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Research report

A simple automated system for appetitive conditioning of zebrafish in their home tanks

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HIGHLIGHTS

- An automated associative conditioning paradigm for zebrafish was developed.
- Groups of fish rapidly learned auditory and visual associations with food.
- Learned associations were retained for at least 2 days after conditioning.
- Memories can be demonstrated when testing fish both individually and in groups.
- The paradigm can be easily adapted for zebrafish of different ages.

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ABSTRACT

We describe here an automated apparatus that permits rapid conditioning paradigms for zebrafish. Arduino microprocessors were used to control the delivery of auditory or visual stimuli to groups of adult or juvenile zebrafish in their home tanks in a conventional zebrafish facility. An automatic feeder dispensed precise amounts of food immediately after the conditioned stimuli, or at variable delays for controls. Responses were recorded using inexpensive cameras, with the video sequences analysed with ImageJ or Matlab. Fish showed significant conditioned responses in as few as 5 trials, learning that the conditioned stimulus was a predictor of food presentation at the water surface and at the end of the tank where the food was dispensed. Memories of these conditioned associations persisted for at least 2 days after training when fish were tested either as groups or as individuals. Control fish, for which the auditory or visual stimuli were specifically unpaired with food, showed no comparable responses. This simple, low-cost, automated system permits scalable conditioning of zebrafish with minimal human intervention, greatly reducing both variability and labour-intensiveness. It will be useful for studies of the neural basis of learning and memory, and for high-throughput screening of compounds modifying those processes.

1. Introduction

Zebrafish (*Danio rerio*) offer numerous advantages for the study of learning and memory. These include ease and efficiency of animal husbandry [1,2]; ready availability of molecular tools to dissect underlying mechanisms; homology of genes with mammals (including humans); and similarity of basic developmental, morphological, and physiological processes shared across the vertebrates [3]. Zebrafish also have a rich repertoire of behaviours, which they execute using relatively simple neuronal circuits and may therefore possess further advantages for reductionist approaches to understanding underlying brain mechanisms [4]. Furthermore, the small size and relative transparency of zebrafish, particularly at early developmental stages and in non-pigmented

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mutant strains, renders them particularly suitable for powerful methods of optical imaging of electrical activity and optogenetic activation or inhibition of specific sets of neurons [5]. A final and increasingly important incentive for studies of learning and memory in zebrafish comes from the use of high-throughput screens, which test for pharmacological and genetic effects on cognition [6,7].

A comprehensive appraisal of an animal's ability to learn and remember requires multiple assays employing different sensory modalities, behavioural responses and forms of learning. A variety of learning paradigms for zebrafish have been described in recent years, with each paradigm possessing particular strengths and limitations. For example, several aversive conditioning paradigms have been developed, including ones that employ electric shock associated with changes in lighting of observation tanks [8–11]. Generally, such use of aversive electrical shocks is highly effective [12], but may also have possible direct effects on patterns of electrophysiological activity in aquatic animals thus confounding further studies of associated brain mechanisms or function [13]. Appetitive paradigms, including the association of food with a particular location or with discrete visual or olfactory cues [14–16], avoid delivery of external electrical stimuli, but the use of food as a reward can be complicated by satiation, limiting the rate of training. It has been suggested that social stimuli retain their reinforcing properties during repeated administrations and might offer alternatives to food rewards [17].

One major issue common to all these paradigms is that they require special mazes or observation tanks. Periods of acclimation are therefore needed before behavioural assays can be performed [14]. Handling during transfer to such special apparatus, including netting, has been shown to increase cortisol levels significantly [18], potentially confounding analyses. Furthermore, most existing paradigms are time- and labour-intensive, requiring large numbers of pairings usually performed manually. Requirements for such extended acclimation and manual execution of experiments reduce the usefulness of such paradigms for high-throughput screening [6].

In this paper, we describe an automated, easily reconfigurable appetitive system that can be used to rapidly condition individuals or groups of both adult and juvenile zebrafish in their home tanks. Fish quickly learned to associate a light or sound with the presentation of food and move toward the feeding location. Fish retain the memory of the association when tested individually or in groups in the days following training. The learned behaviour may involve multiple components including classical conditioning of an innate surface feeding response. In addition, fish also appear to learn and retain a memory for the location where the food is presented. Further optimization of the stimulus presentations and training procedures are likely to improve measures of learning and memory shown here. This system will have broad applicability to future studies of the neural substrates of behaviours and to genetic and pharmacological screens using zebrafish.

2. Methods

2.1. Animals

Wild-type adult zebrafish, 3.5–4.0 cm in length, (PetSmart, Bedford, NS, CAN) and juvenile, AB strain zebrafish, 49 days post-fertilization and 10–14 mm in length (Faculty of Medicine, Zebrafish Core Facility, Dalhousie University, Halifax, NS, CAN), were housed as mixed-gender groups of five fish in 3 L and 1.5 L plastic tanks (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA), respectively, beginning at least two days prior to experimentation. The fish were maintained on a 14:10 h light-dark cycle and in municipal water (28.5 °C) that had undergone reverse osmosis and was then treated with 600 mg Instant Ocean (United Pet Group, Blacksburg, VA, USA) and 26.4 mg sodium bicarbonate (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA) per litre. Each tank was provided with a water flow of 13–14 L per hour while on a maintenance rack. Adult fish were normally fed twice daily using 300–500 μm pellets of Golden Pearl Reef Diet (Brine Shrimp Direct, Ogden, UT, USA). Juvenile zebrafish were fed once daily using 100–200 μm GEMMA Micro Food (Skretting, Westbrook, ME, USA). All experiments were conducted in accordance with the Canadian Council on Animal Care standards and guidelines.

2.2. Experimental apparatus

For training and testing, each home tank containing five fish was moved to a specialized rack partitioned into three arenas, each containing one fish tank (Sup. Fig. S1). Arenas were separated from one another by white corrugated plastic sheets (Coroplast, Granby, QC, CAN), and the back wall of the enclosure was covered in translucent white nylon fabric, which diffused the LED backlighting for each tank (1600 lm LED work lights, Snap-on, Kenosha, WI, USA). While on the training/testing rack, each tank was provided with recirculating water from either a dedicated 40 L reservoir for adults or the maintenance rack system (Pentair Aquatic Eco-Systems, Apopkoka, FL, USA) for juveniles.

A micro controller (Arduino Uno, Arduino, Ivrea, ITA) with an associated motor control board (shield) (Product ID: 1438), auditory wave shield (Product ID: 94) and DS1307 real time clock (Product ID: 264) from Adafruit, New York, NY, USA was used to control automatic feeders and to present auditory and visual stimuli. Arduino programs (sketches) were created in the Arduino integrated development environment [19] utilizing the following libraries to control the experiments: Time [20], TimeAlarms [21], Motorshield [22] and waveHC [23]. See Appendix A for Arduino sketches.

An automatic feeder, produced with a 3D printer (Replicator 2, Makerbot, New York, NY, USA) using biodegradable polylactic acid thermoplastic (stereolithography file downloadable from http://crollab.physiology.dal.ca/automaticfeeder) was placed over an existing hole in the lid of each tank (Sup. Fig. S1). Food was placed in the hopper of each feeder and could be dispensed using a stepper motor (Sparkfun, Niwot, CO, USA) which turned a 5 mm steel drill bit. The bit served as an auger to dispense approximately 10 mg of the adult food or 4 mg of the juvenile food. A white plastic divider was placed at the level of the water, 6.5 cm from the front, to keep the dispensed food floating near the feeder.

Auditory stimuli were presented to the fish using an 8 ohm bone conduction sound transducer (Product ID: 1674) (Adafruit, New York, NY, USA) which was centred laterally and vertically underneath the outflow at the back of each tank (Sup. Fig. S1). The auditory conditioned stimulus consisted of a frequency modulated (FM) half-second ascending and descending tone sweep between 100 and 1000 Hz (Sweep Tone Generator, http://www.audiocheck.net), amplified to half of the maximum output power of the wave shield (0.125 W). This auditory stimulus was selected based on previous evidence showing maximum sensitivity to this range of frequencies [24]. To indicate when the auditory stimulus was administered, a 5 mm red LED (Digi-Key, Thief River Falls, MN, USA) was placed on the lid of each tank, partially occluded by heat shrink tubing to allow detection by video recording equipment (see below) but not by the fish.

The visual conditioned stimulus was presented using a 15 cm light strip with 6 RGB LEDs (Mosaic LED Flexible Light Kit, Sylvania, Danvers, MA, USA). The LED strips were placed against each tank on the support shelf, visible to both the camera and fish (Sup. Fig. S1). For the experiments, the visual conditioned stimulus con-

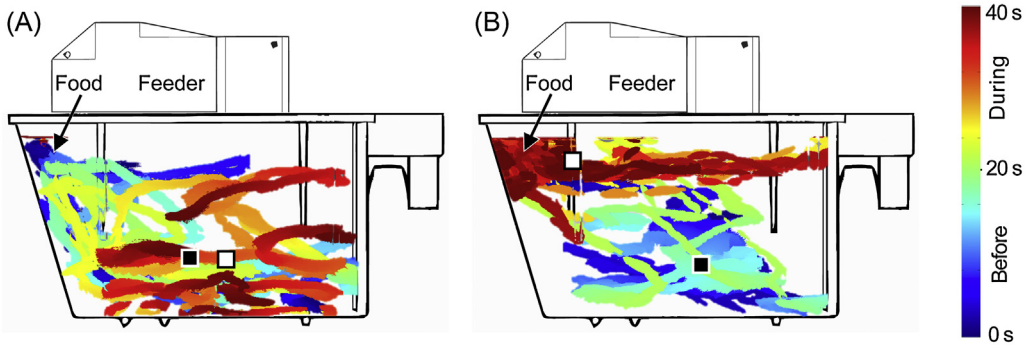

Fig. 1. Instantaneous positions of five zebrafish in a control (A) and experimental (B) tank over the interval from 20 s before and 20 s during the presentation of auditory stimulus during trial 20. The colour scale refers to the time (s) when corresponding areas were occupied by the five fish. The mean positions of the group before, and during the last 10 s of, the auditory stimulus are indicated by black and white squares, respectively. Mean movement in the control tank was 2.10 cm away from the food source and mean movement in the experimental tank was 10.11 cm toward the food source.

sisted of green illumination, and was selected based on the spectral sensitivity of zebrafish [25], with a rated output of 6.3 lm.

2.3. Conditioning

Training consisted of 10 sessions during light hours on each of two consecutive days. Inter-trial intervals of 34–108 min were selected from those produced using a random time generator (Random Time Generator, http://www.random.org). Conditioning was performed by either playing the FM tone sweep or illuminating green LEDs (auditory or visual conditioned stimuli) for a 20-s period. The conditioned stimulus was immediately followed by the presentation of the food reward from the automatic feeder. In trials with control fish, the unconditioned stimulus (food) did not immediately follow the conditioned stimulus, but was instead administered at the midpoint of the auditory and visual inter-stimulus interval except for the last trial in which it was administered 17–54 min later.

After the completion of training for adults, the feeders, plastic dividers and sound transducers were removed from each tank. These tanks were then moved back to the racks on which they were regularly maintained, and normal care was resumed until animals were tested for memory retention. Tanks with juvenile fish were left on the training/testing rack for the duration of experiments.

2.4. Probe trials

Probe trials to test memory retention were conducted at various times after training. Fish were either tested in the groups in which they were trained or tested individually. For adult group testing, the entire tank of five fish was moved from the maintenance rack back to the observation arena, and the upper divider that prevented dispersion of the food was reintroduced as a visual landmark at one end of the tank. If the fish were to be tested for retention of auditory conditioning, the sound transducer was also reattached to the tank. LED strips used for visual conditioning were left adhered to the shelf throughout conditioning and testing. For testing single fish, one animal at a time was removed from each of the maintenance tanks and transferred to a new tank equipped with the food divider and either the sound transducer or LED strip. All adult fish were transferred back to the observation arenas one day before testing in order to re-acclimate them to the apparatus. On the day of testing, fish were exposed to the stimulus to which they were conditioned for 20 s without the food reward to test the association. Each group or individual fish was given only a single probe trial at 2, 4, 8, 16 or 32 days after training.

2.5. Data collection and analysis

A single camera was centred along one side of each tank in the observation arenas such that the outflow was on the right. Experiments were video recorded either in black and white at a resolution of 640 × 480 pixels (HCM5748 camera from Honeywell Video Systems, Louisville, KY, USA) or in colour at a resolution of 1280 × 720 pixels (C930e camera from Logitech, Newark, CA, USA). Surveillance software (iSpy, http://www.ispyconnect.com or Novex, Toronto, ON, CAN) permitted recording time-stamped video files from multiple cameras simultaneously. Videos were recorded at or converted to 6 frames/s and were then trimmed to 40 s clips (VirtualDub, http://www.virtualdub.org) covering the 20 s immediately before exposure to the auditory or visual conditioned stimulus and the 20 s period during presentation of the conditioned stimulus.

The behaviour of groups of fish during acquisition and probe trials was analysed using a program [26] developed in Matlab (The Mathworks Inc., Natick, MA, USA). Average positional values for the group were generated as mean vertical and horizontal coordinates of the individual fish. The behaviour of single fish in probe trials was analysed in ImageJ [27] using the built-in Manual Tracking plugin. We also reanalysed the tracks of individual fish from acquisition groups in three control and three experimental tanks using the Manual Tracking plugin since this plugin generated vertical and horizontal positional values for each fish in each frame and allowed for analysis of factors such as velocity and turn angle of individuals and nearest neighbour analysis for group acquisitions. However, because no significant differences were found in any of these measures, they were excluded from further analysis and only the positional values were examined.

The average vertical (Y) and horizontal (X) positions of the fish in each tank were calculated for the 20 s **before** the presentation of the conditioned stimulus and compared to average coordinates **during** presentation of the stimulus. These coordinates were measured relative to the food source as the common origin. This comparison is similar to what has been previously used to analyse responses of fish to the presentation of odours [28], and to examine effects of stress on the position of fish relative to the bottom of the tank [29]. These horizontal and vertical positions were combined into a single measure using the following equation $\sqrt{(X^2 + Y^2)}$ corresponding to the distance from the food source. The distances during presentation of the conditioned stimulus were then subtracted from the distances before the stimulus. This subtraction was also performed independently for vertical and horizontal positions. Positive subtraction scores for vertical coordinates correspond to upward movements towards the surface, and positive scores for

horizontal coordinates correspond to a lateral movement toward the end of the tank with the food source, regardless of initial positions. Positive combined distance scores correspond to movement towards the food source. However, adult fish exhibited a substantial latency in responding to the conditioned stimulus and therefore average positions were only calculated during the last 10 s of the 20 s stimulus presentation. Juvenile fish showed a shorter latency and therefore average positions were calculated during the last 15 s of the 20 s stimulus presentation.

Linear mixed-effects models were used to analyse the acquisition data. Models included conditioning treatment and trial number as fixed effects, and two random effects for the tanks (both intercept and by-trial slope). Log-likelihood ratio tests compared reduced models with only main effects for conditioning treatment and trial versus the full model including both main effects and the interaction between the two. Differences in Akaike's Information Criterion (ΔAIC) were also examined [30]. Conclusions paralleled those from the log-likelihood test P-values, with full models showing ΔAIC values >10 over the reduced models for all acquisition tests, with the exception of horizontal position and distance for the juvenile fish (ΔAIC = 5.2 and 7.4, respectively). In all cases, residual plots showed no major deviations from normality or homoscedasticity. Two-way full factorial analyses of variance (ANOVAs; with conditioning and probe time factors) and Welch two sample *t*-tests were conducted for the probe trials in adults and juveniles, respectively. All analyses were performed in R [31] with the help of the following packages: nlme [32], effects [33], car [34], ggplot [35], sjplot [36], plotly [37]. P-values are reported in the text but for full statistical analyses see Appendix B (adult auditory data), Appendix C (adult visual data) and Appendix D (juvenile auditory data).

3. Results

3.1. Auditory conditioning and memory retention of adult fish

3.1.1. Acquisition of appetitive conditioning

Both experimental and control fish were observed to swim over much of the depth and length of the tank during the 20 s period before presentation of the FM tone sweep, with the mean position of the fish being near the center of the tank (Fig. 1A, Video S1). During training, the control groups, which were presented food with variable delays following the FM sweep, continued a similar swimming pattern in the 20 s period that the auditory stimulus was presented. In contrast, the experimental fish, which were presented with a food reward directly after each FM sweep, increasingly spent more time near the feeding location during the presentation of the auditory stimulus as training progressed (Fig. 1B, Video S2). Hence, on average, the fish moved closer to the food source during presentation of the auditory stimulus.

Fig. 2A shows this progressive movement of experimental (but not control) fish away from their initial mean position near the centre of the tank and closer to the corner in which food was presented as training proceeded. Analysis of linear mixed effects models confirmed a significant interaction between conditioning and training trial ($\chi^2(1) = 39.45$, $p < 0.001$). The experimental and control group scores appeared to begin to diverge after only a few trials and bootstrapped confidence intervals suggested that by the 5–8th training trial the experimental groups were moving consistently toward the food source during the presentation of the auditory stimulus. The fish at trial 11 (first trial of day 2) showed no apparent forgetting from previous day (Fig. 2A).

Separate analyses of horizontal and vertical components of the movements each showed significant interactions between condition and training trial (vertical: $\chi^2(1) = 25.528$, $p < 0.001$, See Fig. 1A in [38]; horizontal: $\chi^2(1) = 32.471$, $p < 0.001$, See Fig. 2A in [38]) suggesting fish learned to adjust both their depth and horizontal position in the tank in response to the conditioning auditory stimulus.

3.1.2. Memory retention in groups of fish

In order to examine whether the memory for the association between the auditory stimulus and the food reward was retained after the acquisition period, we tested the groups of fish for their responses to the auditory stimulus alone with probe trials at 2 and 16 days following training (represented as circles in Fig. 2B). A two-way ANOVA on the movement of fish towards the feeding location, revealed a significant overall effect of conditioning ($p = 0.001$) and a significant interaction between conditioning and day of retention ($p = 0.007$) but no significant effect of retention day ($p > 0.05$). Hence, our data indicate that the memory is retained for at least 2 days and that there is a decline in the strength of the memory over 16 days. An analysis of only the vertical component of movement showed a significant effect of conditioning (two-way ANOVA, $p = 0.020$) but no effect of retention day or interaction between conditioning and retention day (both $p > 0.05$; See Fig. 1B in [38]). Finally, analysis of the horizontal components of the movement indicated no effect of retention day (two-way ANOVA, $p > 0.05$; See Fig. 2B in [38]), but a significant effect of conditioning ($p = 0.001$) and a significant effect of interaction between condition and day of retention ($p = 0.007$).

3.1.3. Memory retention in individual fish

We considered the possibility that only dominant fish in each group actually learned the conditioned association, with subordinate fish merely following in the shoal. To determine whether all fish trained in groups actually learned and retained memories of the conditioned associations and to attempt a better determination of the duration of memory retention, we performed additional probe trials using single fish at 2, 4, 8, 16 and 32 days post training. An analysis of movement towards the feeding location showed a significant overall effect of conditioning (two-way ANOVA, $p = 0.002$; represented as triangles in Fig. 2B), but no effect of retention day or interaction between retention day and condition (both $p > 0.05$). Two-way ANOVAs on the vertical and horizontal data also showed a significant effect of conditioning ($p<0.001$; See Fig. 1B in [38] & $p = 0.038$; See Fig. 2B in [38], respectively) but no effect of retention day or interaction between retention day and conditioning (all $p > 0.05$). Therefore, the individual fish showed retention of memory during the period of 2–32 days but the data did not permit definitive assessment of the time course of memory decline during this period.

3.2. Visual conditioning and memory retention of adult fish

In addition to examining whether zebrafish could form an association between an auditory stimulus and a food reward, we also examined the performance of fish in a similar visual conditioning paradigm.

3.2.1. Acquisition of appetitive conditioning

As with auditory conditioning, fish came to swim closer to the food source during the presentation of a visual stimulus that was paired with food. Fig. 3A shows this progressive tendency of fish in the conditioning treatment (but not those in the control treatment) to swim closer to the corner of the tank in which food was presented as training progressed. Analysis of linear mixed effects models confirmed a significant interaction between conditioning and training trial ($\chi^2(1) = 31.755$, $p < 0.001$). Bootstrapped confidence intervals suggested that by the 7–10th training trial, the experimental groups were moving consistently toward the food source during the presentation of the auditory stimulus. Separate

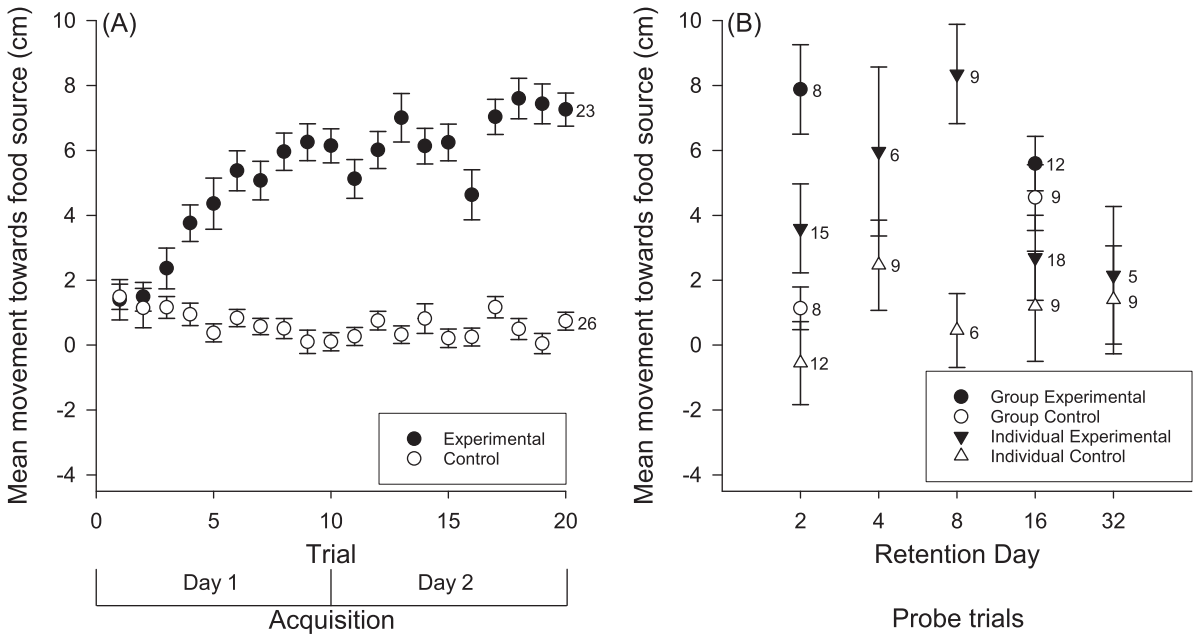

Fig. 2. Movement of adult zebrafish during acquisition and retention of an auditory appetitive paradigm. (A) Adult zebrafish in the experimental group moved towards the food source from their initial positions as a result of conditioning to the auditory stimulus. The magnitude of this response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the auditory stimulus. (B) When tested for retention, both trained groups and individuals moved closer to the food source compared to controls. Data points are mean distance from the food source before the FM tone sweep minus mean distance from the food source during the FM tone sweep. Numbers beside data points represent replicates for individuals (single fish) and groups (each containing 5 fish) in each condition. Error bars = ± s.e.m.

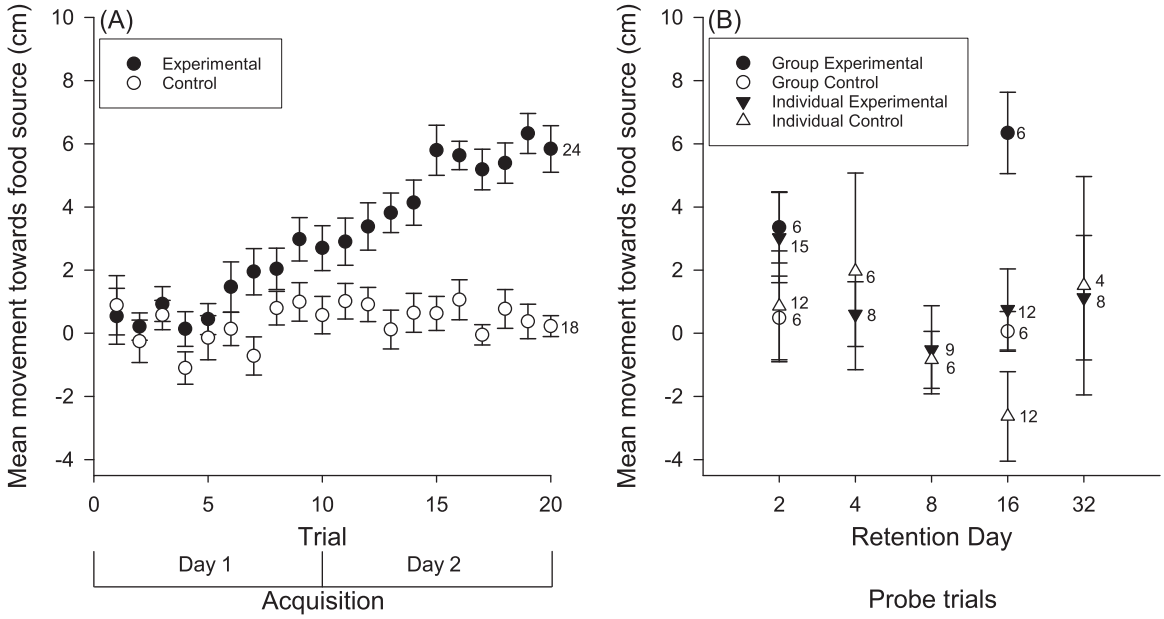

Fig. 3. Movement of adult zebrafish during acquisition and retention of a visual appetitive paradigm. (A) Zebrafish in the experimental group moved towards the food source from their initial positions as a result of conditioning to the visual stimulus. This response increased throughout the training trials. Zebrafish in the control group did not move toward the food source in response to the visual stimulus. (B) When tested for retention, trained groups moved closer to the food source when compared to controls. Individual fish did not move closer to the food source, when compared with controls. Data points are mean distance from the food source before LED illumination minus mean distance from the food source during LED illumination. Numbers beside data points represent replicates for individuals (single fish) and groups (each containing 5 fish) in each condition. Error bars = ± s.e.m.

analyses of vertical and horizontal components of the movements each showed significant interactions between conditioning and training trial (vertical: $\chi^2(1) = 28.233$, $p < 0.001$, See Fig. 3A in [38]; horizontal: $\chi^2(1) = 15.798$, $p < 0.001$, See Fig. 4A in [38]) suggesting fish learned to adjust both their depth and horizontal position in the tank in response to the conditioning auditory stimulus.

3.2.2. *Memory retention for groups of fish*

To examine whether the association between the visual stimulus and the food reward was retained after training, we tested the groups of fish for their responses to the visual stimulus alone with probe trials at 2 and 16 days after training (Fig. 3B). A two-way ANOVA on the movement of fish towards the feeding location revealed a significant effect of conditioning ($p = 0.001$) but no sig-

nificant effect of retention day or interaction between retention day and condition (both $p > 0.05$). An analysis of the vertical components also showed a significant effect of conditioning (two-way ANOVA, $p < 0.001$; See Fig. 3B in [38]) but no effect of retention day or interaction between retention day and condition (both $p > 0.05$). A two-way ANOVA of the horizontal data indicated a significant effect of condition ($p = 0.038$) and a significant effect of day of retention ($p = 0.039$; See Fig. 4B in [38]) but no interaction between retention day and condition ($p > 0.05$).

3.2.3. Memory retention in individual fish

Probe trials were performed from 2 to 32 days post training to determine whether fish trained in groups also retained memories for the conditioned associations with a visual stimulus when tested individually. An analysis on the movement towards the feeding location showed no significant effects of condition, day of retention or any interaction between them (two-way ANOVA, all $p > 0.05$; Fig. 3B). A two-way ANOVA of the vertical data indicated a significant effect of condition ($p = 0.012$; See Fig. 3B in [38]) but no effect of retention day or interaction between retention day and condition (both $p > 0.05$). Analysis of the horizontal data showed no significant effects of condition, day of retention or any interaction between them (two-way ANOVA, all $p > 0.05$; See Fig. 4B in [38]). This weaker retention is probably due to a less robust visual conditioning than what was seen with the auditory conditioning.

3.3. Auditory conditioning and memory retention of juvenile fish

Finally, we examined whether the paradigm that was effective in rapidly conditioning adult fish was also applicable to juvenile fish.

3.3.1. Acquisition of appetitive conditioning

Similar to the behaviour observed during conditioning of adult fish, the juvenile fish also swam closer to the food source during the presentation of the auditory stimulus that was paired with food. Fig. 4A shows the movement of experimental fish closer to the corner of the tank in which food was presented as training proceeded. Analysis of linear mixed effects models confirmed a significant interaction between conditioning and training trial ($\chi^2(1) = 9.4213$, $p = 0.002$). Bootstrapped confidence intervals again suggested that by the 10–13th training trial, the experimental groups were moving consistently toward the food source during the presentation of the auditory stimulus. Separate analyses of horizontal and vertical components of the movements each indicated significant interactions between condition and training trial (vertical: $\chi^2(1) = 16.048$, $p < 0.001$, See Fig. 5A in [38]; horizontal: $\chi^2(1) = 7.1794$, $p = 0.007$, See Fig. 6A in [38]) suggesting fish learned to adjust both their depth and horizontal position in the tank in response to the conditioning auditory stimulus.

3.3.2. Memory retention for groups of fish

We again examined the retention of the memories for the paired associations by testing groups of juvenile fish for their responses to the auditory stimulus alone with probe trials at 2 days after training (Fig. 4B) and found that the 2 day experimental group was significantly different from the control group (Welch two sample *t*-test, $p < 0.046$), thus indicating that the experimental groups retained the memory of the association between the auditory stimulus and the presentation of food. An analysis of the vertical component of the data indicated a significant difference between the control and the experimental data (Welch two sample *t*-test, $p = 0.002$; See Fig. 5B in [38]). Analysis of the horizontal data showed no significant difference between the control and experimental values (Welch two sample *t*-test, $p > 0.05$; See Fig. 6B in [38]).

4. Discussion

In this study, we developed automated appetitive paradigms to condition zebrafish in their home tanks. Our results demonstrated that fish could rapidly learn to associate either an auditory (continuous 100–1000–100 Hz FM sweeps) or visual (illumination of green LEDs) stimulus with the presentation of food. Groups of zebrafish navigated towards the food source upon presentation of the conditioned stimulus. The strength of association increased over the course of 20 acquisition trials, suggesting fish progressively improved in their ability to anticipate the presentation of food as an unconditioned stimulus. In addition, individual and groups of zebrafish were capable of retaining and retrieving the associative memory for at least 2 days post training indicating that they formed a robust long-term memory of the association.

4.1. Conditioning

A question naturally arises regarding what was learned by the fish in these studies. We observed little or no response by the control fish to the FM sweeps or LED illumination as illustrated by their consistent, near-zero movements toward the food source, thereby suggesting that these stimuli were neutral in the absence of any training. In contrast to the naïve (at the beginning of training) and control fish, zebrafish that were trained to associate the auditory or visual stimuli with food rose to the surface in response to the conditioned stimuli (See Figs. 1,3,5 in [38]), thus mimicking the innate consummatory behavior of these surface-feeding fish [39]. We therefore propose that this robust conditioned response to a previously neutral stimulus represents a form of classical conditioning.

In addition to classical conditioning, which resulted in fish travelling upwards to the surface, the fish also travelled laterally towards the feeder in anticipation of food (See Figs. 2, 4, 6 in [38]). This response suggests that they might also learn to associate food with the specific location at which the food was dispensed. This finding is consistent with previous studies that showed zebrafish are capable of forming concurrent double associations both between the timing of salient cues and a food reward, and between the reward and its location [15,40]. It is not clear, however, whether zebrafish formed a cognitive map of their environment [41] or simply formed associations between a specific landmark (such as the plastic divider near the feeder) and the location of the food source [40]. Additional work is needed to determine whether there was a spatial component to the learned response. The conditioned response to go toward one side of the tank was, however, consistently weaker than the response to swim toward the surface, although caution must also be exercised regarding the interpretation of this difference. More salient landmarks, clearly indicating the end of the tank from which the food was dispensed, might be expected to improve the lateral component of the learned response significantly [13,42].

Together, these findings suggest that the responses described in this study are the result of classical conditioning and that a more complex form of learning may also be involved. The ability of fish to anticipate both the timing of food availability and the location of the food would provide a substantial advantage for fish competing for limited resources in their natural environment [43].

A consistent finding of these studies was that the auditory conditioning appeared to be stronger than the visual conditioning. This might appear to be surprising in light of the well-established use of vision in zebrafish food-finding behavior [44,45]. However, it is likely that the relative intensities of the stimuli played a role in the strengths of the responses. The purpose of this study was simply to determine whether zebrafish could be conditioned in their home tanks, without stress of handling and with the use of

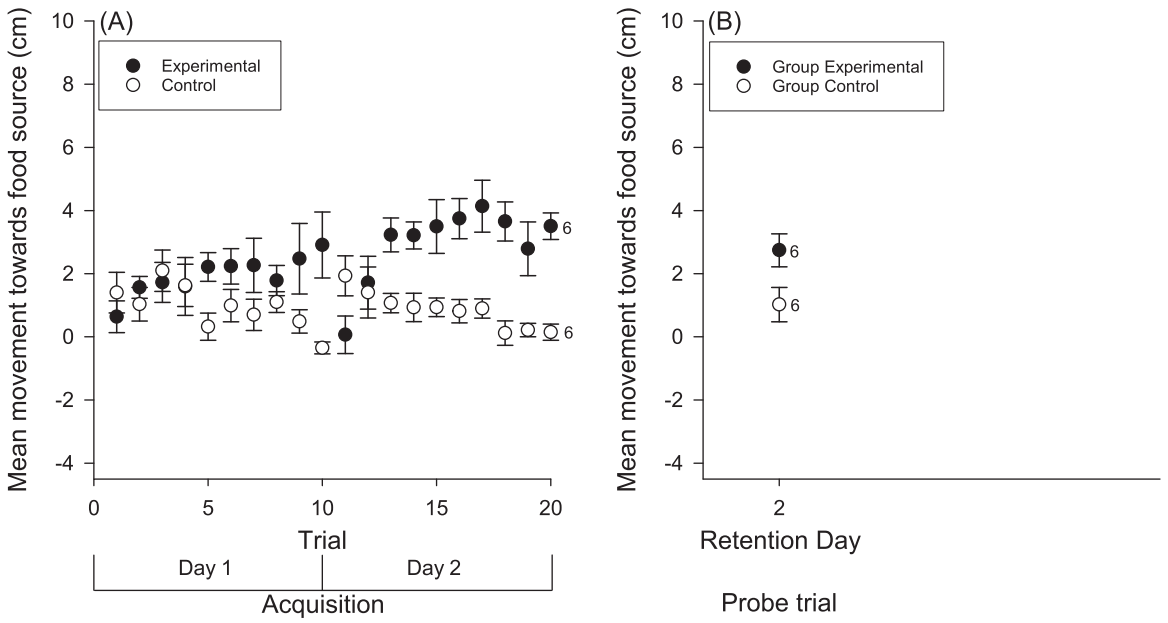

Fig. 4. Movement of juvenile zebrafish during acquisition and retention of an auditory appetitive paradigm. (A) Zebrafish in the experimental group moved towards the food source from their initial positions as a result of conditioning to the auditory stimulus. This response increased throughout the training trials. The control groups did not move toward the food source in response to the auditory stimulus. (B) When tested for retention in groups after 2 days, trained fish moved closer to the food source compared to controls. Data points are mean distance from the food source before the FM tone sweep minus mean distance from the food source during the FM tone sweep. Numbers beside data points represent replicates for groups of 5 fish in each condition. Error bars = ± s.e.m.

different modalities of sensory stimuli. No attempt was made to balance the salience of the stimuli between the paradigms; stimulus intensities were chosen simply within the bounds of what the fish were known to be able to detect [24,25] but not cause large startle responses (unpublished data). Further research will be needed to test the effectiveness of different tones and colours together with durations and intensities of stimuli to optimize conditioning.

4.2. Rate of acquisition

A number of associative paradigms have been developed to condition zebrafish with between 1 and 40 pairing trials administered over the course of 1 to 5 days using aversive stimuli, such as electrical shocks [8,10,12] or disturbances in the water [46]. Appetitive paradigms, which minimize undesired stress-induced reactions [13], often require large numbers of pairings, ranging from 20 to 400 trials for up to 8 days [14–16,47,48].

The appetitive paradigms described in this paper conditioned groups of fish in less than 2 days with 10 trials delivered per day. A significant conditioned response was observed by 5–13 pairings for auditory and visual conditioning. Again, these results were obtained using somewhat arbitrary inter-trial intervals, stimulus intensities and durations, and food amounts. Optimization of these parameters is likely to improve the rates of learning even further [46]. We suggest that the rapidity of learning demonstrated in our studies is the consequence of both monitoring natural food-finding responses normally exhibited by zebrafish, and the use of home tanks to eliminate stressors, which are inherent features of many other paradigms involving zebrafish. Other paradigms often involve transferring fish into new experimental tanks, inducing handling stress [18] and necessitating long acclimation periods [49]. We minimized stress by conducting experiments in home tanks using the same or equivalent water temperature, composition and flow as in conventional facilities where zebrafish are reared and/or maintained. Setting up new experiments and testing fish after long retention periods involves only the moving of whole tanks with pre-existing groups of fish from the maintenance racks to the observation racks, followed by overnight acclimation. Even this minor inconvenience could be avoided by installing the apparatus we have described permanently around each tank.

4.3. Memory retention

Despite the growing number of learning paradigms for zebrafish, few studies have examined memory retention beyond 2–3 days after training. For instance, there is evidence that zebrafish can maintain associative memory of food with either a visual [17] or olfactory [15] stimulus in place preference paradigms for 1 and 2 days, respectively. Another study observed retention of an aversive association between a visual stimulus and shock after 3 days [50]. Evidence does exist, however, for spatial memories lasting for 10 days [42].

Our study provides further evidence for long-term memory in zebrafish. Two-way ANOVAs indicated significant main effects during retention periods ranging up to 16 or 32 days after training and demonstrated memories persisting at least 2 days. As with other aspects of this study, future optimization of procedures will likely result in more definitive retention curves. For example, initial conditioning of fish to high performance criteria, rather than simply to arbitrary numbers of training trials, will probably provide more accurate estimates of maximum duration of memory retention in these animals.

Our study also demonstrates a novel aspect of memory retention in zebrafish. When animals behave as members of social groups, it can be unclear, without detailed analyses [51,52], how individuals contribute to the behaviour of the group as a whole (here, a shoal of zebrafish). Do all or most fish in the shoal learn the conditioned associations or do only a few fish learn and the rest of the shoal follows those leaders? It was also possible that outside of the context of the shoal, no individual fish would exhibit memories of the conditioning acquired as a group. Here we exploited the efficiency of quickly training groups of fish [53] to demonstrate unambiguous memory retention by individuals. It should be possible in future to correlate such measures of individual perfor-

mance with changes in brain activity, to elucidate neural substrates of learning and memory [5]. Individual testing could also provide a foundation for genetic and pharmacological screens for factors affecting long-term memory consolidation and retrieval.

4.4. Advantages of automation

Though automated systems offer conspicuous advantages over paradigms utilizing manual pairings, few automated paradigms have yet been developed for zebrafish [48,54,55]. Automation offers a more controlled environment by minimizing possible confounds such as the presence of experimenters who could potentially act as predictors for the food reward [48], and by providing more precise and consistent delivery of the food reward. Our automated feeding system also overcame a potential problem of satiation [14], as food was dispensed in small amounts throughout conditioning. The feeder can dispense a wide variety of commercially available fish food of varying sizes, making it suitable for different fish of different ages, as demonstrated here with both adult and juvenile fish.

Importantly, automation also offers the opportunity to easily scale operations up to efficiently condition large number of animals. The automated apparatus described here is easily constructed, and can be added to existing tanks without modifications. In addition, the apparatus has the capacity to present a wide variety of tones and coloured lights as conditioned stimuli. Durations and intervals can be easily programmed in Arduino sketches. Thus, the paradigms can be used not only to test cognitive abilities of fish in high-throughput screens but also to test for fine sensory discrimination in studies of psychophysics.

In addition to describing easily constructed and inexpensive apparatus to produce conditioning, we also demonstrated that behavioural responses in zebrafish can be reliably measured by analysing movement in two dimensions using cameras and subsequent analyses through ImageJ and Matlab. These are readily available and cost effective alternatives to commercially available tracking software packages used in many other conditioning paradigms [14,16,17,40,49].

5. Conclusions

With increasing use of zebrafish as models for the study of learning and memory, it becomes important to establish paradigms to test a fuller range of this animal's behavioural repertoire. Demand for high-throughput screening further requires efficient training procedures that can produce robust learning and long-lasting memories. The appetitive paradigms described here meet these demands by quickly and reliably conditioning zebrafish to associate neutral auditory or visual stimuli with food in under 20 trials over less than 2 days. The paradigms, with our inexpensive automated apparatus, can easily be run using groups of either adult or juvenile fish in their home tanks, thus eliminating the need for specialized tanks and extended periods of acclimation. After training, fish can be removed from the observation arenas and moved back to maintenance racks while awaiting probe trials for memory retention. In this way, we have shown that fish trained in groups can learn individually, demonstrating long-term memories lasting at least 2 days. We anticipate that these automated paradigms will find widespread use for rapid, high-throughput investigations of learning and memory in zebrafish.

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Appendices A–D Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.09.044.

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