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Research report

Parametric analyses of anxiety in zebrafish scototaxis

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ABSTRACT

Scototaxis, the preference for dark environments in detriment of bright ones, is an index of anxiety in zebrafish. In this work, we analyzed avoidance of the white compartment by analysis of the spatiotemporal pattern of exploratory behavior (time spent in the white compartment of the apparatus and shuttle frequency between compartments) and swimming ethogram (thigmotaxis, freezing and burst swimming in the white compartment) in four experiments. In Experiment 1, we demonstrate that spatiotemporal measures of white avoidance and locomotion do not habituate during a single 15-min session. In Experiments 2 and 3, we demonstrate that locomotor activity habituates to repeated exposures to the apparatus, regardless of whether inter-trial interval is 15-min or 24-h; however, no habituation of white avoidance was observed in either experiment. In Experiment 4, we confined animals for three 15-min sessions in the white compartment prior to recording spatiotemporal and ethogram measures in a standard preference test. After these forced exposures, white avoidance and locomotor activity showed no differences in relation to non-confined animals, but burst swimming, thigmotaxis and freezing in the white compartment were all decreased. These results suggest that neither avoidance of the white compartment nor approach to the black compartment account for the behavior of zebrafish in the scototaxis test.

1. Introduction

Scototaxis, a preference for dark environments in detriment of brightly lit ones, is a behavioral trait that is present in many species of teleost fishes [1–6], and it has been suggested that it represents a species-specific defense pattern that exploits crypsis with the substratum as a strategy for avoiding predators [4–6]. As such, scototaxis can represent "anxiety" or "fear"-like behavior in these species, including the zebrafish [5,7–10].

All procedures described so far to the establishment of dark preference use free exploration of a black and white tank, measuring time spent in both compartments as a proxy for preference, and using diverse measures of total locomotion as a control for activity effects [3–7,9–13]. As it happens with unconditioned models in rodents [14], the definition of what are the controlling stimuli in the scototaxis task is still needed [10]. Without extensive analyses, it is not clear what are the reinforcers which are involved in scototaxis – whether a particular behavior observed in this model is driven by positive (approach the dark compartment) or negative (avoid the light compartment) drives.

An approach to determining the underlying motivations which control behavior in a given task is "behavioral validation" [15,16], a strategy in which analysis of the organization of the ethogram of the animal in the test situation reveals ethologically relevant dimensions of defensive behavior. This approach was taken by Blaser et al. [10], using the dark–light tank in zebrafish. The authors established preference for a dark environment in this species, and proceeded to confine subjects to each compartment and record their behavior while confined. After confinement, animals were then exposed to the preference test once again. Animals were separated in "high-avoidant" and "low-avoidant" using a median split of their combined pre- and post-test scores for time spent in the white compartment. Confinement to the white compartment elicited freezing in high avoidance animals, but not in low-avoidance subjects;

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erratic movement presented a weak relationship with avoidance, and thigmotaxis and locomotor behavior were poor predictors of high- versus low-avoidance. Based on these results, Blaser and colleagues suggested that freezing is a viable measure of white environment-induced anxiety, while erratic movement, thigmotaxis and locomotor behavior are not.

In this work, we attempt a similar approach with the scototaxis test in zebrafish, evaluating intra- and intersession habituation of white avoidance and locomotor behavior, as well as the effects of forced exposures to the white compartment on spatiotemporal measures of the test. We observe no signs of intra- or intersession habituation of white avoidance, with intersession habituation of locomotor activity. We also analyzed habituation and effects of forced exposures to the white compartment on burst swimming, thigmotaxis and freezing. In the intrasession experiment, thigmotaxis shows a U-shaped function, while freezing shows an inverted U-shaped function, but burst swimming does not change across the session. In the intersession habituation experiment with 15-min inter-trial intervals, no habituation was observed in ethogram measures. In the intersession habituation experiment with 24-h inter-trial intervals, burst swimming habituated, but thigmotaxis and freezing did not. All ethogram measures decreased after animals were forcefully exposed to the white compartment three consecutive times.

2. Methods

2.1. Subjects

Subjects were 50 adult wild-type zebrafish of mixed genders, purchased from a local aquarium supply store. Subjects were housed in collective 30 L tanks, in groups of 20–25 individuals, for 3 months prior to experiments, at Laboratório de Neurociências e Comportamento at Universidade Federal do Pará. Tanks possessed a recirculation filtration system using mechanical, biological, and chemical filtration. The temperature of the tanks was held at $25 \pm 2\,^{\circ}C$, and the room was maintained on a 12/12 h light/dark cycle (photoperiod starting at 0700). Subjects were fed once daily on a diet of pellet ration (Oscar Gold©). Housing conditions and experimental protocols were approved by Universidade Federal do Pará's Research Ethics Committee, and complied to ASAB/ABS [17] and SBCAL/Brazil standards [18].

2.2. Apparatus

The apparatus used in all experiments was the same as that described elsewhere [5,6]. Briefly, a tank (15 cm × 10 cm × 45 cm) constructed with both black and white matte acrylic was used; half of the tank comprised a white compartment (15 cm × 10 cm × 22.5 cm), and the other half comprised a black compartment. The colored material chosen was not reflective, to avoid shoaling tendencies. The tank contained sliding central doors, colored with the same color of the aquarium side, defining a central compartment with 15 cm × 10 cm × 10 cm. During experiments, the water column was kept to 10 cm, and the tank was rotated in 180° after each trial, so as to eliminate orientation effects. The tanks were illuminated by environmental light (60 W light bulb, located at 1.80 m above the tank), which kept the illumination uniform and constant between trials.

2.3. Experiment 1

In Experiment 1, 10 subjects were individually placed in the central compartment of the preference tank for a 5-min acclimation interval. After that, both doors between the black and the white compartments were removed, allowing the fish to make and initiate choice and then to freely explore the two compartments. The subjects' behavior was video-recorded with a Sony DCR-DVD610 camera for the entire 15-min of the preference test, and digital MPEG2 files transferred to a computer for viewing; records for each measure (see Section 2.7 below) were made for each 3-min. Even though the confinement in the central compartment for acclimation allowed for contact with both sides of the tank, data from those animals that did not cross the midline in the 900 s session were discarded, to guarantee that animals sampled both alternatives (black and white compartments) [19].

2.4. Experiment 2

In Experiment 2, 10 subjects were tested in the standard preference test, as described in Section 2.3. After the first trial ended, animals were transferred back to the acclimation tank, where they stayed for a 15-min interval. After that interval, subjects were individually placed in the central compartment for the 5-min acclimation interval, and subsequently tested again. When the second trial ended, animals were then transferred back to the acclimation tank, and the whole procedure was iterated. Subjects were exposed to a total of 3 trials, comprising 60 min per subject.

2.5. Experiment 3

In Experiment 3, the same procedure used in Experiment 2 was applied, with the exception that inter-trial intervals had duration of 24-h, and 5 trials were done.

2.6. Experiment 4

In Experiment 4, 10 subjects were individually transferred to the white compartment of the aquarium for the first "flooding" trial, and were not allowed to leave the white compartment. A "flooding" trial lasted 15-min, and trials were repeated two more times (with a 3-min interval between them) before animals were tested for dark preference. After "flooding" trials were completed for a single subject, they were transferred to the central compartment of the apparatus, and tested in the standard protocol, as described in Section 2.3.

2.7. Spatiotemporal and behavioral measures

All measures were recorded using a video camera (Sony DCR-DVD610) positioned above the tank, and transcribed using X-Plo-Rat 1.1.0 [20]. Spatiotemporal measures included *Proportion black* (%): the proportion of the trial spent in the black compartment; *Proportion white* (%): the proportion of the trial spent in the white compartment; *Shuttle* (*n*): the total number of times that the center-point of the animal crossed through the center of the tank that divided the black and white compartments.

Behavioral measures (swimming ethogram) were recorded solely for behavioral units taking place in the white compartment. The swimming ethogram was partially based on Blaser et al. (in press), and included; *Duration burst swimming* (%): the proportion of the time spent in the white compartment used in unsteady, transient swimming with a duration of <2 s [21]; *Duration thigmotaxis* (%): the proportion of the time spent in the white compartment used in sustained swimming at a distance of up to 2 cm of the nearest wall; *Duration freezing* (%): the proportion of the time spent in the white compartment that the animal is immobile (moving less than 0.75 cm/s).

2.8. Statistical analysis

Since normality was not assumed, preference for the black compartment was established using Mann–Whitney's *U*-test for proportion white against the null hypothesis that it is equal to 50%. Habituation was assessed using linear regressions followed by *F*-tests for the slopes against the null hypothesis that the slope equals zero. In Experiments 2 and 3, the consistency and stability of multiple testing across individuals was assessed by intra-class correlation coefficients. Spatiotemporal measures from Experiment 4 were analyzed using Mann–Whitney's *U*-test, while ethogram measures were analyzed using one-way Kruskal–Wallis analyses of variance.

3. Results

3.1. Experiment 1

Fig. 1 shows the results for the intrasession habituation experiment. No habituation effect was observed for either spatiotemporal measures (proportion white: $r^2 = 0.38$, slope = 0.12 ± 0.092, $F_{[1,3]} = 1.8$, NS; shuttle: $r^2 = 0.58$, slope = 0.13 ± 0.061, $F_{[1,3]} = 4.1$, NS) or ethogram measures (burst swimming: $r^2 = 0.00$, slope = 0.00 ± 0.10, $F_{[1,3]} = 0.00$, NS; thigmotaxis: $r^2 = 0.00073$, slope = -0.033 ± 0.71, $F_{[1,3]} = 0.0022$, NS; freezing: $r^2 = 0.029$, slope = -0.13 ± 0.45, $F_{[1,3]} = 0.088$, NS). In terms of the cumulative spatiotemporal measures of preference, animals spent significantly more time in the black compartment than in the white compartment ($U_{[df=9]} = 100$, $p = 0.0001$; data not shown).

3.2. Experiment 2

Fig. 2 presents results for the intersession habituation experiment with 15-min inter-trial intervals. Across trials, shuttle frequency habituated ($r^2 = 1.0$, slope = -19 ± 0.58, $F_{[1,1]} = 1100$, $p = 0.0193$), but not burst swimming, thigmotaxis or freezing (burst swimming: $r^2 = 0.94$, slope = -3.5 ± 0.87, $F_{[1,1]} = 16$, NS; thigmotaxis: $r^2 = 0.75$, slope = 0.50 ± 0.29, $F_{[1,1]} = 3.0$, NS; freezing: