducted using SPSS 15.0 for the PC. Repeated Measures Variance Analysis (ANOVA) was used to investigate the effect of training (between individual factor with two levels: paired, unpaired) and trial (repeated measure factor with two levels: trial 1 and trial 10). The question whether zebrafish spent above chance percent of time in the proximity of the stimulus fish was answered using one-sample one-tailed $t$-tests. The question whether the paired training group spent significantly more time near the conditioned stimulus (red cue card) compared to the unpaired training group was tested using a two sample one-tailed $t$-test. For the latter analysis logarhythm transformation was employed to homogenize variances and to meet the normal distribution criterion of parametric tests.

The first question we studied was whether experimental zebrafish showed preference for the stimulus fish, i.e. whether they stayed in close proximity to them during training. ANOVA showed that the paired and unpaired group did not differ (training $F(1, 37) = 0.24$, $p > 0.60$) but a significant reduction of the percent of time spent in the proximity of the stimulus fish between trial 1 and trial 10 ($F(1, 37) = 14.22$, $p < 0.001$) occurred, and the interaction between training and trial was non-significant ($F(1, 37) = 0.45$, $p > 0.50$). The above suggests that responding to the stimulus fish habituated with time, i.e. the preference to stay close to the stimulus fish decreased in experimental zebrafish. Second, this decrease was similar in the paired and the unpaired experimental groups. Importantly, although significantly diminished, the percent of time experimental zebrafish spent in the proximity of the stimulus fish remained above chance (18.8%) even at trial 10 (paired group at trial 1 $t = 4.40$, d.f. = 19, $p < 0.001$; unpaired group at trial 1 $t = 3.01$, d.f. = 20, $p < 0.01$; paired group at trial 10 $t = 1.70$, d.f. = 17, $p = 0.05$; unpaired group at trial 10 $t = 1.84$, d.f. = 20, $p < 0.05$). Thus the presence of stimulus fish is a positive stimulus that may have rewarding properties.

To test this question we investigated how zebrafish responded to the presentation of the cue card alone a day after training was completed. During this "Probe Trial" we expected experimen-
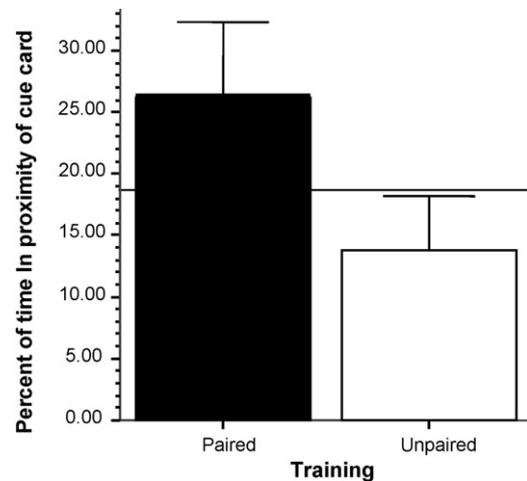


Fig. 2. Percent of time zebrafish spent in the proximity of the red cue card (conditioned stimulus) during the probe trial. Means ± S.E.M. are shown. Random chance (18.8%, refers to the "proximity" area as a percent of the total area of the maze) is indicated by the horizontal line. Note that during the probe trial only the red cue card is presented and stimulus fish are absent. Note that zebrafish in the paired group spent significantly more time near the cue card as compared to the zebrafish in the unpaired group.

tal zebrafish in the "paired" group to stay close to the cue card and zebrafish in the unpaired group to show no such preference. Indeed this is exactly what we have found (Fig. 2). The paired group spent significantly more time in the proximity of the cue card than the unpaired group did (one-tailed $t$-test: $t = 1.89$, d.f. = 36, $p = 0.033$). Notably, the paired group spent approximately the same amount of time near the cue card during the probe trial as they did during the last (10th) training trial in the proximity of the stimulus fish. Briefly, the paired group acquired the association between the cue card and the stimulus fish. Thus, the presentation of a group of conspecifics was rewarding and could support acquisition of memory in an associative learning task in zebrafish.

The reason why this is noteworthy, is as follows. Associative learning tasks have been conducted successfully with a variety of species using a variety of motivating stimuli. In appetitive tasks the most frequently used reward has been food. Food can be precisely administered using automated pellet or liquid dispensers for laboratory rodents, and indeed numerous learning tasks, including associative as well as operant tasks, have been successfully conducted using these species. Delivery of food reward, however, is somewhat more difficult in zebrafish. It is a small species that can consume a large amount of food at a time. It can also stay healthy and active without eating for several days. Thus, unlike mammals whose metabolism is high, zebrafish may not need to forage at regular short intervals and consequently food reward may not consistently support acquisition in a learning task in this species. Furthermore, precise delivery of small amounts of food in a localized manner as required in most learning tasks is technically more difficult in the water environment. Food may dissolve or diffuse in the water. If left unconsumed, it may increase organic waste and decrease water quality. Thus, an alternative reinforcer may be useful. Sight of conspecifics may be a practical way to reward zebrafish. Importantly, it may also

allow the development of high throughput paradigms because the timing of presentation of this visual stimulus can be precisely controlled by computers. One may be able to operate guillotine doors obstructing the view to the stimulus fish. In addition, we (unpublished data) and others [23] have successfully used computer images of zebrafish and elicited shoaling or reproductive behavioral responses to the images in experimental zebrafish. Computer images can be presented at will and in a precisely controlled manner, making this rewarding stimulus ideal for automated, and thus higher throughput, learning tasks in zebrafish.

The last point we stress is the preliminary nature of our results. There are numerous questions that remain unanswered and there are numerous technical and procedural details that will have to be explored and fine tuned before a stimulus fish reward-based learning paradigm can be reliably employed. First, we do not know why the preference for staying close to the stimulus fish decreased from the 1st to the 10th trial. It is possible that as the experimental zebrafish became habituated to the apparatus and handling procedure, their level of fear diminished and thus their motivation to swim close to their conspecifics decreased [14], a working hypothesis that will be tested in the future. Optimization of stimulus presentation will also be explored including the ideal number of stimulus fish according to the most preferred shoal size [25], the most optimal gender composition [21], body size, and movement characteristics of the stimulus fish shoal, and whether visual stimuli alone are sufficient or whether tactile stimuli (vibrations below the audible frequency range that are detected by the lateral line of fish) may also contribute to shoaling decisions.

In summary, there are numerous questions one will have to address before presentation of conspecifics as a rewarding stimulus will be utilized optimally in learning paradigms in zebrafish. Nevertheless, the current data suggest that indeed such a stimulus can act as a reward and support acquisition and that future research addressing the above questions is worth the effort.

### Acknowledgements

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