

Video Article

Behavioral Approaches to Studying Innate Stress in Zebrafish

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Abstract

Responding appropriately to stressful stimuli is essential for survival of an organism. Extensive research has been done on a wide spectrum of stress-related diseases and psychiatric disorders, yet further studies into the genetic and neuronal regulation of stress are still required to develop better therapeutics. The zebrafish provides a powerful genetic model to investigate the neural underpinnings of stress, as there exists a large collection of mutant and transgenic lines. Moreover, pharmacology can easily be applied to zebrafish, as most drugs can be added directly to water. We describe here the use of the 'novel tank test' as a method to study innate stress responses in zebrafish, and demonstrate how potential anxiolytic drugs can be validated using the assay. The method can easily be coupled with zebrafish lines harboring genetic mutations, or those in which transgenic approaches for manipulating precise neural circuits are used. The assay can also be used in other fish models. Together, the described protocol should facilitate the adoption of this simple assay to other laboratories.

Introduction

Stress responses are altered behavioral and physiological states resulting from potentially harmful or aversive stimuli. Stress responses are conserved throughout the animal kingdom, and are critical for the survival of an organism¹. Decades of research have greatly expanded our knowledge of some of the genetic and neuronal mechanisms underlying stress states. Today, areas of the brain such as the amygdala and the striatum², and genetic factors such as *corticotropin releasing hormone (crh)*, and the *glucocorticoid (gr)* and *mineralocorticoid receptors (mr)* have been studied extensively^{3,4,5,6}. Despite these critical findings, much remains unknown about genetic and neuronal regulation of stress. As such, many stress related disorders suffer from a lack of therapeutics.

Genetically amendable model organisms provide a useful tool in the study of genetic and neuronal control of behavior. Fish models, in particular, are extremely powerful: they are small organisms with short generation times, their use in a laboratory setting is facile, their genomes are easily modified, and, as a vertebrate, they share not only genetic, but also neuroanatomical homology with their mammalian counterparts^{7,8}. Standard assays for measuring stress can be paired with zebrafish lines harboring genetic mutations, or those in which manipulation of precise neuronal subsets is possible, and the effects of single genes or defined neurons can be assessed rapidly and efficiently.

Behaviorally, stress responses can be characterized in fish as periods of hyper-activity or prolonged periods of inactivity (akin to 'freezing')⁹, reduced exploration¹⁰, rapid breathing, reduced food intake¹¹, and a place-preference for the bottom of a tank¹². For example, when placed into an unfamiliar tank, adult zebrafish and other small fish models show an initial preference for the bottom half of the tank, yet, over time, the fish begin exploring top and bottom halves with near-equal frequency¹². Treatment of adults with drugs known to reduce anxiety cause fish to explore immediately the top half^{10,13}. Conversely, drugs that increase anxiety cause fish to show strong preference for the bottom half of the tank^{12,14,15}. Thus, reduced exploration and preference for the bottom half of the tank are simple and reliable indicators of stress.

Like most vertebrates, stress responses in fish are driven by activation of hypothalamic-pituitary-inter-renal axis (HPI; analogous to the hypothalamic-pituitary-adrenal [HPA] axis in mammals)^{14,16}. Hypothalamic neurons expressing the hormone corticotropin-releasing hormone (CRH) signal to the pituitary, which in turn releases adrenocorticotrophic releasing hormone (ACTH). ACTH then signals to the inter-renal gland to produce and secrete cortisol, which has a number of downstream targets¹⁶, one of them being negative feedback of the *crh*-producing hypothalamic neurons^{3,17,18,19}.

Here, we describe a method to assess behavioral measures of innate stress. For the behavior, we detail protocols using the novel tank diving test^{12,14}. We then demonstrate, as an example, that a known anxiolytic drug, buspirone, reduces behavioral measures of stress.

Protocol

The protocol has been approved by the Institutional Animal Care and Use Committee at Florida Atlantic University.

1. Preparation

1. Designate an isolated room for performing behavioral studies, or close off a section of a room so that it is isolated.
NOTE: The room should be undisturbed and have low traffic to avoid disrupting normal behavior of the fish.
2. Move the following materials and equipment into the behavioral room: (i) a camera and lens, (ii) an infrared filter which can be attached to the lens, (iii) a camera stand, (iv) a computer with camera acquisition software, (v) a firm and stable table to perform the assay on, (vi) infrared lights (IR lights; 850 nm or 940 nm), (vii) a white acrylic diffuser, which is longer than the length of the recording tank (viii) 1.8 L trapezoidal plastic assay tank (referred to as the 'novel tank'; the one used here is 12 x 18 inches), and (ix) a bucket of fish system water.
NOTE: For the novel tank, our lab uses commercially available plastic vessels, which are trapezoidal in shape. The dimensions of the tank are roughly 6 in x 9 in (detailed dimensions are provided in **Figure 1A**). The diffuser board we use is slightly larger than the novel tank (12 in x 18 in). Novel tank experiments have been performed with tanks having differing shapes, such as those which are rectangular or those with different trapezoidal dimensions^{20,21}. Typically, fish behavior is similar in all tanks regardless of their dimensions: for all containers, fish initially prefer the bottom half, yet over time begin exploring top half with greater frequency.
3. Attach the infrared filter to the camera lens. The wavelengths of the infrared light strips typically range from 850 nm to 940 nm. The filter is a long pass filter that restricts light of wavelengths less than 720 nm from transmitting through to the camera.
4. Select suitable parameters for the camera acquisition software. For most recordings, set the camera acquisition to a rate of 30 frames-per-second, and recording duration to 10-min.
NOTE: These parameters may differ, depending on the experiment. For example, to study habituation in a novel tank^{22,23}, longer recordings may be required.

2. Setup

NOTE: The steps in this section describe setting up the novel tank assay. A diagram of the end product is given in **Figure 1B**.

1. Place the novel tank in the middle of the table.
2. Position the infrared lights behind the tank and place the white acrylic sheet or diffuser screen in between the tank and LED light source.
 1. Place the diffuser so that it maximally spreads the light coming from the LEDs, and the intensity of the light is enough to illuminate the novel tank. The closer the board is to the light source, the brighter the lights will be, yet the less it will diffuse. By contrast, placing the diffuser board away from the light source will reduce light intensity, but spread the light better.
3. Fill approximately three quarters of the novel tank with fish system water.
NOTE: System water is generated using reverse osmosis of tap water, followed by dosing such that conductivity equals $900 \pm 100 \mu\text{S}$, that pH is neutral (7.2), and that the temperature is $27 \pm 1^\circ\text{C}$.
4. Attach the camera to the camera stand and connect the camera to the computer. Open up the video acquisition software and adjust the camera to face the front of the tank and ensure the entire novel tank can be seen and that there are no obscured areas in the video. Adjust the tank and the infrared lights such that there is sufficient and even illumination throughout the tank when observed through the camera.
NOTE: Before proceeding to experiments, it can often be useful to perform a trial run, in which video of a fish is captured and tracking is performed. This will ensure that the setup is sufficient for experimentation of behavior.

3. Novel tank test setup

1. Prepare a 250 mL beaker pre-filled with fish system water, and at least two holding tanks.
2. On the morning of the test, transfer at least 10 test adult zebrafish to be used for each experimental condition (controls and experimental adults) from fish facility into a holding tank, transfer them to the behavior room, and allow them to acclimate for at least one hour.
NOTE: A power analysis should be performed before experimentation, yet in our hands, an $n = 10$ is usually sufficient to detect statistical significance. Moreover, the holding tank should contain no more than five individuals per liter of water. An acclimation of one hour is sufficient as zebrafish adults have been shown to habituate within 30 minutes of a new tank²². Also, behavioral rhythms are affected by circadian processes, and thus experimental replicates done on different days should be performed within the same hours. We typically perform all experiments between the hours of 11:00 am and 6:00 pm.
3. Label the tanks such that the condition or genotype of the animals is blind to the experimenter.
NOTE: Experiments can easily be blinded to the experimenter by labeling tanks using a letter or number system (i.e., one tank is labeled 'A', another 'B', etc.). A party not involved in the experiments labels the tanks with such a system, and masks the identities from the experimenter until after post-analysis is complete.
4. Using a net, gently place a single adult in the pre-filled beaker from step 3.1. Allow the adult fish to acclimate in the beaker for 10 minutes.
NOTE: Record the sex of the adult, as it might be important post-analysis to look for sex-specific differences.
5. After acclimation in the beaker, introduce the fish into the novel tank (set up in section 1) by gently pouring out the water and adult from the beaker.
6. After introducing the adult into the novel tank, start the camera recording, and move away from the setup to prevent additional distress to the fish.
7. After the recording has finished, remove the individual from the novel tank and place into a new holding tank.
NOTE: A different holding tank from the one in step 3.2 should be used to prevent repeated testing on the same individuals.
8. Repeat steps 3.4 to 3.7 for each adult until all animals have been tested.
NOTE: In addition to blinding conditions or genotypes, randomize trials. Use a random number generator or any tool that allows one to randomize between the trials. This should be done before experimentation so that each trial is determined before the experiments begin.
9. At the end of all tests, return the fish back to the fish facility.

4. Pretreatment with drug

NOTE: The aim of the following steps is to compare the behavior of an individual before and after the use of drugs. This comparison is achieved by first performing a novel tank test as in step 3.4 to 3.6, followed by drug treatment, and then a second novel tank test (**Figure 3A**).

1. Prepare a stock solution of the drug, including positive and negative controls.
NOTE: If the drug has previously been used in the literature, find an appropriate working dose and use this. For example, for buspirone in the representative results, we make a 100x stock solution and use 0.05 mg/mL as the final concentration, as described in the literature^{13,20}. If suggested dose is unknown, a dose response curve should be performed by examining several concentrations. Set up more beakers with serial dilutions of drug. If the drug is not dissolvable in water, use dimethyl sulfoxide (DMSO) as a solvent.
2. Dilute drugs to working concentration in 250 mL beakers with system water. For example, if a 100x solution was made, dilute 1:100 in system water. Set up a beaker with only system water as a control.
NOTE: If DMSO was used as a solvent in step 3.1, use an equal volume of DMSO in the control beaker.
3. With the help of another researcher, mask the identities of the drug and control beakers to ensure that the tester is blind to the treatment conditions until post-analysis.
NOTE: A number or letter system may be used.
4. Perform a novel tank test by following steps 3.1 to 3.6 to obtain a baseline behavioral stress response.
5. After the baseline recording, use a net to immediately remove the adult fish from the novel tank and place it in the beaker dosed with drug or vehicle, as described in step 4.2. Allow the adult to stay in the beaker for 10 min.
NOTE: Ensure that the net does not touch the water in the beakers to prevent cross-contamination of drugs. Ensure proper dosage and administration time depending on the drug used. A 10 min treatment time might not work for all drugs.
6. After treatment, use a net to remove the adult from the beaker in step 4.5 and place it in another beaker filled with fresh system water only. This is the washout period to minimize further dosing during the second novel tank test. Allow the adult to stay in the wash out beaker for an additional 10 min.
NOTE: Separate nets should be used for each drug condition to prevent any unwanted cross treatment with drug. The washout period may be skipped if the experimenter wishes.
7. Perform the novel tank diving test a second time by removing that adult from the beaker in previous step, place it in a new novel tank, and follow steps 3.5 to 3.6.
8. After the second novel tank test, remove the individual into a separate holding tank. Pour away the system water in the second novel tank and fill it with fresh system water for the next test. This step prevents cross-contamination of any drug.
NOTE: Depending on the half-life of the drugs, fresh beakers containing drugs should be made every 3 hours. For buspirone, make fresh solutions every 3 hours. In addition, following the note in step 3.8, the trials should be randomized between controls and drug treatments.
9. At the end of all trials, return individuals back into the fish facility.
NOTE: Depending on type of drug used, the effects of these treatments on individuals can be long lasting. Therefore, do not use these individuals in other experiments.

5. Video analysis

1. After all trials, load video files into tracking software of choice.
NOTE: We typically use commercially available tracking software, yet several freely available software packages can be used. The steps to achieve tracking may differ according to the software package used.
2. Using a still frame from the video, define imaginary boundaries around (i) the entire novel tank arena that is filled with water, and boundaries around (ii) the top third, (iii) middle third, and (iv) bottom third of the tank. Use these to establish time that the fish spent in each portion of the novel tank.
3. Calculate x-y displacement per frame for each arena defined in step 5.2.
4. Define top, middle, and bottom areas of the tank. Each region should be similar in size. A brief method for determining these regions is to calculate the length of the tank in the y-direction, and divide this by 3.
NOTE: Variations to the general protocol do exist. For example, some labs use halves instead of thirds¹⁴.
5. Determine the time spent in each arena, the distance, and the velocity.
NOTE: Most tracking packages will automatically calculate these for the user. However, if the tracking software does not, these can be calculated easily from the x-y displacement values. For example, distance can be determined using the formula:

$$\text{distance} = \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2}$$

and velocity can be determined following the formula:

$$\text{velocity} = \frac{\text{distance}}{t}$$

6. Repeat steps 5.2 to 5.4 to acquire tracks and measurements for all trials.
NOTE: Variations to this general protocol

6. Testing for normality

1. Perform statistics before proceeding to calculate statistical differences. Check to see if the data is normally distributed using a Shapiro-Wilk test.
2. If the null hypothesis that the data is normally distributed is rejected (i.e., the data does not follow a Gaussian distribution), perform all tests using non-parametric tests. Conversely, if the data is found to follow a normal distribution, proceed to use parametric tests.
NOTE: We use commercially available software to perform statistics; however, the R programming language can also be used. A Shapiro-Wilk analysis can be performed using R's shapiro.test function.

Representative Results

Examining stress in zebrafish

To examine stress behavior over time in wild-type zebrafish, we tested adult fish from the AB strain²⁴ in the novel tank test. AB adults were subjected to the protocol as described above. Briefly, fish were given a 1-h acclimation period in a tank in the behavior room. An individual was placed in a beaker for 10-min, and then placed gently in an unfamiliar tank (novel tank) filled with fresh system water. Locomotor activity was recorded for 10-min, and tracking was performed offline using commercially available software. Comparison of locomotor activity between the first and last minute showed dramatic differences (**Figure 2A,B**). When first introduced into the tank, fish spent the majority of the time in the bottom (**Figure 2B**), yet over time, adults had a gradual increase in the amount of time spent in the top (**Figure 2B,C**). Total duration spent in the top in the first minute compared to the last minute revealed significant differences (6.29 ± 8.21 s vs. 23.23 ± 9.02 s; paired t- test, $p < 0.05$) (**Figure 2C**). By contrast, total distance traveled between first and last minute revealed no significant differences (440.4 ± 110.3 cm vs. 405.5 ± 49.71 cm; paired t- test, $p = 0.375$) (**Figure 2D**). Because innate preference was different between the first and last minute, and not distance traveled, we believe the change in behavior represents a stress response, and not merely a change in locomotor activity. These results demonstrate that zebrafish exhibit an easily measurable innate stress response. This approach also establishes a foundation to compare stress differences between different groups of animals, and assess the differences in stress between them.

Effects of anxiolytic drugs on stress behavior in zebrafish

Zebrafish are a powerful system for screening drugs, since application of drug can be applied in non-invasive ways by simply adding to the water^{25,26,27}. To validate that bottom dwelling in zebrafish represents an innate stress response, we compared behavior in groups of adult zebrafish tested before and after exposure to an anxiolytic drug; as a control, we handled a separate of adults similarly, yet applied only vehicle (system water) instead of drug. We used the 5HT_{1A} receptor agonist buspirone, which is not a controlled substance and is prescribed to human patients suffering from generalized anxiety disorder²⁸. Buspirone has been validated to cause reduction in behavioral stress responses in various fish and mammalian models^{10,13,21,29,30,31,32,33,34}. As described in the protocol, zebrafish were first recorded in the novel tank test, then retrieved and placed in a beaker of drug or vehicle for 10 min. Fish were then given a 'wash-out' period, where they were placed in a new beaker for 10 min, and then re-recorded in the novel tank test (**Figure 3A**).

Analysis of locomotor paths revealed little difference before and after treatment for groups of adults exposed to vehicle alone (**Figure 3B**). By contrast, adults exposed to buspirone spent a large amount of time in the top compared to the locomotor paths of the same fish before drug exposure (**Figure 3B,C**). Quantification of duration of time spent in the top revealed no significant differences between pre- and post-treatment in control animals (183.9 ± 90.46 s before vs. 113.8 ± 81.88 s after; one-way ANOVA followed by Sidak's multiple comparisons test, $p = 0.4254$), yet animals exposed to buspirone spent significantly more time in the top relative to pre-treatment, and control individuals after treatment (Buspirone: 201.4 ± 49.95 s before vs. 552.2 ± 42.97 s after; one-way ANOVA followed by Sidak's multiple comparisons test, $p < 0.0001$; Control vs. Buspirone post-treatment: $p < 0.0001$.) (**Figure 3C**). To examine whether the differences were due to less locomotion in general, we measured total distance traveled. These data revealed no significant differences for any of the groups (4134 ± 601.9 cm before vs. 3471 ± 766 cm after for control; Kruskal-Wallis test, $p > 0.05$; 3904 ± 301.3 cm before vs. 3644 ± 566.3 cm after for buspirone; Kruskal-Wallis test, $p > 0.05$) (**Figure 3D**). Taken together, these data demonstrate that bottom dwelling in adult zebrafish is a measure of innate stress, and establish a foundation for further pharmacological experiments in adult zebrafish.

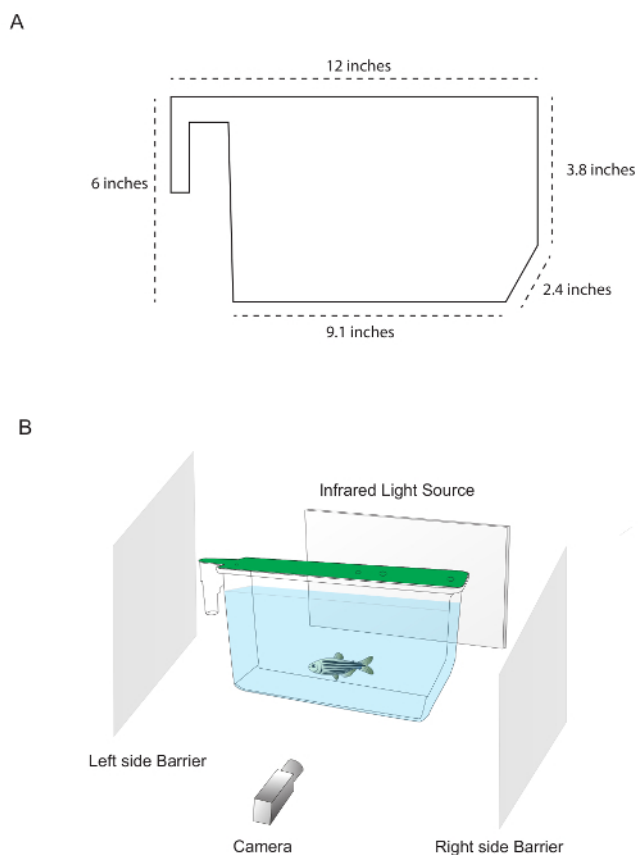


Figure 1. Diagram of the novel tank setup. (A) Dimensions of the 1.8 L trapezoidal novel tank as seen from the recording side of the tank. (B) Diagram of the setup including positions of the infrared lights, camera, and barriers used to minimize human interference. [Please click here to view a larger version of this figure.](#)

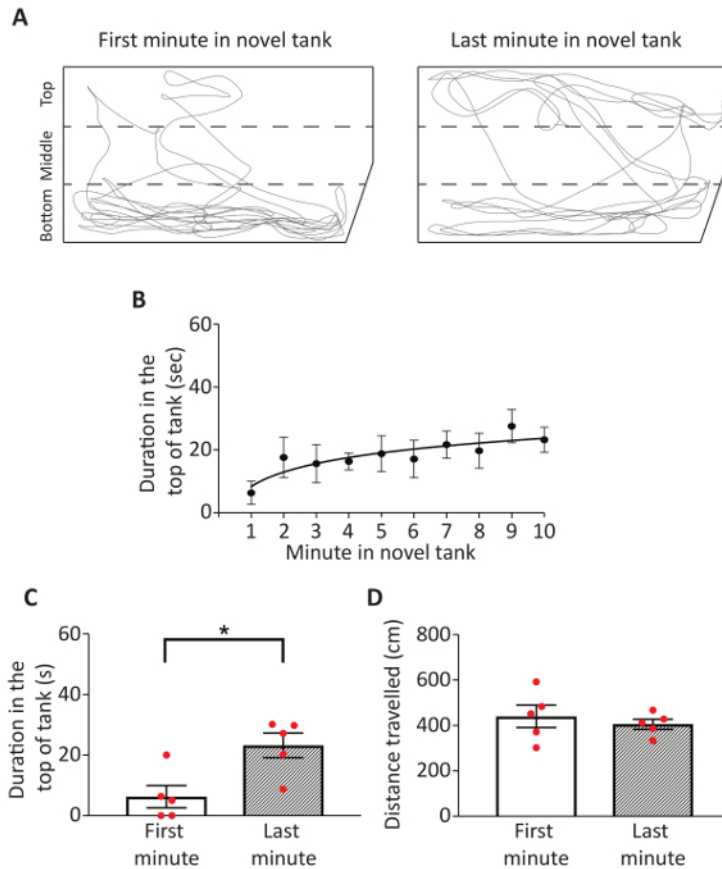


Figure 2. Examining innate stress responses in wild-type zebrafish. (A) Representative swim paths of an individual adult in the novel tank test in the first minute (left) and last minute (right) of a 10-min recording. Imaginary lines defining the top, middle, and bottom zones of the tank are indicated with dotted lines. (B) Quantification of total time spent in the top zone for each minute of the 10-min recording. (C & D) Comparisons of total duration spent in the top zone (C) and total distance traveled (D) in the first and last minute of all trials. Paired t - tests were used for analysis since the data passed the Shapiro-Wilk test for normality. $n = 5$; $^* p = 0.028$. Error bars indicate standard error of the mean. [Please click here to view a larger version of this figure.](#)

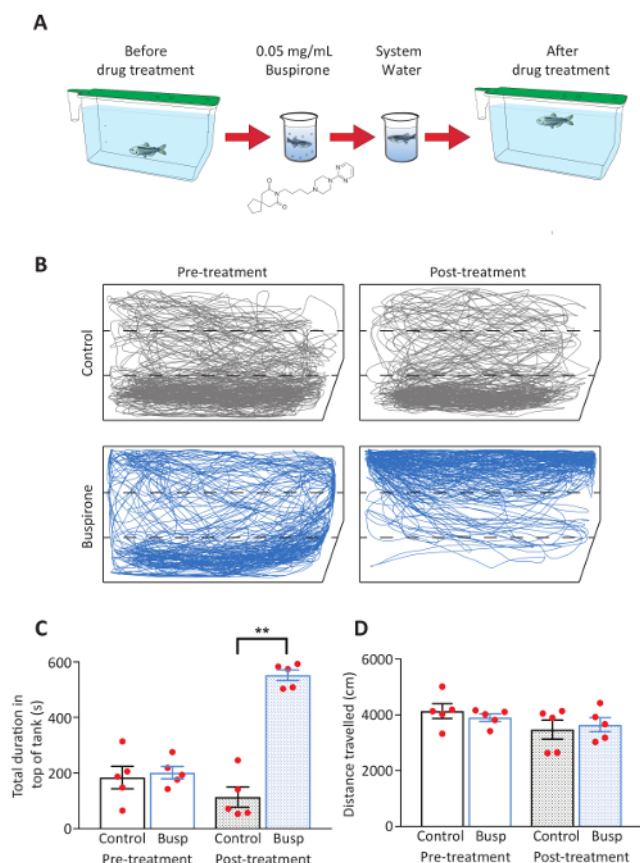


Figure 3. Examining the effects of anxiolytic drugs on stress behavior. (A) Schematic of experimental flow. (B) Representative swim paths pre- and post- treatment of an individual from a control individual treated with system water only, and another individual treated with buspirone in the novel tank test. Dotted lines define separation of top, middle, and bottom zones of the tank. Grey tracks represent the control individual, and blue tracks represent the buspirone-treated individual. (C & D) Comparisons of total duration spent in the top zone (C) and total distance travelled (D) between control (Ctrl) and buspirone-treated (Busp) trials. A test for normality using the Shapiro-Wilk test was first done. Where the test for normality failed, Kruskal-Wallis test followed by Dunn's multiple comparisons test was used (C); and if the data passed normality, one-way ANOVA followed by Sidak's multiple comparisons test was used for analysis (D). $n = 5$ for each condition; ***, $p = 0.001$. Error bars indicate standard error of the mean. [Please click here to view a larger version of this figure.](#)

Discussion

Zebrafish exhibit a robust stress response in a novel tank

Here, we describe a simple behavioral approach for examining stress responses in adult zebrafish, and validate the approach as a simple measure of stress using pharmacology.

The novel tank test is a widely used test for examining innate stress in zebrafish and other species of fish^{12,14,21,35,36}, and zebrafish has been shown to be a powerful model to examine the pharmacological effects of anxiety-related drugs. Similar to humans, these studies have demonstrated that drugs such as buspirone, nicotine, fluoxetine, and scopolamine have anxiolytic effects in zebrafish^{12,13,14,37}. Moreover, drugs such as scopolamine that are typically not used to treat anxiety in humans can have additional anxiolytic effects³⁷. Drug screens demonstrating these anxiolytic effects in zebrafish can facilitate the study of side effects and their pharmacological mechanisms. Further, the zebrafish has a comparable stress response pathway to humans, hence pairing the assay with quantification of the release of cortisol after a stressor or drug treatment can be used to validate the behavioral responses¹⁴. Finally, we wish to point out that this assay is not specific to zebrafish, and has also been extended to other fish species such as the Mexican blind cavefish, *Astyanax mexicanus*²¹. It is likely that the assay can be extended to cichlids³⁸, mosquitofish³⁹, killifish⁴⁰, and other piscine systems.

An important advantage of the novel tank test is its ecological relevance; as the assay measures innate preference for the bottom half of the tank, the response is likely one that would occur in the wild. In addition to the novel tank test, other behavioral assays that have been used in other model organisms can be used to further validate the behavioral stress response, such as an open field test or a light/dark assay^{41,42}. These assays are based on the tendency for an animal to follow the sides of the arena (thigmotaxis), and preference for exploration in the dark (scototaxis) after being exposed to a stressful cue^{42,43}. In addition, electric shock has been used to measure either innate or conditioned fear responses^{9,44,45}, though the ecological relevance of this approach is unclear.

When one is considering an assay for his/her study, it is important to take into account innate bias within strains or species. In addition to maintaining and reducing environmental fluctuations in behavioral assays, keeping the genetic background of the test adults consistent will be

vital since research has shown variability within and between individuals of the same genotype^{41,46}. A comprehensive review of the advantages, disadvantages, validity of each common behavioral assay used to study anxiety, and also variations in behavior within common wildtype lines can be found elsewhere⁴¹.

Zebrafish as a model for examining stress

Zebrafish are becoming a popular model for examining genetic and neuronal pathways that modulate precise behaviors^{47,48}, and recently developed brain atlases allow for mapping neurons regulating behavior with precision^{49,50,51,52,53}. The approach we describe here to measuring innate anxiety is able to harness powerful genetic and neural circuits tools in zebrafish. Two foreseeable approaches rely on the large collection of mutant lines and transgenic driver lines. Mutant lines, for example, will facilitate investigators to examine the role that precise genes have in modulating stress. Additionally, transgenic Gal4/UAS and QF/QUAS system have been extensively applied to zebrafish^{54,55}, and when crossed to UAS or QUAS effector lines, the function of precise neuronal circuits can be manipulated and behavior assessed. These approaches provide a complement to genetic mutant lines, and permit investigation of how precise neural circuits contribute to stress.

Novel techniques for examining neural activity can be fully integrated with this assay. Quantification of *c-fos* mRNA or protein are widely used to examine neuronal activity⁵⁶. This gene is an immediate early gene, whose transcription is activated by neuronal activity. Newer approaches based on similar methodology have been developed. For example, the extracellular-signal-regulated kinase (ERK) was recently developed for examining neuronal activity in zebrafish⁵⁰. The ERK protein exists in nearly all cells of the central nervous system. Upon neuronal activation, the ERK peptide become phosphorylated. Moreover, reliable antibodies for both un-phosphorylated ERK (total ERK, tERK) and phosphorylated ERK (pERK) have been developed and work well in zebrafish. Thus, by co-labeling with antibodies specific to tERK and pERK, neuronal activity can be reliably measured. Using this approach, adults that significantly display more bottom dwelling in the novel tank test can be removed after recording, stained for either *c-fos* or tERK/pERK, and resulting brain sections imaged.

Taken together, these approaches should facilitate a facile approach for dissecting the genetic and neuronal mechanisms underlying stress in zebrafish. Moreover, due to the high conservation of genetic and neuronal pathways in zebrafish and mammals, we expect these methods to reveal conserved mechanisms underlying stress behavior.

Disclosures

The authors declare that they have no competing or financial interests.

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