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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-