Chapter 13

Neurophenotyping of Adult Zebrafish Using the Light/Dark Box Paradigm

Adam Stewart, Caio Maximino, Thiago Marques de Brito, Anderson Manoel Herculano, Amauri Gouveia Jr., Silvio Morato, Jonathan M. Cachat, Siddharth Gaikwad, Marco F. Elegante, Peter C. Hart, and Allan V. Kalueff

Abstract

The light/dark box test, traditionally used to quantify rodent anxiety-like behavior, has recently been applied to the adult zebrafish (*Danio rerio*). Utilizing the fish's scototaxis (aversion to bright areas and natural preference for the dark), this paradigm can be used to assess levels of anxiety in adult zebrafish. The light/dark box is a simple and time-efficient one-trial test that does not require pre-training the animals. Importantly, this novelty-based paradigm may also represent a useful tool for studying the pharmacological modulation of zebrafish behavior. Summarizing the experience with this model in several laboratories, here we outline a protocol for the neurophenoptyping of zebrafish anxiety-like behavior using the light/dark paradigm.

Key words: Zebrafish, Light/Dark box, Scototaxis, Anxiety, Novelty-based paradigm.

1. Introduction

Various novelty-based paradigms, some of which are comprehensively covered in this book, have been developed to quantify zebrafish behavior (1–7). The light/dark paradigm, traditionally used in animal (rodent) behavioral research (8–11), has only recently been applied to zebrafish (12–15). Nevertheless, this test, based on the innate fish preference for the dark (scotophilia or scototaxis), is receiving growing popularity in neurobehavioral laboratories (2, 14, 16, 17).

A.V. Kalueff, J.M. Cachat (eds.), *Zebrafish Neurobehavioral Protocols*, Neuromethods 51,
DOI 10.1007/978-1-60761-953-6_13, © Springer Science+Business Media, LLC 2011

Previous research in rodents has shown that while anxiolytic manipulations can facilitate exploratory activity (i.e., increased entries and duration in the light part), anxiogenic drugs cause the opposite effect (8, 9, 11, 18). Given the amazing translatability of zebrafish models into rodent and human neurophenotypes (1, 2, 19), the possibility to adapt a scototaxic paradigm to zebrafish was logical (*see* (15) for details). Prior evidence has shown that scototaxis may contribute to predator avoidance in nature, as adult zebrafish stand out clearly when swimming amidst a light background. This further underscores their inherent anxiogenic response evoked when confined to a white background (12).

Several modifications have been made to produce a zebrafish paradigm that parallels the rodent light/dark assays (2, 12, 17). The utility of the zebrafish light/dark box is further strengthened when used in conjunction with video-aided analysis, which can assist in tracking and quantifying animal behavior. Here we describe a simple protocol for using the light/dark model to assess stress- or drug-evoked alterations in adult zebrafish anxiety.

2. Methods and Materials

2.1. Animals and Housing

Adult zebrafish (e.g., wild-type short-fin, 6–8 month-old; ≈50:50 male:female ratio) can be obtained from a local commercial distributor, and should be given at least 20 days to acclimate to the animal facility. Animals can be housed in groups of approximately 20–30 fish per 40-L tank. Tanks should be filled with filtered facility water, with both room and water temperatures maintained at ≈25°C and water pH at 7.0–8.0. Illumination can be provided by ceiling-mounted fluorescent light tubes (e.g., 1000 lux) on a 12–12 or 10–14 h cycle, consistent with the zebrafish standard of care (20).

2.2. Apparatus

Several modifications of the light/dark paradigm, used by our laboratories, will be discussed here. One modification, used at Tulane University, USA (Modification I), represents a rectangular Plexiglas tank (15 height × 30 length × 16 cm width) that rests on a level surface, and divided into two equal vertical portions (**Fig. 13.1a**), demarcated by black and white coloration (2). It differs from the rodent apparatus in that it is sealed to prevent leakage, filled with water to a height of 12 cm, and does not have a wall (with a sliding door) between the compartments. In this modification, fish can freely swim between the light and dark compartments of the apparatus.

Another, more sophisticated, modification of this test was successfully used by Brazilian laboratories (Modification II,

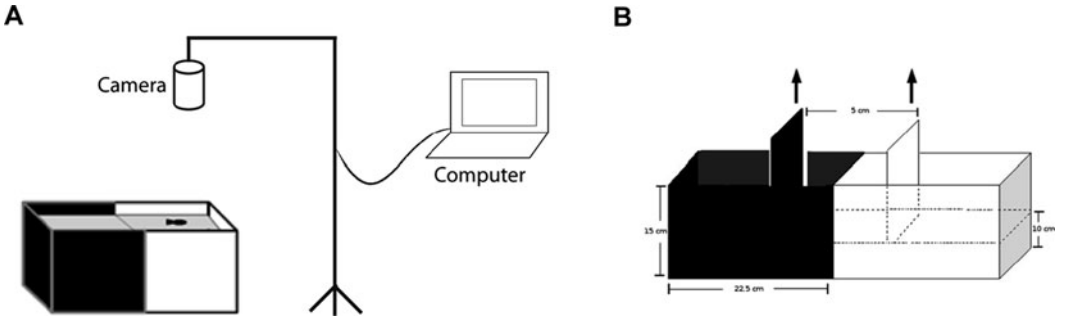

Fig. 13.1. The light/dark paradigm for characterization of adult zebrafish behavior. **a** – Typical experimental set-up used in Tulane University, USA (Modification I), allowing for video-recording for subsequent analysis using video-tracking software. Note the camera need only be centered above the *white* half, as the *black* half will not be analyzed. **b** – Typical light/dark box test used in Brazilian laboratories (Modification II, as described in (13)).

Fig. 13.1b). This modification, applied to zebrafish and some other fish species (13, 15), represents an acrylic tank of equal measures (15 height × 45 length × 10 cm width) with half black/half white walls and bottom colored, and filled with water to a height of 10 cm. The colored material chosen should be non-reflective, in order to avoid the tendency of animals to behave in relation to their own reflection. Unlike Modification I, this apparatus contains sliding central doors, colored with the same color of the aquarium side, thereby defining a central compartment with 15 height × 10 length × 10 cm width (**Fig. 13.1**).

During experiments, the tank must be rotated after each trial, so as to eliminate orientation effects. The tanks are illuminated by environmental light (e.g., by a 60-W light bulb, located at 1.80 m above the tank top), which kept illumination uniform and constant between trials (**Fig. 13.1**).

2.3. Experimental Setup

The light/dark box should be positioned for optimal lighting while avoiding all glare from the room's light source. Since the brightness of the apparatus is a fundamental feature of this paradigm, use a light meter (e.g., 840006 by Sper Scientific, AZ) to ensure that all areas of the apparatus are illuminated with the same intensity. For a light-sensitive assay such as this, optimal and homogeneous lighting conditions are important for this protocol. The results are also sensitive to the light amounts; animals tested under low-light levels (250 lux) spend more time in the white compartment than animals tested under high-light levels (500 lux) (**Fig. 13.2**). Additionally, unlike other behavioral tests, manual scoring is complicated by the nature of this apparatus, as the experimenter would have to lean over the apparatus to gather the data. This can be problematic for the testing, since the experimenter could cast a shadow or startle the fish. However, the use of a webcam and computer can alleviate this problem, as it allows

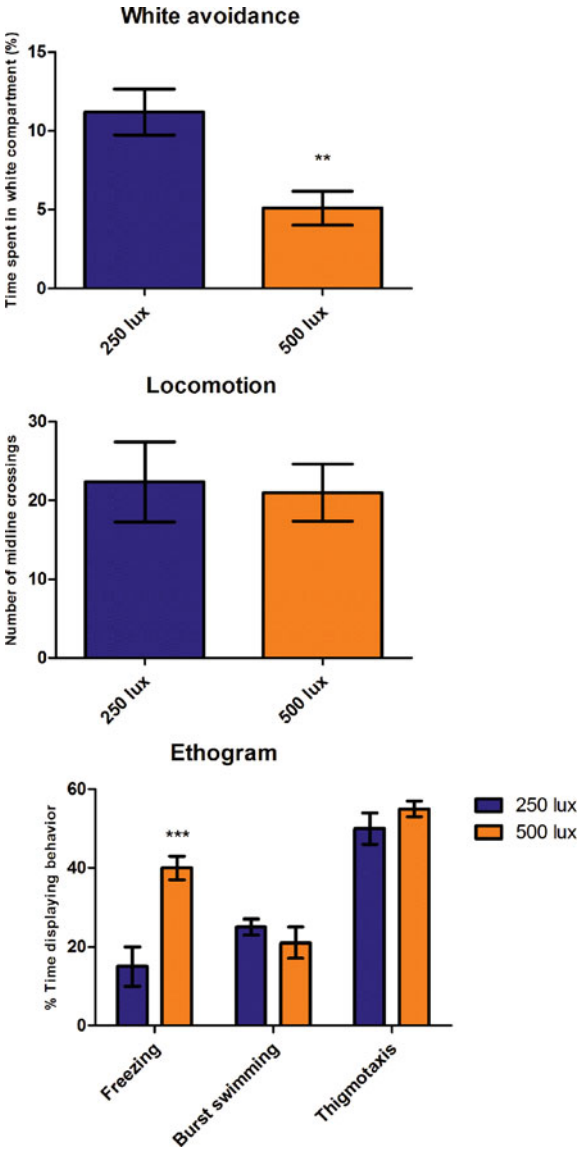

Fig. 13.2. Lighting levels alter the results of the zebrafish light/dark box test. Animals tested in Modification II of this test for 15 min under high illumination levels (500 lux) spend less time in the white compartment and freeze more while there ($n = 10$), Maximino et al., unpublished data; $^{***}p<0.001$, $^{**}p<0.01$.

for remote observation via the computer screen, as well as allows option for later video-aided analysis (**Fig. 13.1a**).

2.4. Behavioral Endpoints

Behavioral scoring can be performed manually to quantify the latency to enter (s), time spent (s), average entry duration (s), and the number of entries to the white half of the apparatus (due to the dark background, zebrafish behavior in the black compartment cannot be detected, and, therefore, is not assessed in this paradigm). To further characterize zebrafish light/dark

preference, the white:total time spent ratios can be calculated for both cohorts. Video-tracking programs, such as Ethovision XT 7 (Noldus Information Technology, Netherlands; *see* Chapter 1 in this book), can also be used to analyze variety of additional endpoints, such as distance traveled, velocity, meandering, turning angle, angular velocity, or time spent moving.

3. Procedure

3.1. Acclimation and Pre-treatment

Transport the animals from their holding room to the experimental room for acclimation 1 h prior to testing. During this time, if the study involved pharmacological manipulations, prepare 3–4 L beaker(s) in order to administer the drug via immersion. Fill each beaker with ~3 L of exposure solution, maintained at the same temperature as the holding room (drug concentration is determined by referring to prior literature and/or pilot study). After the acclimation period (and when the drug is fully dissolved), the fish are individually transferred to the exposure beaker filled and treated for the optimal exposure time (lengths of treatment will vary with the drug, but is generally in intervals of 10, 20, or 30 min).

3.2. Light/Dark Box Testing

Fill the light/dark apparatus with 10–12 cm of room-temperature filtered water. After the necessary pre-treatment time has elapsed, begin video-recording and carefully move the fish to the light/dark box. If using Modification I, introduce the fish into the black half (facing the wall), and video-record for 6 or 10 min, while manually scoring the behaviors. Recording times may be extended, however, 6- or 10-min trials appear to be optimal for most experiments. If using Modification II, introduce the fish by netting it from the maintenance tank and transferring it, as quickly as possible, to the central compartment; in this case, keep the sliding doors on for 3–5 min, for acclimation, then remove them to allow the animal to explore the apparatus. Standard 15 min testing sessions have been used for this modification. If endocrine data are collected, euthanize the fish by immersion in 500 mg/L Tricaine (*see* Chapter 11, this book). Store each fish individually in Eppendorf tubes, denoting its treatment group and store at –80°C for later cortisol extraction. For details on troubleshooting, refer to Notes 1–3.

3.3. Video-Aided Analysis

As already mentioned, zebrafish behavioral endpoints may be evaluated using video-aided analysis. Transfer videos to computer for subsequent analysis using video-tracking software. Define the arena to overlap with the outline of the apparatus, and define the zone to encompass only the white portion of the test. Accord-

ingly, set the program to track objects that are darker than the background. In addition to evaluating the endpoints recorded manually, other indices can be specified to include the time spent (s) in the white zone, distance (m) traveled, velocity (m/s), and immobility (freezing) frequency and duration. Traces of the path taken by the animal can also be generated (**Fig. 13.3a**; refer to chapters on visualizing and video-tracking zebrafish behavior in this book). For details on troubleshooting, refer to Notes 4–7.

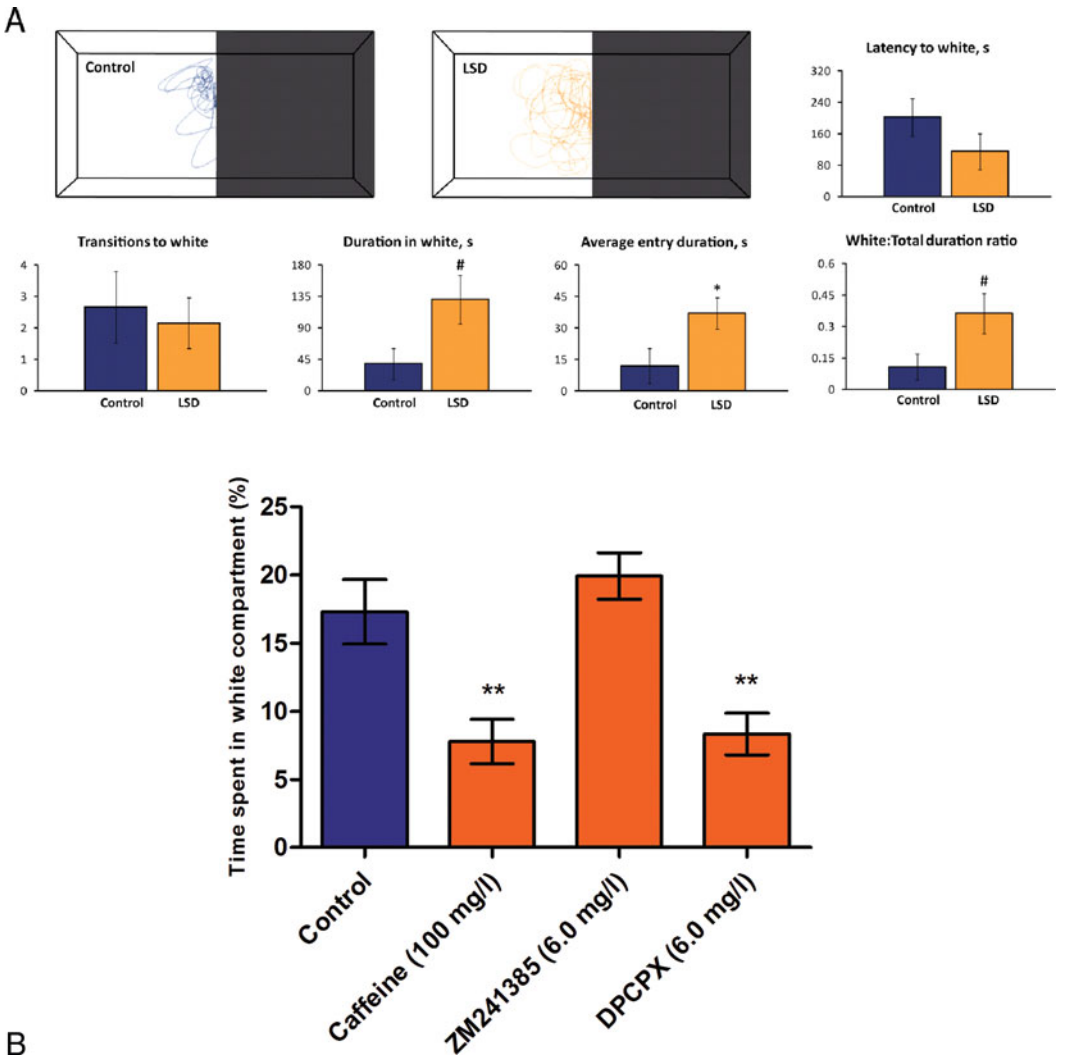

Fig. 13.3. Behavioral effects of selected pharmacological agents in the light/dark box test. **a** – effects of Lysergic acid diethylamide (LSD) (250 μg/L) on zebrafish tested in the 6-min light-dark box test, Modification I ($n = 12$); data is based on (14). Representative traces were generated by Ethovision XT7 software using the top view video-recording; only light part of the box and a small part of the dark part are shown in this panel. **b** – effects of several adenosine receptor antagonists (caffeine, nonselective antagonist, 100 mg/L; ZM241385, A_{2A} receptor antagonist, 6 mg/L; DPCPX, A_1 receptor antagonist, 6 mg/L) on zebrafish tested in a 15-min test (Modification II); $n = 12–14$; Maximino and Herculano, unpublished data; $^{**}p<0.01$, $^{*}p<0.05$, #p=0.05–0.1 (trend) vs. control.

3.4. Statistical Analysis

Use the Mann-Whitney *U*-test for comparing two groups. Student's *t*-test may be used for normally distributed data. Our group has devised a useful template to calculate statistics and generate graphs for zebrafish manual or video-tracking data, which can be downloaded from our laboratory's website at: www.kaluefflab.com/science.html. For more than two groups, use an Analysis of Variance (ANOVA), followed by an appropriate post-hoc test (e.g., Tukey, Dunn, Newman-Keuls, or Dunnet tests). In general, *n*-way ANOVA may be applied, with commonly used factors being: treatment, dose, sex, strain, time, trial, or age.

4. Notes

1. *Zebrafish display atypical and/or varied behavioral phenotypes* Different zebrafish strains can have varying baseline levels of anxiety (1), which could result in the failure to cross into the white half of the apparatus. Alternatively, it may represent a behavioral hyperactivity, or disinhibition to regard the white half as aversive. Sensory deficits, such as impaired vision, will also produce atypical data in this test. Likewise, altered cognitive functions will produce abnormally low (good memory) or high (poor habituation) exploration of the white area. Finally, variations in responses can also be seen among the standard wild-type strain, with both low- and high-avoidant fish often present in the same cohort (21). Low-avoiding fish can be particularly problematic because of their heightened tendency to quickly habituate to the white half of the tank. In general, to rule out nonspecific factors, a careful examination of zebrafish cognitive, neurological, and sensory phenotypes is recommended in case if atypical behavioral responses are observed in the light/dark paradigm. Additionally, the time of the trial may have to be adjusted to obtain more reliable data (*see* above).

2. *Fish consistently fail to cross into the white half during the trial* Generally, the presence of the experimenter in the room during testing may startle the fish, causing a heightened anxiety-like behavior, especially if a webcam and computer setup are not employed. Also, differences in water temperature or excessive net stress prior to testing can also induce a state of decreased locomotion. Furthermore, after ruling out strain variation (*see* above), the pharmacological agent itself may need to be considered. For instance, some fish may often remain in the dark half for an entire 6-min trial. If they are treated with an anxiogenic drug, which leads to an even greater aversion to cross into the white, the drug-

evoked effects will be masked by high background anxiety (floor/ceiling effect). To compensate, consider extending the trial duration (e.g., to 30 min), which will encourage more active animal exploration.

3. *Fish displaying abnormally high thigmotaxis*
   Fish spending too much time (~30% of the total test time) against a particular wall, or walls in general, of the apparatus (maximum distance of ~2 cm from the wall) may be responding to its own reflection (15). Consider changing the material from which the apparatus is made, to avoid confounding variables. The experimenter should keep track of the thigmotactic fish, and thigmotaxis itself should be analyzed (either by recording its frequency and duration in individual fish, or by recording the number of fish that displayed it) (15).

4. *Software not detecting fish*
   This lack of object detection can be resolved by altering one or several setting as well as ensuring adequate lighting. Notably, it is essential that the subject be defined as darker than the background (*see* Chapter 12 for details).

5. *Fish freezes in white compartment, after first choice*
   Occasionally, fish can freeze after they choose the white compartment, no longer exploring the apparatus for the whole trial duration. This is especially common for stressful manipulations; or if there is noise, vibration or movements in the experimentation room. Data from this animal should be discarded from analysis. The experimenter may keep track of the freezing fish, and freezing behavior itself should be analyzed (either by recording its frequency in individual fish or recording the number of fish which froze) (15). However, careful attention should be paid to the frequency/patterning of this behavior to ensure external factors (discussed above) are not inducing excess freezing.

6. *Software not producing data on fish*
   Verify the detection settings and ensure that the software is able to track the fish in the white half of the tank. However, it is most likely that the fish for these particular trials did not cross into the white half during the trial (this is especially common among control cohorts).

7. *Fish jumping out of the tank*
   Infrequently, the animal "jumps" out of the test tank. When this occurs, the experimenter must rapidly pick up the animal and discard it. Behavioral data from such fish should be excluded from the analyses (15).

5. Anticipated Results

The observed behavioral responses and indices of zebrafish anxiety assessed in the light/dark box test should generally parallel those observed in the novel tank and open-field models. However, some differences in pharmacological results with variations of these tests have been observed, suggesting that these models may target different aspects or subtypes of anxiety (6). In line with this, anxiety levels can be attenuated or exaggerated depending on drug exposure. For example, exposure to anxiolytic agents will cause an increase in transitions to and time spent in the white half of the tank. A decreased latency to cross into the white half should also be expected. Although not specific to this apparatus, the bouts and duration of freezing, as well as erratic movements, should also be decreased by a reduction in anxiety (**Fig. 13.3**). Conversely, the opposite is expected with the administration of anxiogenic compounds. For example, acute treatment with methylmercury is anxiogenic in zebrafish, as assessed by light/dark preference (**Fig. 13.4**).

In addition to pharmacological modulation, other manipulations can be used in this model. For example, rearing in enriched environment for 2 months increases the time spent in the white compartment of the test tank, compared with those reared in an impoverished environment (15). Thus, positive stress-reducing factors such as environmental enrichment can decrease zebrafish anxiety-like behavior in this test, strikingly paralleling similar findings in rodents (15, 22–24).

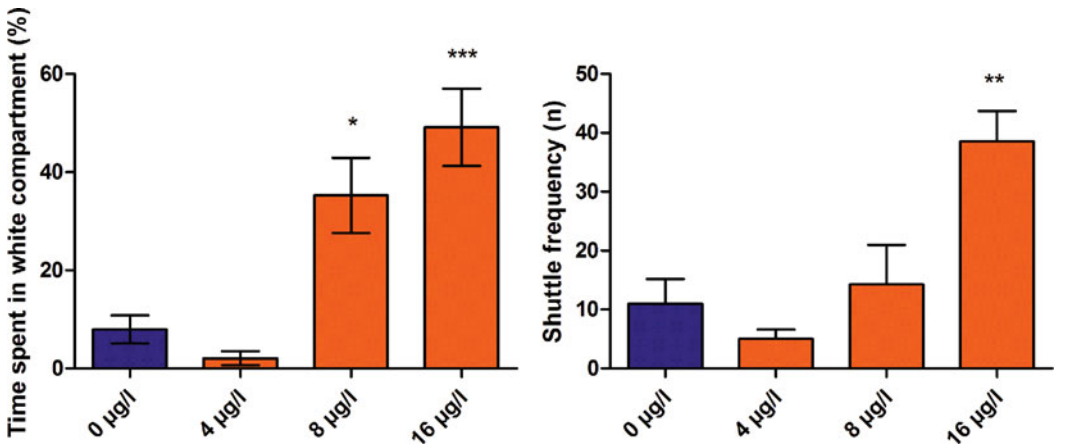

Fig. 13.4. Effects of methylmercury chloride exposure (4, 8, and 16 µg/L for 24 h) on the time spent in the white compartment and total locomotion in the 15 min light/dark box (Modification II) in adult zebrafish ($n = 10–14$). Maximino et al., unpublished data; ***$p<0.001$, **$p<0.01$, *$p<0.05$ vs. control.

6. Summary

The light/dark box test is emerging as a promising behavioral assay to quantify anxiety-like behavior in adult zebrafish. Overall, this test serves as a useful addition to the array of novelty-based paradigms, being unique in its ability to assess light/dark aversion. Importantly, the quantification of scototaxis may serve as a reliable tool in neurophenotyping research and high-throughput drug screens. Rodent literature has demonstrated that the light/dark test is especially useful for phenotyping mutant strains, a utility that has recently been confirmed in zebrafish (21). However, the evaluation of different strains using this paradigm has yet to be undertaken. For example, this may be particularly useful to address some conflicting reports in the literature (not discussed here) that some zebrafish cohorts may prefer the white and avoid the dark. While fish sex, age, social status and strain differences, as well as their interactions with various environmental factors (such as lighting of the apparatus and/or the testing room), can indeed modulate zebrafish light/dark box behavior, we have not observed aberrant white preference in our zebrafish experiments, where robust scototaxis was the most prominent and consistent phenotype evoked.

In addition to its use in adult zebrafish, the light/dark paradigm has recently been applied to larvae, although in a different model. Notably, unlike adults, larval zebrafish are *phototactic*, as they prefer lighter areas (25) and move toward well-lit areas when presented with a choice (26). As such, larvae locomotion patterns have been studied under a range of lighting conditions with varying durations. For example, when subjected to an extended period of darkness, larvae locomotor activity is high at first and then decreases to a low level. In an extended light duration, their activity gradually increases to a stable level, but can be also be pharmacologically modulated in both light and dark conditions (27). Logically, the "reversed" light/dark box test could be developed for zebrafish larvae, and further research is needed in this field.

Overall, the light/dark box is a simple and fast one-trial test that does not require pre-training the animals. This paradigm offers a promising and sensitive tool to complement the other tests measuring anxiety-like behavior in zebrafish.

Acknowledgments

The study was supported by Tulane University Intramural funds, Provost's Scholarly Enrichment Fund, Newcomb Fellows

Grant, LA Board of Regents Pfund and NARSAD YI awards, Zebrafish Neuroscience Research Consortium (ZNRC), as well as CAPES/Brazil.

References

1. Egan, R.J. et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.* **205**(1), 38–44 (2009).
2. Stewart, A. et al. The developing utility of zebrafish in modeling neurobehavioral disorders. *Int. J. Comp. Psychol.* **23**(1), 104–121 (2010).
3. Levin, E.D., Bencan, Z., & Cerutti, D.T. Anxiolytic effects of nicotine in zebrafish. *Physiol. Behav.* **90**(1), 54–58 (2007).
4. Gerlai, R. et al. Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacol. Biochem. Behav.* **67**(4), 773–782 (2000).
5. Wong, K. et al. Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behav. Brain Res.* **208**(2), 450–457 (2010).
6. Sackerman, J. et al. Zebrafish behavior in novel environments: effects of acute exposure to anxiolytic compounds and choice of Danio rerio line. *Int. J. Comp. Psychol.* **23**, 43–61 (2010).
7. Blaser, R. & Gerlai, R. Behavioral phenotyping in zebrafish: comparison of three behavioral quantification methods. *Behav. Res. Methods.* **38**(3), 456–469 (2006).
8. Bourin, M. & Hascoet, M. The mouse light/dark box test. *Eur. J. Pharmacol.* **463**(1–3), 55–65 (2003).
9. Hascoet, M., Bourin, M., & Dhonnchadha, B.A. The mouse light-dark paradigm: a review. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **25**(1), 141–166 (2001).
10. Fraser, L.M. et al. Measuring anxiety- and locomotion-related behaviours in mice: a new way of using old tests. *Psychopharmacology* (Berlin), **211**(1), 99–112 (2010).
11. Shimada, T. et al. The modified light/dark transition test in mice: evaluation of classic and putative anxiolytic and anxiogenic drugs. *Gen. Pharmacol.* **26**(1), 205–210 (1995).
12. Blaser, R.E., Chadwick, L., & McGinnis, G.C. Behavioral measures of anxiety in zebrafish (Danio rerio). *Behav. Brain Res.* **208**(1), 56–62 (2010).
13. Maximino, C. et al. A comparative analysis of the preference for dark environments in five teleosts. *Int. J. Comp. Psychol.* **20**, 351–367 (2007).
14. Grossman, L. et al. Characterization of behavioral and endocrine effects of LSD on zebrafish. *Behav. Brain Res.* **214**(2), 277–284 (2010).
15. Maximino, C. et al. Scototaxis as anxiety-like behavior in fish. *Nat. Protoc.* **5**(2), 209–216 (2010).
16. Maximino, C. et al. Parametric analyses of anxiety in zebrafish scototaxis. *Behav. Brain Res.* **210**(1), 1–7 (2010).
17. Serra, E.L., Medalha, C.C., & Mattioli, R. Natural preference of zebrafish (Danio rerio) for a dark environment. *Braz. J. Med. Biol. Res.* **32**(12), 1551–1553 (1999).
18. Malmberg-Aiello, P. et al. Mouse light/dark box test reveals anxiogenic-like effects by activation of histamine H1 receptors. *Pharmacol. Biochem. Behav.* **71**(1–2), 313–318 (2002).
19. Shin, J.T. & Fishman, M.C. From Zebrafish to human: modular medical models. *Annu. Rev. Genomics Hum. Genet.* **3**, 311–340 (2002).
20. Westerfield, M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th ed. (University of Oregon Press, Eugene, 2007).
21. Takao, K. & Miyakawa, T. Light/dark transition test for mice. *J Vis Exp.* **1**, 104 (2006).
22. Prior, H. & Sachser, N. Effect of enriched housing environment on the behaviour of young male and female mice in four exploratory tasks. *J. Exp. Amin. Sci.* **37**, 57–68 (1994).
23. Chapillon, P. et al. Rearing environmental enrichment in two inbred strains of mice: 1. Effects on emotional reactivity. *Behav. Genet.* **29**(1), 41–46 (1999).
24. Roy, V. et al. Environmental enrichment in BALB/c mice: effects in classical tests of anxiety and exposure to a predatory odor. *Physiol. Behav.* **74**(3), 313–320 (2001).
25. Neuhauss, S.C. Behavioral genetic approaches to visual system development and function in zebrafish. *J. Neurobiol.* **54**(1), 148–160 (2003).
26. Mueller, K.P. & Neuhauss, S.C. Behavioral neurobiology: how larval fish orient towards the light. *Curr. Biol.* **20**(4), R159–R161 (2010).
27. MacPhail, R.C. et al. Locomotion in larval zebrafish: influence of time of day, lighting and ethanol. *Neurotoxicology* **30**(1), 52–58 (2009).