Original Article

Zebrafish Preference for Light or Dark Is Dependent on Ambient Light Levels and Olfactory Stimulation

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Abstract

Zebrafish have been shown to have preference for light or dark environments depending on the ambient light level and the presence or absence of food odor. We used a cylindrical tank, half of which was surrounded by a black surface and the other half by white, to elicit a choice from individual wild-type, adult zebrafish. One treatment group was exposed to food odor and the other to water (as a control) at the beginning of the trial. During 10-min trials, the light level was increased each minute over a fivefold range in steps from 1.34×10^{17} photons/s/m^2 at the beginning to a final light level of 8.31×10^{17} photons/s/m^2. We demonstrate that the preference of the zebrafish for the light or dark half of the cylinder is dependent upon ambient light levels as well as olfactory stimulation. These results provide a potential explanation for the contradictory observations that, when given a choice, adult zebrafish prefer brighter light environments (Gerlai *et al.*, 2000) or darker light environments (Serra *et al.*, 1999). Thus, we present data useful in designing more powerful and reliable behavioral assays for use with zebrafish as well as further information about the effect of olfactory stimulation on zebrafish visual behavior.

Introduction

ZEBRAFISH ARE AN IMPORTANT model species in studies of genetics, physiology, and developmental biology[1] and in medical science.[2–4] Because of its potential use in behavioral genetic studies,[5,6] as well as behavioral assays of the effects of drugs,[7,8] disease,[9] or genetic mutations,[10] zebrafish are also becoming more popular as a model for behavioral studies.[11–14]

Two studies, by Gerlai *et al.*[15] and Serra *et al.*,[16] tested the effectiveness of a simple behavioral assay in zebrafish: the preference for a light or dark environment. The fish tested by Gerlai *et al.*[15] appeared to prefer a light environment, whereas those tested by Serra *et al.*[16] demonstrated the opposite preference. The simplicity of this assay and the strength of the response make it a potentially attractive and widely applicable test, but first these contradictory results reported need explanation.

Zebrafish visual behavior has been well documented,[17] and several studies suggest that it is affected by olfactory stimulation[18–20] (Stephenson, Partridge, and Whitlock, Unpublished data). Behavioral tests of this hypothesis have been restricted to determining whether the threshold of light required for a response to a visual stimulus is lower after exposure to olfactory stimuli[20] (Stephenson, Partridge, and Whitlock, Unpublished data).

This experiment therefore tests two hypotheses: (1) Zebrafish exhibit a preference for a relatively light or dark environment depending on the ambient light level; (2) olfactory stimulation alters zebrafish light/dark preference behavior at light levels above the threshold required for a response to a visual stimulus.

Materials and Methods

Fish origin and maintenance

The zebrafish used in this study were from the breeding population at the University of Valparaíso, Chile, and were naive to any form of behavioral experiment. The genetically defined "New Wild-type" line, derived from the University of Oregon AB line (http://zfin.org/; Zebrafish International Resources Center [ZIRC]), was used throughout this work. Twenty wild-type zebrafish (10 females and 10 males) were kept at 26°C–27°C in 40-L aquaria filled with deionized (reverse osmosis) water to which Instant Ocean® Sea Salt was added to reach a conductivity of 400 micro-Siemens/cm^2 and sodium bicarbonate was added to reach pH 7.3 (hereafter referred to as "fish water"). Fluorescent room light provided a 13.5:10.5 (L:D) photoperiod (light phase: 9 am to 10.30 pm); light level at the surface of the water in the communal home tanks ranged from 619 to 705 lx. When fish were held in

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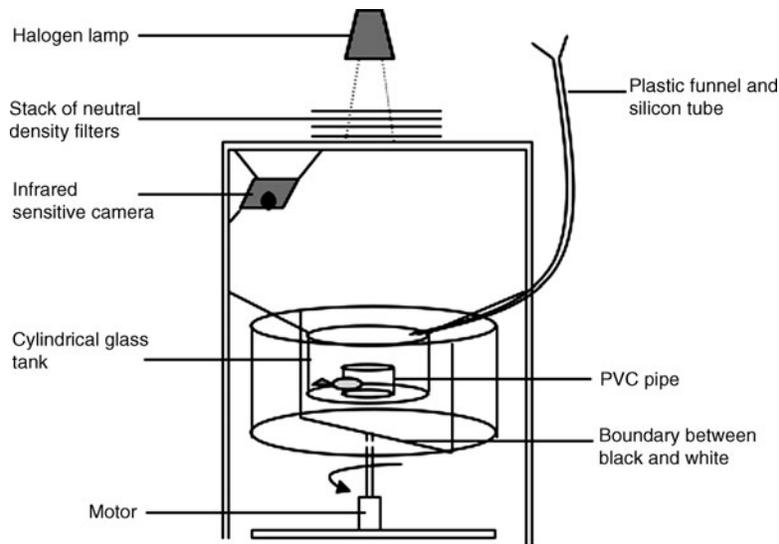[2]Centro de Neurociencia, Centro de Genomicas Celular, Universidad de Valparaíso, Valparaíso, Chile.

FIG. 1. The apparatus used during the experiment.

individual tanks (described below), the light level at the surface of the water of these tanks ranged from 502 to 767 lx, as measured using a digital luxmeter (Mastech MS6601; Precision Mastech Enterprises). Fish were fed a mixture of dried chironimid larvae (hereafter referred to as "bloodworms") and Tetramin® flakes at 9.30 am and 4.00 pm during the experimental period.

Apparatus

The apparatus (Fig. 1) consisted of a cylindrical glass tank (diameter: 18 cm; depth: 10 cm) suspended from a Dexion® steel frame. The tank was surrounded by a cardboard drum (diameter: 28 cm; depth: 14 cm), which could be rotated in either direction at a constant speed of 10 rpm by a motor. Half of the drum was painted white and the other half black. The base of the drum was painted white and half covered with a semicircle of black fabric. Both halves reflected in the infrared. The fish could be viewed and behavior was recorded using an infrared-sensitive video camera (Siemens Pro-100L CCIR B/W camera AVC308C/F36) mounted overhead. The camera was supported from the top of the frame, three sides of which were covered in black cardboard. The top and one side were draped in two layers of blackout fabric. A 10 cm×10 cm hole was cut in the fabric covering the top of the frame, over which were laid 15 cm×15 cm squares of neutral density (ND) filters (Lee 209 and 299; nominal absorbances 0.3 and 1.2, respectively). A halogen light (12 V, 35 W; 220–240 V, 50 Hz) was positioned directly above the hole.

To calculate the irradiance values, a cosine corrector (model CC-3-UV; Ocean Optics) was placed at the level of the surface of water and connected to a spectrometer (model QE65000; Ocean Optics) via a 400-μm solarized optical fiber (item QP400-2-SR; Ocean Optics). The spectrometer was connected to a computer running Spectrasuite (Ocean Optics) data acquisition software, the output of which was used to calculate the total irradiance between 380 and 780 nm for the experimental setup with no filters in place. The spectral absorbance of both filters (Lee ND filter 209 and 299) was measured using a dual-beam scanning spectrophotometer (model UV2101-

PC; Shimadzu Corp.), and the spectral absorbances between 380 and 780 nm of each filter were used to estimate, by calculation, the spectral irradiance under each filter combination used during the trials. The irradiance spectra resulting from each combination of filters used in this experiment are shown in Figure 2.

Olfactory stimuli were introduced to the experimental tank as odors in water. Control water and the food stimulus had
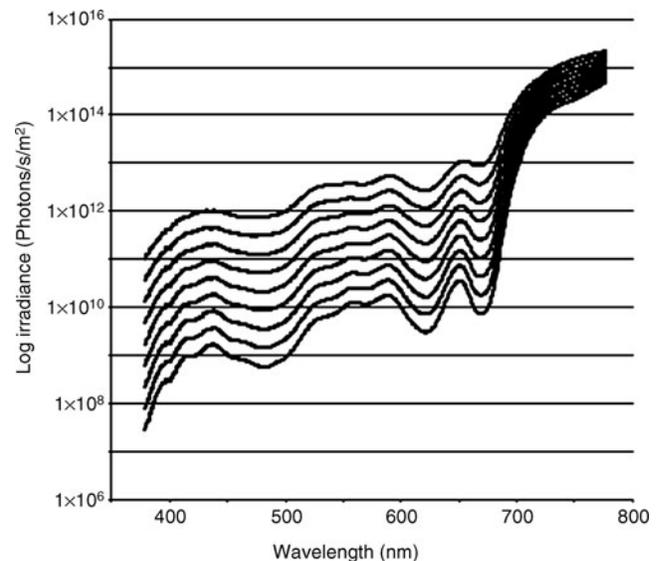

FIG. 2. The irradiance spectra of the filter combinations used during the experiments. The transmission spectra of the Lee 209 (0.3 ND) and 299 (1.2 ND) filters were measured in a spectrophotometer and were used to calculate the irradiances presented here, as described in the text. Trials began with the light level represented by the lowest line and proceeded with the removal of one Lee 209 (0.3 ND) filter, resulting in light levels increasing in the steps represented on this graph. Trials ended at the light level represented by the top line.

separate input apparatus. Two lengths of new silicon tubes were fitted with small funnels and hung above the Dexion frame. The tube to be used was fed through a hole in the cardboard surrounding the frame and hung just above the surface of water in the experimental tank. The hole for the tube was covered with two layers of fabric to block out light.

Stimulus preparation

The same mixture of dried bloodworms and Tetramin flakes was used to produce the food odor and feed the fish. About 0.1 g of food was mixed into 1 L of fish water and left overnight so that food particles settled on the bottom of the container. The stimulus water required for the duration of the experiment was extracted from the top and was uncontaminated with visible food particles. One liter of fresh fish water was left overnight in a plastic container similar to that used to make the food odor. The containers used to make and store each stimulus were covered to prevent contamination from airborne particles.

Experimental protocol

Individuals to be tested were held overnight in individual 1-L transparent tanks. Fish were not fed during their isolation. All trials were performed between 9.30 am and 4.30 pm over 3 days. One liter of fresh fish water was poured into the experimental tank, to a depth of 4 cm. A naive fish was placed in the tank and the fabric was arranged to ensure no light reached the tank from any source other than the 10 cm × 10 cm hole. The hole was covered with two layers of Lee ND 299 filter and eight of Lee ND 209 filter. The lamp was turned on, giving a total irradiance of 1.34×10^{17} photons/s/m^2, and the fish were left 25 min for acclimatization and dark adaptation. The experimental room was heated constantly to 26°C–27°C, and thus the temperature of the water in the experimental tank did not vary from the fish's home tank.

Each trial began with 1 min of recording before stimulus input. During this minute, the drum was briefly rotated at the start of recording and at 15 s intervals thereafter during the experiment (i.e., four times in total). Rotational direction (clockwise or anticlockwise) was selected such that the fish were confronted with the boundary between black and white in the shortest time. Throughout the trials, rotation was used to control for any nonlight-related bias for a particular area of the tank and to encourage the fish to reassess their position in the chamber. During the second minute, 10 mL of stimulus water (either food odor or control water) was injected into the tank. The sequence of rotations of the first minute was repeated in the third and subsequent minutes of the trial. After the third minute, the light level was increased by about 0.3 log units by the removal of one Lee ND 209 filter per minute. At the end of each trial, the fish were transferred to a communal home tank and the stimulus input tube was rinsed thoroughly with fish water. The tank was washed thoroughly with 70% ethanol, tap water, and fish water.

Data analysis

The video records were analyzed by an observer ignorant of the stimulus to which the test fish had been exposed. The time spent on the black and white sides at each light level was converted into a proportion of time spent on the black half. This nonnormal variable was arcsine square root transformed and used as the response variable in repeated measures analysis of variance in SYSTAT (Systat Software). After transformation, Kolmogorov–Smirnov and Levene tests showed that 95% of the data conformed to the assumptions of normality and homogeneity of variance. The factors sex of the fish, block, and time of day were removed sequentially from the full model. The final model comprised light level as a repeated measure, olfactory stimulus (food or control water), and the interaction between them. The software did not allow *post-hoc* tests to be performed on repeated measures analysis. We therefore used two-sample *t*-tests in Excel (Microsoft®) to show the light levels at which the food odor-exposed and control groups differed significantly (illustrated in Fig. 2 and in Table 1) and to ascertain the effect of stimulus input on the response variable between minutes 1 and 3. One-sample two-tailed *t*-tests were performed in R (version 2.10.1, GUI 1.31; S. Urbanek & S. M. Iacus, © R Foundation for Statistical Computing, 2009) to test for a difference between the mean proportions of time spent on each half of the drum and 0.5 (results given in Table 1). Any significant difference between the mean and 0.5 indicates that fish spent significantly more time on one half of the drum than the other, which implies a preference. We report the actual *p*-values throughout the results. Bonferroni correction would remove the significant *t*-test results in Table 1, but as this correction is highly conservative we suggest that the uncorrected values are more informative. Although the analyses were performed on transformed data, we present the proportional data in the figure for clarity.

Results

Light level had a significant effect on the amount of time fish spent on the black half of the drum ($F_{8,144} = 12.695$, $p < 0.001$), as did the exposure to food odor ($F_{1,18} = 4.273$, $p = 0.05$). There was not, however, a significant difference in the way that fish exposed to food odor or plain "fish water" stimuli responded to the increase in light level ($F_{8,144} = 0.784$, $p = 0.618$).

Figure 3 illustrates the effect that exposure to food odor has on zebrafish preference for light or dark. At lower light levels, the control fish (Fig. 3, dashed line) display a preference for the white half of the drum. For example, at 2.10×10^{17} photons/s/m^2, the mean proportion of time spent on the black half of the drum was 0.33, which is significantly less than half the time ($t = -3.422$, df = 9, $p = 0.008$; Table 1). This preference reverses as light level increases: at 6.56×10^{17} photons/s/m^2, the mean proportion of time the control fish spent on the black half of the drum was 0.72, significantly more than 0.5 ($t = 5.341$, df = 9, $p < 0.001$; Table 1). The fish exposed to food smell (Fig. 3, solid line), however, did not significantly prefer either half of the drum at lower light levels, but at higher light levels their preference for the black half of the drum was equal to that of control fish (at 6.56×10^{17} photons/s/m^2, mean = 0.74; difference from 0.5: $t = 5.652$, df = 9, $p < 0.001$; Table 1).

Neither the sex of the fish ($F_{1,17} = 1.004$, $p = 0.330$), the block of the experiment in which it was tested ($F_{9,7} = 0.737$, $p = 0.673$), nor the time of day the trial was conducted ($F_{1,16} = 1.139$, $p = 0.302$) affected the fish's behavior in the trial.

TABLE 1. RESULTS OF POST-HOC T-TESTS TO ILLUSTRATE THE DIFFERENCE IN PROPORTION OF TIME SPENT
ON THE BLACK HALF OF THE DRUM AT EACH LIGHT LEVEL BETWEEN THE GROUPS OF FISH EXPOSED
TO FOOD ODOR AND CONTROL WATER

Light level ($photons \times 10^{17}/s/m^2$)	Treatment	N	Mean	Standard deviatio n	Comparison between treatments		Difference from 0.5	
					t statistic	p-Value	t statistic	p-Value
1.34	Food odor	10	0.56	0.16	0.203	0.841	1.317	0.220
	Control	10	0.55	0.12			1.432	0.186
1.68	Food odor	10	0.56	0.18	2.037	0.057	1.438	0.184
	Control	10	0.42	0.13			−1.474	0.175
2.10	Food odor	10	0.51	0.15	2.611	0.018	0.239	0.816
	Control	10	0.33	0.16			−3.422	0.008
2.63	Food odor	10	0.56	0.20	1.058	0.304	1.005	0.341
	Control	10	0.47	0.18			−0.462	0.655
3.29	Food odor	10	0.49	0.12	0.699	0.493	−0.160	0.877
	Control	10	0.45	0.15			−1.021	0.334
4.13	Food odor	10	0.56	0.16	0.568	0.577	1.180	0.268
	Control	10	0.52	0.16			0.361	0.726
5.20	Food odor	10	0.63	0.20	0.840	0.412	2.089	0.066
	Control	10	0.56	0.15			1.404	0.194
6.56	Food odor	10	0.74	0.13	0.337	0.740	5.652	<0.001
	Control	10	0.72	0.13			5.341	<0.001
8.31	Food odor	10	0.77	0.10	0.645	0.527	8.625	<0.001
	Control	10	0.74	0.14			5.214	<0.001

Whether or not the means were different from 0.5, which would indicate a preference for one half of the drum, was also tested using *t*-tests, and the results are presented here. Highlighted values indicate where the difference between the two groups was significantly different from 0, or when the means were significantly different from 0.5 ($p < 0.05$).

The stimulus input (between the first and third minute of the trial) alone had no effect on the amount of time the fish spent on the black half of the drum. There was no difference between the response recorded in the first and third minutes of each trial (food odor: $t(9) = 0.517$, $p = 0.617$; control water: $t(9) = −0.214$, $p = 0.835$) and there was no difference between the sexes in this respect ($t(19) = −0.647$, $p = 0.525$).

Discussion

Ambient light and olfactory stimulation affect zebrafish light environment preferences. Although the light conditions experienced by the fish tested by Gerlai *et al.*[15] and Serra *et al.*[16] have not been reported, it is very unlikely that these studies used exactly the same level of ambient light. Serra *et al.*[16] used a half-white/half-black tank for their preference test, whereas Gerlai *et al.*[15] used a tank of uniform color, half of which was covered with paper. A recent review suggests that the explanation for the discrepancy between these two findings lies in this difference between the methodologies. Although zebrafish may prefer black backgrounds to minimize their visibility, they may avoid enclosed, cave-like places that could harbor predators.[21] By manipulating the light level, however, the present study was able to replicate both sets of results, despite the fact that the methodology was more similar to that employed by Serra *et al.*[16] It is likely that zebrafish perception of the dark or light areas of a tank changes under different ambient light levels, and thus, both these explanations are important.

The reason for the light dependence of the preference behavior is unclear. Although both halves of the drum received the same amount of light from above, the amount reflected by the black half would have been less. However, most of the light experienced by a fish is from above, via Snell's window,

and the difference in total ambient illumination due to the proximity of the light or dark parts of the drum is likely to be small. Nevertheless, the pattern of behavior observed here partly supports the hypothesis that zebrafish modify their behavior in an attempt to maintain a particular state of dark/ light adaptation. Adaptation to bright light after dark adaptation takes several minutes in adult zebrafish.[22] The hypothesis that adaptation to small changes in light level takes
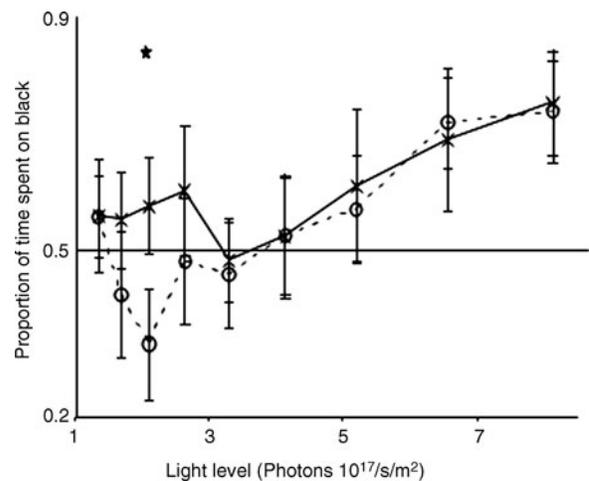

FIG. 3. Plot of the proportion of 1 min fish spent on black at each light level. Each line represents 10 control fish (dashed line) or 10 fish exposed to food odor (solid line), and error bars are the 95% confidence intervals. Asterisk denotes the light level at which the two treatment groups were significantly different ($p < 0.05$).

as long is untested, but if it were the case it would suggest a reason why zebrafish appear to prefer darker environments as light levels increase: to maximize visual sensitivity while their eyes adapt to the higher light levels.

The initial preference demonstrated by the control fish for the white half of the drum does not lend support for the above hypothesis but may be explained behaviorally, suggesting that the results are due to a combination of fish visual behavior and physiology. During the first few minutes of the trial, fish did not appear to prefer either side of the drum, possibly because they had become accustomed to the experimental tank at a dim light level. As light level increased, control fish spent more time on the white half of the drum. The fish may have become wary of the black half until the ambient light level reached the point at which they could visually confirm that it did not conceal a predator. As the light level continued to increase, fish may have preferred the black half in order to minimize their visibility against the background.[23] Zebrafish larvae alter the distribution of melanin pigment in their skin to ambient light levels and to match backgrounds[24]; this ability continues to adulthood (personal observation). Preference for the black half of the drum at higher light levels could also optimize their visual sensitivity, as discussed earlier.

It is possible that the pattern observed here is due to a time- or familiarity-dependent change in fish behavior. We feel that this is unlikely, as we and other authors[20] have observed that in cylindrical tanks zebrafish tend to swim in circles around the edge of the tank, particularly after extended periods and familiarity with such an enclosure. This behavior was observed here at light levels that did not elicit a preference. At other light levels, fish desisted from swimming in circles and swam along the side of the tank but turned before exiting their preferred side. Continuous swimming in one direction throughout a trial could not have therefore produced the complex pattern of preference for light or dark sides of the tank that was observed here.

It is not possible to distinguish between the behavioral and physiological explanations of the effect of food odor on the recorded light environment preference behavior. The fish exposed to food odor did not show any avoidance of the black half of the drum during the lower light levels used during the trial, unlike the control fish (Table 1). These fish are likely to have been hungry, and the detection of food odor may influence the trade-off between foraging and the risk of predation from a predator potentially hidden in the black half.[25] Hungry fish of several different species have been shown to be less responsive to the risk of predation than their well-fed counterparts (*Semotilys atromaculatus* and *Rhinichthys atratulus*[26]; *Oncorhynchus kisutch*[27]; *Salmo salaar*[28]; *Gasterosteus aculeatus*[29]; *Theragra chalcogramma*[30]; *Pimephales promelas*[31]). Olfactory stimulation could, however, have directly enhanced their visual sensitivity to such an extent that the fish exposed to food odor could see that the black half of the drum did not conceal a predator at lower light levels than the control fish. Indeed, we have data suggesting that zebrafish visual sensitivity is dependent on odors (Stephenson *et al.*, In preparation). The observation that both the control and food-odor exposed groups preferred the black half of the drum toward the end of the trial suggests that once the absence of food has been confirmed, the fish reverts to a behavior that minimizes its risk of predation, or that the effect of olfactory stimulation on visual sensitivity is short-lived.

Although this experiment provides an insight into the factors determining zebrafish preference for dark or light environments, further work is required. The preferences fish display at light levels outside the range tested here, and the optimum light level for eliciting the strongest preference, need to be determined. In addition, the significance of the spectral distribution on irradiance in natural environments could usefully be compared with that of this and future laboratory experiments. The effect of the difference in methodology between the studies by Serra *et al.*[16] and Gerlai *et al.*[15] should also be considered. Further experiments are required to confirm the adaptive significance of this behavior and the role of food odor in its modification. Quantifying the difference in light level between the black and white halves of the drum, elucidating the difference in light environment preference between hungry and satiated fish, as well as potential effects of other odors would test the hypothesized explanation of the results given here. As long as the olfactory environment is controlled, however, and a suitable ambient light level is selected, zebrafish display a strong and consistent preference for either light or dark surroundings.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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