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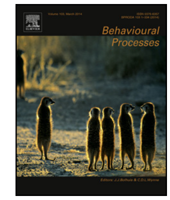

How *Betta splendens* finds its way

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ABSTRACT

This study investigated the Siamese fighting fish *Betta splendens* performance in associating a stimulus with a specific cue when distractors are present. After trained to associate a specific color cue to a stimulus (conspecific) in a tank containing three colored distractors, the fish was challenged to locate the exact place where the stimulus fish was presented. With only color cues as guides, the Siamese fighting fish spent most of its time close to the color where the stimulus fish was previously presented, regardless of the distractors. However, fish trained to associate an empty place (no cues) to a stimulus fish, and then tested to localize the specific zone where the stimulus was shown, succeeded to locate the place even without any obvious cues/distractors for orientation. This study confirms that Siamese fighting fish show good conditioned learning and cannot be distracted by other stimuli. In addition, an unexpected good performance in the absence of cues may suggest the Betta's ability to orientate by using another sensorial modalities, as magnetic orientation. Collectively, the results of this study confirm Betta as a valid and reliable model for learning and memory tests, and suggest more studies should be developed for the better understanding of the fish's spatial orientation mechanisms.

1. Introduction

Finding the way is a widespread problem for all animal kind. Resources are usually found separated in space, and distances and directions need to be properly processed for successful orientation. After Tinbergen's (1951) pioneer study of digger wasps, many other reports approached the way animals encode information about the environment for spatial traveling and recall of relevant places.

Spatial orientation allows efficient travel between places because it requires encoding of the location of cues in a particular environment. Thus, animals use distal and local cues, and many other signals in a stimulus–response (S–R) association so as to navigate, learn new routes, and track known resource spots. While the natural environment offers many more spatial cues than an animal actually uses when guiding their way through, the majority of the studies on spatial learning use a single cue as the unconditioned stimulus (US) to be associated to a conditioned stimulus (CS) (Al-Imari and Gerlai, 2008; Braubach et al., 2009; Broglio et al., 2010; Karnik and Gerlai, 2012). This paper addresses US-CS using multiple cues as distractors with the purpose of testing the animal's ability to distinguish the US and ignore distractors.

A delayed matching to sample is usually observed when distractors are present (Wilkie, 1983; Fitzgeorge et al., 2011; Buckolz et al., 2014). Evidence suggests that animals exposed to a to-be-remembered stimulus hold it in memory and respond faster during a probe when no other stimuli are present (Wilkie, 1983; Shettleworth and Westwood, 2002). Thus, distractors affect the ability to learn and properly remember, since it inserts confusion to what was seen and where. According to Haworth et al. (2014), distractors that are always at the same location do not affect the animal's reaction as much as distractors placed in random locations. The presence of distractors was used to test learning and memory mainly in humans (Chun and Jiang, 1998; Zehetleitner and Müller, 2010; Schlagbauer et al., 2014) and birds (Wilkie, 1983; Shettleworth and Westwood, 2002; Haworth et al., 2014). However, it is still not clear whether spatial learning takes place with extraneous stimuli that are simply ignored or whether these extraneous stimuli can significantly affect orientation. This study addresses this issue in a freshwater aggressive species, the Siamese fighting fish *Betta splendens*.

The fish *B. splendens*, native from small turbid streams and lakes of Southeast Asia, need advanced spatial navigation to recognize places in order to obtain food, locate conspecifics (opponents and mates), and avoid predators (Braddock and Braddock, 1955; Roitblat et al., 1982; Verbeek et al., 2008). Such ecological and social features seem to have favored the selection of spatial skills in this species. Therefore, the fish *B. splendens* was used as a model to

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approach place learning based on single US-CS in which extraneous stimuli were used as distractors. By using a phylogenetically primitive animal, this study may assist to shed a light on the evolutionary mechanism of brain route formation for learning and memory.

2. Material and methods

2.1. Animals

Twenty seven Siamese fighting fish males, *B. splendens* Regan, 1910 were used as experimental fish for this study. Other six *B. splendens* were used as unconditioned stimulus. All fish were adult males (6–8 months) obtained from an Ornamental fish farm in Natal, RN, Brazil, and held in high-density racks. Water quality at the housing racks was maintained by filtration (mechanical, biological, carbon filter). Fish were housed individually in 1 L acrylic tanks, allowing water circulation between fish. Water temperature was kept at 28 °C and photoperiod set 12L:12D cycle. Fish were fed flake food (38% protein, 4% lipid, Nutricom Pet) and *Artemia salina*.

2.2. Experimental lab and maze

The laboratory used for the tests was an 18 × 10 m room with all walls painted in white. Windows were closed and covered with white polystyrene. Six 2 × 2.6 m white dividers were placed in the middle of room forming a barrier that restricted the experimental area to an 18 × 5 m space. In this space, three tanks were arranged in line, each one 5 m away from the other. The ceiling of the room was also painted in white and fluorescent tubes (1.5 m long) organized in lines illuminated the room.

The tank used was a squared (100 × 100 cm) open-field glass tank with 4 small tanks (20 × 10 cm) lying at the center of each wall and equidistant from the corners. The open-field tank had all walls covered with black sheet to prevent the fish from seeing outside. A start box (10 cm diameter) was placed in the middle of the tank and each experimental fish was released by removing the start box with a hook at the beginning of each trial. The tank and the smaller aquaria were filled with system water (same water from the holding racks) for a depth of 15 cm and it was changed everyday to ensure water quality. The learning test was divided in three phases: (1) habituation, (2) training, and (3) probe.

2.3. Spatial learning

In the habituation phase (1), 15 fish were individually allowed to explore the testing tank for 5 days. On the first and second days, fish were placed in the start box for 1 min and after release, fish could swim for 1 h. The same procedure was performed on the other days, but fish were allowed to explore the tank for 20 min on the third and fourth days and 5 min on the fifth day. After each trial, fish were moved back to their holding tank. For each habituation period, fish were rotated throughout a tank positioned in a different place inside the lab.

After habituation, the training phase (2) took place. For that, each small tank inside the open-field received a color card (20 × 10 cm) attached to the back wall, each one in a different color: blue, green, yellow and red. A stimulus fish (*B. splendens*) was placed in one of the four small tanks in order to serve as an unconditioned stimulus while the card color served as a conditioned stimulus. From the 15 experimental fish, each 5 fish were trained to find the stimulus fish in a different color tank, thus, 5 fish received the stimulus associated with the green background, while another 5 fish found the stimulus in the yellow tank, and the other 5 fish within the red tank. The other color cards were randomly distributed to the other 3 small tanks and served as distractors. The experimental fish experienced 4 trials of 5 min per day during 5 days (total of 20 trials). Three open-fields with a different color arrangement were used, thus every trial, fish were introduced in one of the open-fields and experienced a different display of the color tanks. By rotating the experimental fish through the 3 open-fields, association with cues other than the color could be avoided. All the behavioral tests were video-recorded from an overhead camera (SONY® DCR-SX45).

The probe (3) for the associative learning took place 24 h after the last training section. All procedures and conditions were the same as in the training phase, except that no stimulus fish was presented and the experimental fish was allowed to explore the open-field for only 5 min after released from the start box. The exploring period (5 min) was recorded for learning analysis. This group was expected to spend time checking the color cards (distractors) before finding the correct card previously associated to the stimulus fish.

As a control group, 12 fish were submitted to the same procedure above cited, but no color cards were added to the experimental tank. For this group, the habituation phase (1) followed the exact same procedure described for the spatial learning task (see above). After that, experimental fish underwent the training phase (2). For that, one small tank inside the open field received the stimulus fish; no color cues were used. From the 12 experimental fish, each 4 fish were trained to find the stimulus fish in a different place: 4 fish received the stimulus at the north tank, another 4 fish found the stimulus at the south and the other 4 fish found it at the west tank. Three open-field tanks were used for the tests and every trial, fish were introduced in a different tank. By rotating the fish through different open-fields, possible external cues could not be used as guides, although the stimulus was always in the same coordinate position (north, south or west). The experimental fish was subjected to 4 trials of 5 min per day for 5 days (total of 20 trials). All the behavior was recorded from an overhead camera. The probe (3) was recorded on the day after the last training section, and no stimulus fish was presented. The experimental fish could explore the tank for 5 min during which recording was performed for later analysis. This group was expected not to find the correct position where the stimulus fish was present due to the total absence of cues that could guide the fish.

2.4. Statistical analysis

The behaviour of the experimental fish was analyzed using the Any-Maze® video tracking software. The open field tank was divided in areas: 4 equal areas located around each small aquarium (1050 cm^2 each) plus the central and corner areas (5000 cm^2). The time the fish spent in each area was calculated for the training phase, both for the associative learning and spatial learning. The time spent in the central and corners areas were calculated as a unique area because these areas could not be associated to any stimuli or cues. The percentage of time spent in the tank areas were compared by Friedman ANOVA test, since data showed dependence and non normal and homoscedastic distribution (according to Shapiro–Wilk and Levene tests respectively). A probability level of $p < 0.05$ was used as an index of statistical significance.

3. Results

Fig. 1a shows the performance of the fish on the training phase, in which fish were allowed to explore an open field tank where four smaller aquaria were placed, each one in a different color and one of them presenting a stimulus fish to attract the attention of the experimental fish. The time spent at the area where the stimulus fish was presented was higher than any other area and above any random chance (Friedman ANOVA day 1: $\chi^2 = 29.98$, $p < 0.001$; day 2: $\chi^2 = 17.52$, $p < 0.001$; day 3: $\chi^2 = 14.73$, $p = 0.002$;