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Research report

A new training procedure for studying discrimination learning in fish

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ABSTRACT

The study of animal cognition and its neurobiological bases often requires the adoption of associative learning procedures. Though fish are increasingly being used as a model system in behavioral neuroscience, the availability of adequate learning protocols can be a limiting factor in this field of research. This study describes a novel training procedure to explore visual discrimination in fish. Subjects were singly housed in rectangular tanks. At intervals, two stimuli were introduced at opposite ends of the tank and food was delivered near the stimulus to be reinforced. Time spent near positive stimulus in probe trials was taken as a measure of discrimination performance. To validate the method, we replicated two published studies that used operant conditioning to investigate the mechanisms of numerical discrimination in mosquitofish. Our data indicate a complete overlap of the results obtained using the two different methods. The pros and cons of the new procedure are discussed in respect of traditional associative learning paradigms.

1. Introduction

During the past decade, an ever-increasing number of studies have used fish as a model system in behavioral neuroscience. Zebrafish, guppies, goldfish, and stickleback, among others, have been successfully employed to study learning, memory, and visual perception, to screen new drugs, to identify the function of brain genes, and to model human psychopathology and neurodegenerative diseases [1–6].

Many of these studies require visual discrimination. Although procedures of classical and operant conditioning used in avian and mammalian species can be adapted for fish [7–9] to learn to discriminate, they have several limitations. For example, with fish, water cannot be used as a reward, and food deprivation is not as effective as with warm-blooded vertebrates. Consequently, fish can only be administered a few trials per day and experiments may last several weeks.

Recently, some studies have used social reinstatement as a reward. Sovrano et al. [10] studied the ability of fish to use the geometry of the environment for spatial reorientation. In this study, one fish was placed in an unfamiliar place and could only rejoin its shoalmates by choosing the correct exit door. More recently, the same method was employed to study numerical abilities in mosquitofish [11,12]. Al-Imari and Gerlai [13] used social reinforcement successfully to train zebrafish to choose the arm associated with a red cue card in a four-arm radial maze. However, fish tend to become used to these procedures and the reinstatement tendency decreases after repeated testing. Hence, even with social reinforcement, the number of consecutive trials that can be performed is usually limited and the process of learning can last several weeks [12,14]. In addition, the conditioning procedure requires that each subject is moved back and forth between the housing tank and the test apparatus several times a day, a procedure that is potentially very stressful for fish.

This study presents a novel procedure for training small fish to discriminate between two visual stimuli. In brief, for the entire experiment, each fish resides in a small tank that serves as testing apparatus. The stimuli to discriminate are repeatedly placed at the two ends of the tank while food is delivered in the proximity of the rewarded stimulus. The capacity to discriminate is measured as the time spent near the reinforced stimuli during probe trials without a reward.

To compare the new procedure with existing methods, we replicated recent published experiments that used operant conditioning to assess the limits of numerical discrimination in mosquitofish [11,12]. As some authors [15–18] have suggested that discrimination of small numbers (ranging from one to four) may be based on other mechanisms than discrimination of large numbers (>4), we performed separate experiments for the two numerical ranges. In the first two experiments, we studied the influence of ratio and the influence of total size of the set on large number discrimination, respectively. In the third experiment, we studied the limit of discrimination in the small number range. In the fourth experiment, we tested whether extended training can improve the ability of fish to discriminate numerosities.

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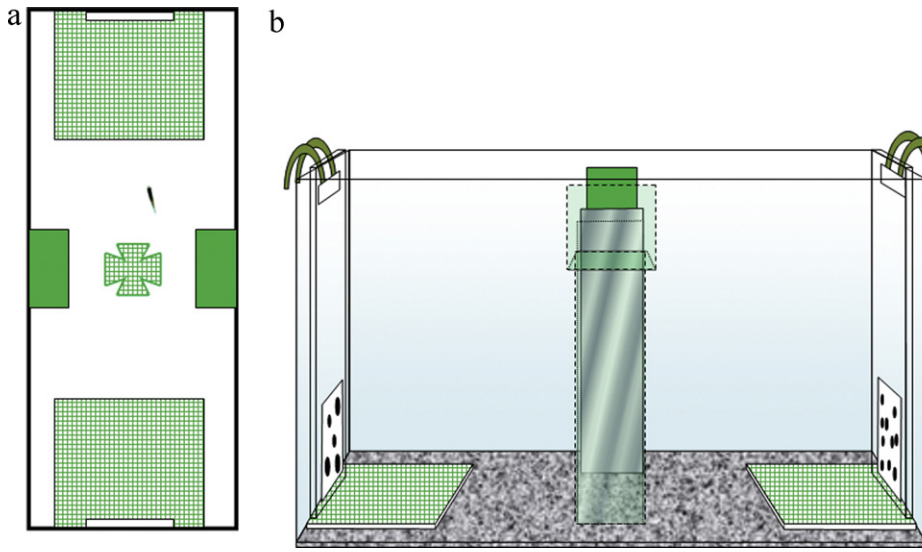

Fig. 1. Experimental apparatus. Subjects were housed in the experimental tank (a: aerial view, b: lateral view) for the entire experiment. Stimuli (two groups of dots differing in numerosity) were presented at the bottom of the tank. (For interpretation of the references to color in the text, the reader is referred to the web version of the article).

2. Materials and methods

2.1. Experiment 1: Influence of numerical ratio in large number discrimination

This experiment investigated whether the discrimination of two large numbers worsens as the numerical ratio between the numerosities decreases, as previously reported using operant conditioning [12]. To this end, fish were observed in their capacity to discriminate: 7 vs. 14, 8 vs. 12, and 9 vs. 12, which yielded ratios of 2:1, 3:2, and 4:3, respectively.

2.1.1. Subjects

Subjects were 11 adult female mosquitofish (ranging from 4 to 6 cm in length) of the species *Gambusia holbrooki*. Fish were initially collected from Valle Averto, a system of brackish water ponds and ditches in the Venetian lagoon basin. They were transported to the Laboratory of Comparative Psychology at the University of Padua and maintained for one month in 150 one-stock aquaria containing mixed-sex groups (15 individuals with approximately a 1:1 sex ratio). Aquaria were provided with natural gravel, an air filter, and live plants. Both stock aquaria and experimental tanks were maintained at a constant temperature of 25 ± 1 °C and a 14:10 h light:dark (L:D) photoperiod with an 18-W fluorescent light. Before the experiment, fish were fed twice daily to satiation with commercial food flakes and live brine shrimp (*Artemia salina*).

2.1.2. Apparatus and stimuli

The experimental apparatus was composed of a $50 \times 19 \times 32$ cm tank. It was filled with gravel and 24 cm of water. The long walls were covered with green plastic material, and the short walls were covered with white plastic material. To reduce the potential effects of social isolation (Miletto Petrazzini et al., in preparation), two mirrors (29×5 cm) were placed in the middle of the tank, 3 cm away from the long walls. An artificial leaf (9×8 cm) was placed between the mirrors to provide some shelter for the subject. In correspondence with the sides in which stimuli were presented, two 'choice areas' were defined by white rectangles (14×12 cm) covered by a green net (Fig. 1).

Stimuli were inserted in a 6×6 cm square and were presented at the bottom of a 6×32 transparent plexiglass panel. They were groups of black geometric figures differing in size on a white background. Different numerical contrasts were presented: 5 vs. 10 and 6 vs. 12 (2:1 ratio) in the training phase; 7 vs. 14, 8 vs. 12, and 9 vs. 12 (2:1, 3:2, and 4:3 ratios, respectively) in the test phase. Stimuli selected for the experiment were extracted from a pool of 24 different pairs for each numerical contrast. The size and position of the figures were changed across sets. Numerosity usually co-varies with several other attributes such as the cumulative surface area, the overall space occupied by the sets, or the density of the elements, and human and non-human animals can use the relative magnitude of these non-numerical cues to estimate which group is larger/smaller [19–21]. Cumulative surface area was controlled to reduce the possibility that fish could have used non-numerical cues: in one-third of the stimuli, the two numerosities were equated for cumulative surface area (100%); in another third of the stimuli, cumulative surface area was controlled by 85%, and, in a final third of the stimuli, cumulative surface area was controlled by 70%. In addition, since density and overall space encompassed by the stimuli are inversely correlated, half of the set was controlled for the overall space, whereas the second half was controlled for the density of the elements (Fig. 2).

Eleven identical experimental tanks were used. They were placed close to each other on the same table and lit by two fluorescent lamps (36 W). A video camera was suspended about 1 m above the experimental tanks and used to record the position of the subjects during the tests.

2.1.3. Procedure

The experiment was divided into two different phases: training and test. During the training phase, we presented an easy numerical ratio (2:1) with the purpose of training the fish to the new task and selecting those fish successfully accomplished the task. In the test phase, we then assessed fish accuracy as the numerical ratio changed.

2.1.3.1. Training. In the two days preceding the start of the training, 11 fish were singly inserted into the experimental tanks in order to familiarize them with the tank. During this period, fish were fed twice a day. Artemia nauplii were inserted in the morning and in the afternoon near the two short walls.

On days 1–3, fish received four trials per day (three consecutive days, for a total of 12 trials). Each trial consisted of inserting the two stimuli hanged on the short walls. Two numerical contrasts were presented in a pseudo-random sequence: 5 vs. 10 and 6 vs. 12. Six fish were reinforced to the larger quantity and five fish to the smaller

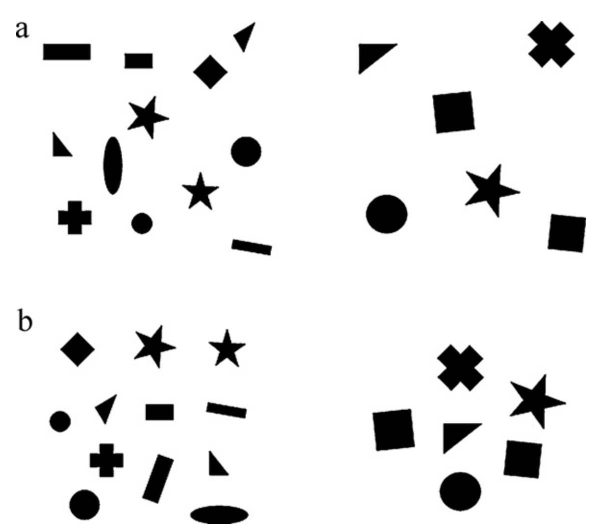

Fig. 2. Example of stimuli. To prevent the possibility of using cumulative surface area instead of number, figures included in the smaller group were enlarged in size and those included in the larger one were reduced, thus pairing the quantity of black in the two sets. The overall space occupied by the groups was controlled by matching the space encompassed by the most lateral figures (a); density was controlled by equalizing the inter-stimulus distance (b).