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Behavioral approaches to studying innate stress in zebrafish

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November 12th, 2018

Drs. Alisha DSouza and Aaron Berard
Journal of Visualized Experiments
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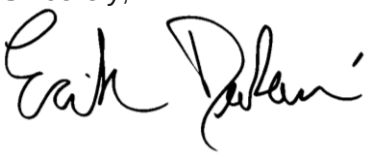
Dear Drs. DSouza and Berard:
Please find a revised manuscript titled "**Behavioral approaches to studying innate stress in zebrafish**" by Chin et al., which we are resubmitting for your consideration to the *Journal of Visualized Experiments*.

The protocol provides steps for measuring innate stress in adult zebrafish using a widely used experimental approach, the novel tank test. The previous submission of this manuscript was reviewed by five reviewers, most of which were favorable. We have now addressed all concerns raised by reviewer's and the editorial staff of *JoVE*. Along with the revised manuscript, we are including a detailed response to each reviewer and editorial staff's concerns. We hope this addressed all of the points raised previously.

We look forward to hearing from you. If you have any questions, please feel free to contact me.

Sincerely,



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TITLE:

Behavioral Approaches to Studying Innate Stress in Zebrafish

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KEYWORDS:

zebrafish, anxiety, stress, novel tank, behavior, pharmacology, buspirone

SUMMARY:

This manuscript describes a simple method to measure stress behaviorally in adult zebrafish. The approach takes advantage of the innate tendency that zebrafish prefer the bottom half of a tank when in a stressful state. We also describe methods for coupling the assay with pharmacology.

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 organism[1]. 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 striatum[2], and genetic factors such as *corticotropin releasing hormone* (*crh*), and the *glucocorticoid* (*gr*) and *mineralocorticoid receptors* (*mr*) have been studied extensively[3–6]. 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 exploration[10], rapid breathing, reduced food intake[11], and a place-preference for the bottom of a tank[12]. 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 frequency[12]. 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 adrenocorticotropic releasing hormone (ACTH). ACTH then signals to the inter-renal gland to produce and secrete cortisol, which has a number of downstream targets[16], one of them being negative feedback of the *crh*-producing hypothalamic neurons[3,17–19].

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

1.1. 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.

1.2. 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.

1.3. 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.

1.4. 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**.

2.1. Place the novel tank in the middle of the table.

2.2. Position the infrared lights behind the tank and place the white acrylic sheet or diffuser screen in between the tank and LED light source.

2.2.1. 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.

2.3. 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 ± 100 µS, that pH is neutral (7.2), and that the temperature is 27 ± 1 °C.

2.4. 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**

3.1. Prepare a 250 mL beaker pre-filled with fish system water, and at least two holding tanks.

3.2. 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 tank[22]. 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.

3.3. 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.

3.4. 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.

3.5. 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.

3.6. 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.

3.7. 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.

3.8. 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.

3.9. 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**).

4.1. 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.

4.2. 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.

4.3. 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.

4.4. Perform a novel tank test by following steps 3.1 to 3.6 to obtain a baseline behavioral stress response.

4.5. 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.

4.6. 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.

4.7. 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.

4.8. 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.

4.9. 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

5.1. 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.

5.2. 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.

5.3. Calculate x-y displacement per frame for each arena defined in step 5.2.

5.4. 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 thirds [14].

5.5. 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:

$$\text{distance} = \sqrt{(□_□ - □_{□-1})^2 + (□_□ - □_{□-1})^2}$$

and velocity can be determined following the formula:

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

5.6. 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

6.1. Perform statistics before proceeding to calculate statistical differences. Check to see if the data is normally distributed using a Shapiro–Wilk test.

6.2. 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 strain[24] 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[25–27]. 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 disorder[28]. Buspirone has been validated to cause reduction in behavioral stress responses in various fish and mammalian models[10,13,21,29–34] . 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 $\pm$ 90.46 s before vs. 113.8 $\pm$ 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 $\pm$ 49.95 s before vs. 552.2 $\pm$ 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 $\pm$ 601.9 cm before vs. 3471 $\pm$ 766 cm after for control; Kruskal-Wallis test, $p > 0.05$; 3904 $\pm$ 301.3 cm before vs. 3644 $\pm$ 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 legends

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.

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 spend 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.

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 traveled (**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.

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[12–14,37]. Moreover, drugs such as scopolamine that are typically not used to treat anxiety in humans can have additional anxiolytic effects[37]. 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 responses[14]. 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 cichlids[38], mosquitofish[39], killifish[40], 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 elsewhere[41].

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[49–53]. 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 activity[56]. 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 zebrafish[50]. 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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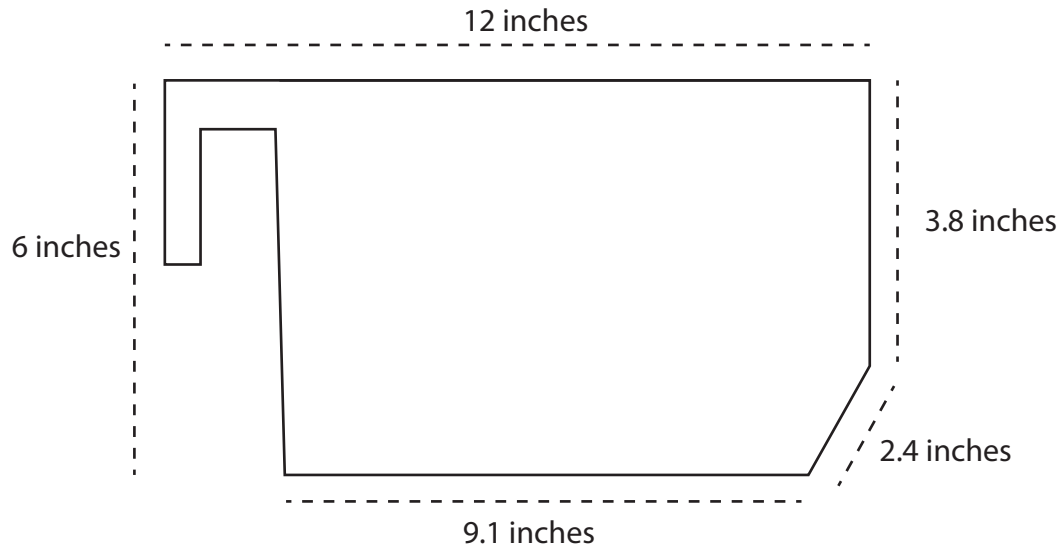
Figure 1

A



B

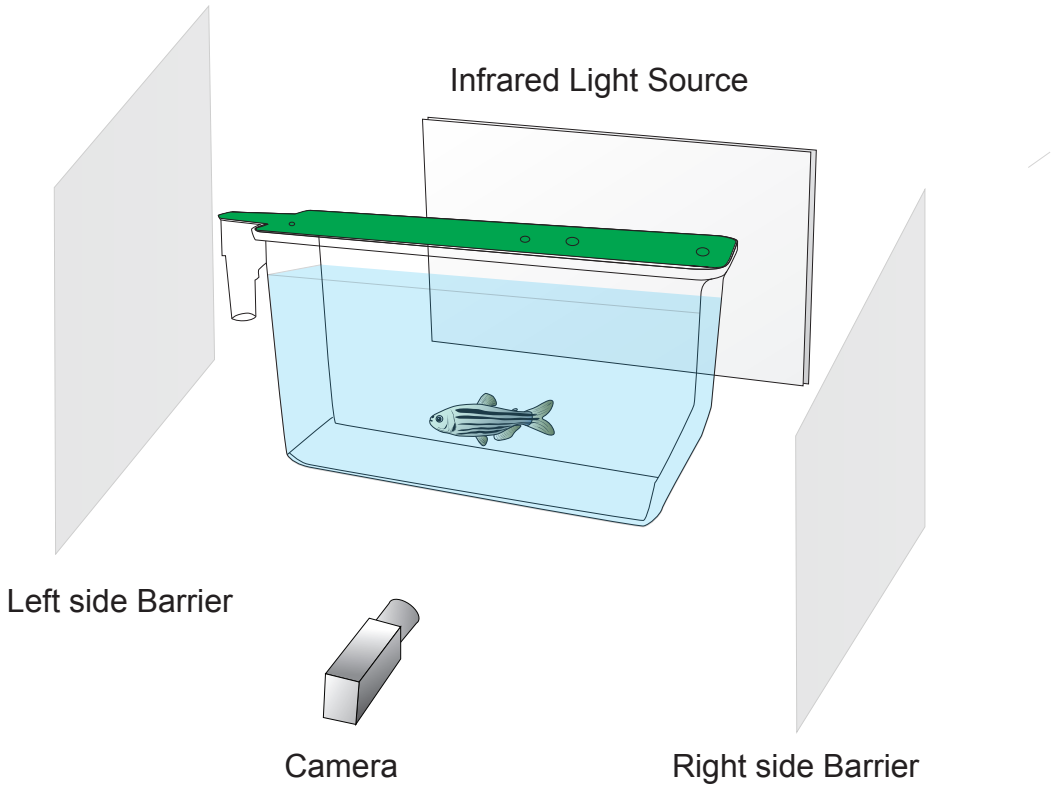


Figure 2

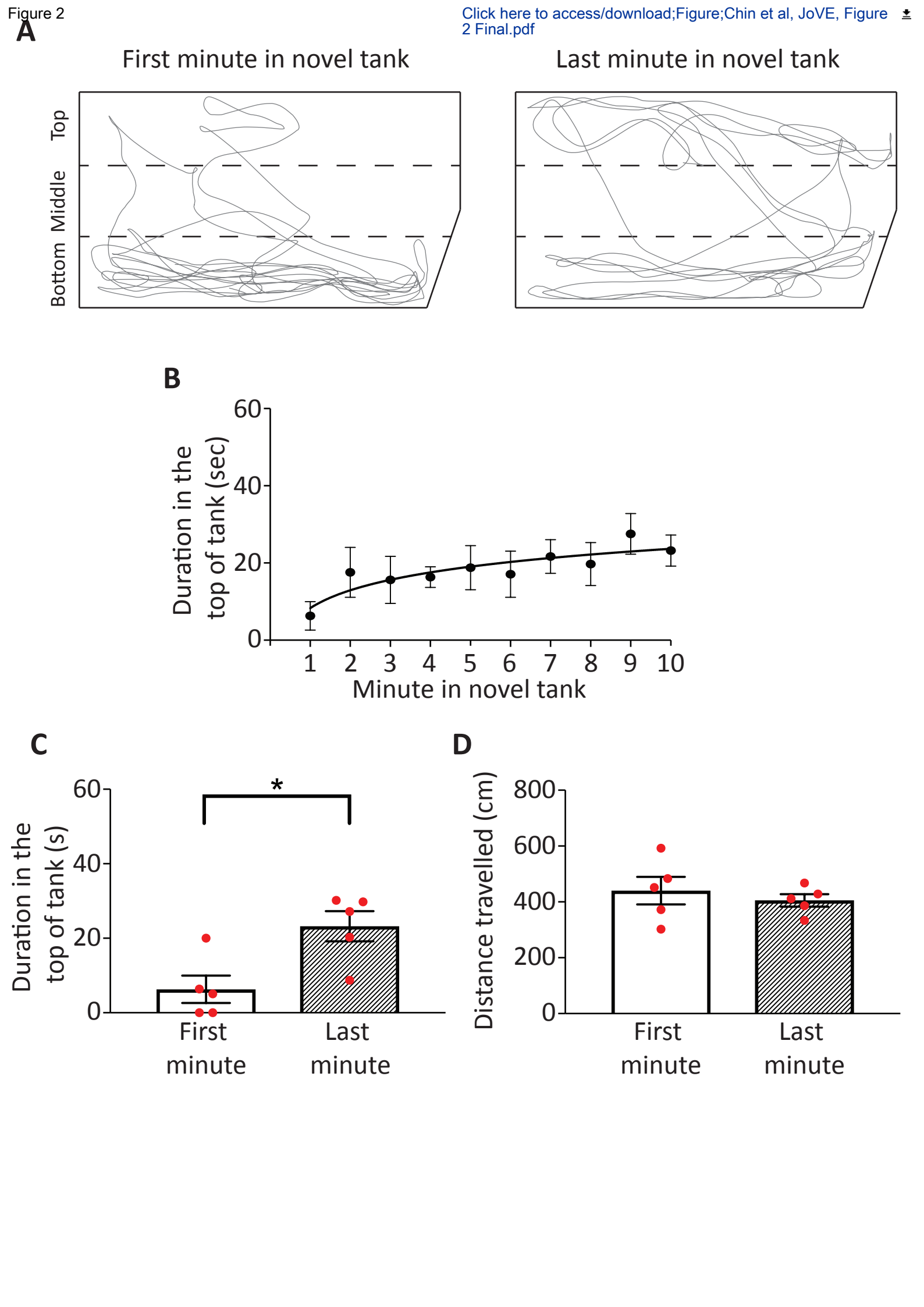


Figure 3

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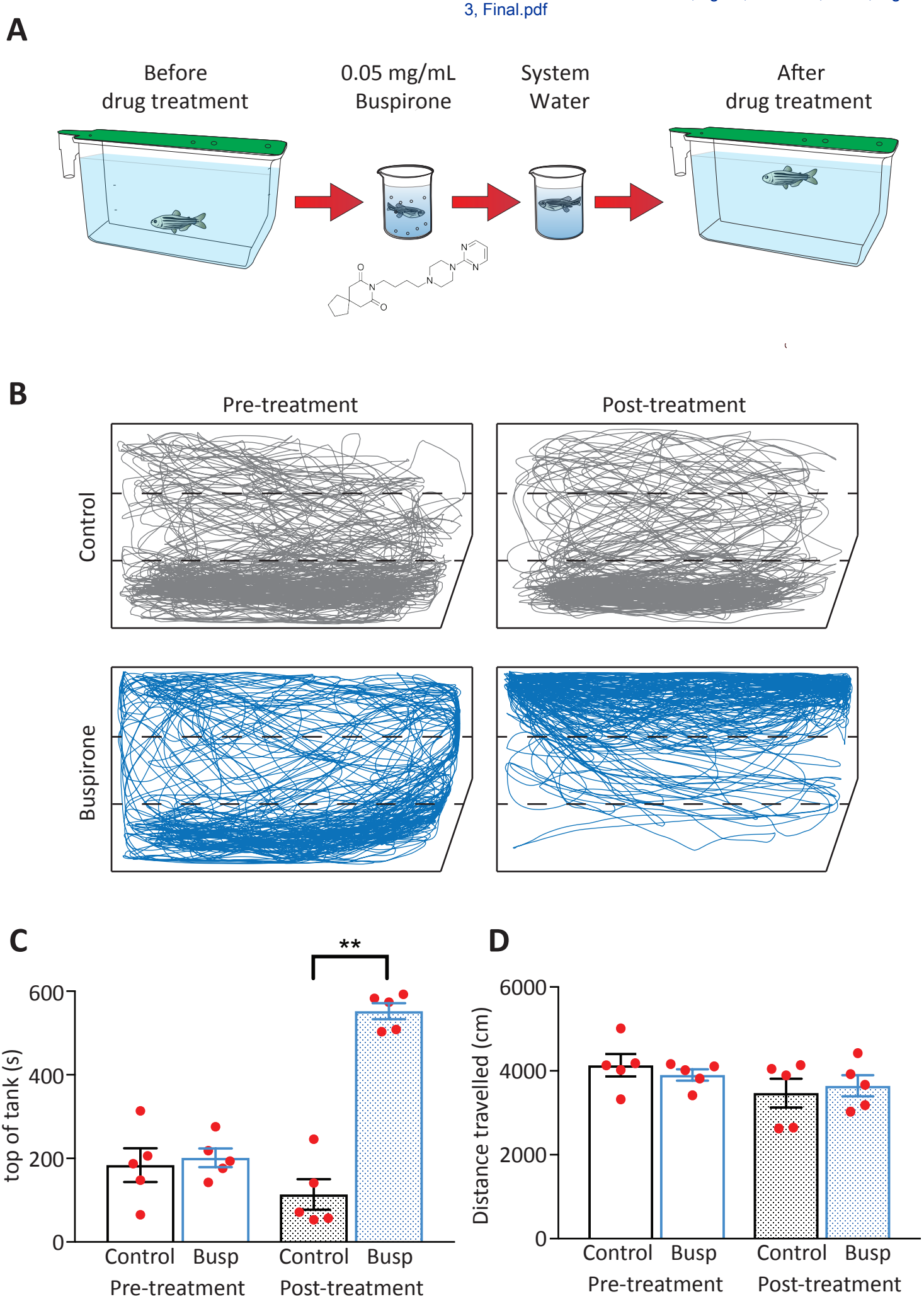

Table of Materials

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Reagent / Resource	Source
Camera	
Infrared filter	Edmund Optics
Video Acquisition Program	
Infrared LED lights	
Assay tank	Aquaneering
Stand and clamp, or standard tripod for camera	
250mL beaker	
Tracking software	
Buspirone chloride	Sigma-Aldrich
Randomized trial generator	

Identifier	Comments
	We use Point Grey Grasshopper3 USB camera with lens from Edmund Optics. Use programs such as Virtualdub or FlyCapture because the acquisition framerate can be set.
Part number ZT180	Size: M3 1.8 liter
	We use Ethovision XT 13 from Noldus Information Technology
B7148	
	We use the RANDBETWEEN function in Microsoft Excel

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We thank all authors, as well as the editorial team at JoVE for the time and care with which this manuscript was reviewed. We have made substantial revisions to the manuscript, and believe that we have addressed most, if not all, of the reviewer's concerns. The major revisions to the manuscript were as follows:

1. Several reviewers noted that the protocol should have more detail, and be easier to follow. We have expanded greatly the protocol section.
2. The editorial board notes that we were not allowed to specify Ethovision, the name of the tracking software. This is unfortunate, because without directing the reader to the name of the software, the steps (many of which are embedded in the Ethovison platform) do not make sense. We have instead given a general approach for tracking, and stated that we use proprietary software, but freely available versions do exist. We hope this change makes it easier for labs that do not have access to Ethovision to perform these experiments.
3. Several reviewers requested more information on the tank, and the setup. Also, we had previously stated that our tanks were purchased from Aquaneering, yet we had to take this out also because Aquaneering is proprietary. To make clear the set-up and the tanks, we have added a new figure with dimensions of the tank, and a schematic of set-up. We hope this helps.
4. A few reviewers requested details on the statistics we used, including tests for normality and power. We have added a statistics section at the end of the protocol, explaining that the experimenter should test their data for normality, and, depending on the result, which sets of statistical measures should be used.
5. We have expanded the introduction and discussion to place this assay in the wider context of the literature, of other available assays for monitoring stress, and with ecological relevance of the approach detailed here.

Whereas the 4 points above represent major changes, detailed responses to each reviewer's concerns can be found below.

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Changes to be made by the author(s) regarding the manuscript:
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We have read through the manuscript and ensured no typographical or grammatical errors.

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We have removed all cases of the name Aquaneering, Ethovision and ViewPoint.

5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All pronouns have been removed from the protocols section.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have revised the protocol section to only include action steps.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We have revised the protocol to add significantly more detail. Moreover, we have now added a statistics section detailing how to run post-behavior analysis.

8. 1.2: Please specify recommended width of the container.

We have now added the dimensions of the tank we use in the manuscript, and have also included a diagram of the tank in figure 1A. The drawing in Fig. 1A is annotated with approximate lengths.

9. 1.3 and 1.4: What is the type of infrared lights? What is the size of a white board? Where is the camera located? Please add such details. A schematic showing the setup may be helpful.

We have specified that the lights are light emitting diodes (LED), and have noted the wavelength range. We have also included details for the size of the diffuser board, and the position of the camera. We hope these additional, together with the diagram of the setup in figure 1, will address these concerns.

10. 2.1: How many test subjects are transferred? What tank is used?

We have now made clear that only one test subject at a time is transferred. We have also specified that approximately 10 adult fish per experimental condition should be used, in accordance with what is typically done in the literature.

11. 2.3: Does novel tank setup refer to the tank prepared in step 1? Please specify.

We have added the phrase "…1.8 L trapezoidal plastic assay tank (referred to as the 'novel tank')…" in step 1.2 to make clear which tank we are referring to.

12. 3.2: Please specify the type and concentration of drug to be used in the protocol. Please specify the vehicle solvent used.

We added the sentence, "If the drug is not dissolvable in water, use dimethyl sulphoxide (DMSO) as a solvent" in section 4.1.

13. 3.5 and 3.6: How about controls? Are they going through the same procedure? Are controls and drug-treated fish put in the same novel tank?

We have added the sentence, "Repeat steps 2.4 to 2.7 for each adult until all animals in both experiential and control conditions have been tested" to the end of section 3 to make clear fish are tested individually, but both control and experimental fish undergo the same protocol.

14. 4.2, 4.5, etc.: Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Done

15. Please include single-line spaces between all paragraphs, headings, steps, etc.

Done

16. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done. We optioned to highlight the drug treatment, since it incorporates parts from section on novel tank diving alone (i.e., drug treatment is just two rounds of novel tank with drug treatment in between the two trials.

17. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Done

18. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are

given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Done

19. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Done

20. Figure 1B and 1C, Figure 2C: Please change "sec" to "s" for time unit.

Done

21. Figure 2C and 2D: Please define error bars in the figure legend.

Figure 2 is now Figure 3. We have included the sentence," Error bars represent s.e.m.".

22. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

We have reformatted the in-line references to follow the requested format.

23. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We have reformatted the references to follow the format list above.

24. References: Please do not abbreviate journal titles.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Following below is the comment about the manuscript entitled "Behavioral approaches to studying innate stress in zebrafish". The authors present an adequate description of the experimental procedures of a protocol widely used in the literature. This article has significant value in this research field, since zebrafish is becoming a popular and useful animal model for behavioral neuroscience studies. Furthermore, an adequate compilation of data is always helpful and allows to have summarized the results accessible in the current literature. The manuscript is well written and may be recommended for publication in this journal.

We appreciate this reviewer's favorable assessment of our work, and we thank them for the thoughtful suggestions for improving the protocol. We have addressed all comments below, and feel that the article is stronger now.

Major Concerns:
For revision, the following changes are recommended:
1) The effects of buspirone in zebrafish is well known. I suggest adding these references to corroborate with the results observed in the manuscript.

Thank you for this suggestion. As the reviewer notes, we and others have used buspirone. These references have now been added, along with a brief statement acknowledging the long-standing use of buspirone in zebrafish research.

2) The protocol proposed by the authors is not usual. Normally, the zebrafish are exposed in beaker containing the treatment and subsequently undergo the behavioral test. Why do authors propose this new methodology? Is there an advantage?

We thank the author for bringing this to our attention. In the past, we have performed drug treatment followed by direct assessment of novel tank behavior, as is typically done in zebrafish research. We have begun, however, to perform a 'wash-out' period to ensure that only trace amounts of the drug is transferred into the novel tank with the fish. This step may not be necessary, and we now (i) give rationale for the additional step and (ii) acknowledge that the step may be skipped if the experimenter wishes. These details have ben provided to the reader in the note following step 4.6.

3) I appreciate the observation in the item 2.2. But I suggest adding the randomization and blinding procedures. Masca et al. (2015) and Gerlai (2018), examining the problems of reproducibility in biomedical research, state that one of the main reasons for irreproducible research is the lack of blinding/masking. The author did not describe that randomization was performed to allocate the animals to the treatment groups (control x buspirone). What was the method used for random allocation in the treatment groups? Were experimenters blind to treatment? Were data analysts blind to treatment? These questions need to be addressed and clearly stated in the methods section.

Thank you for bringing this to our attention. We overlooked these details. Experiments were run blind to drug condition (i.e., the experimenter did not know whether they were administering placebo or drug solution), and were randomized. These steps are now included in the revised protocol in steps 3.3 and 4.3.

4) I suggest that the authors review the ARRIVE guidelines (Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010 Jun 29;8(6):e1000412) to improve the description of the methodology.

We thank the reviewer for bringing these guidelines to our attention, as we had previously not been aware. These guidelines have been helpful to us in re-writing the manuscript to address the reviewers concerns.

5) The statistical section is lacking in the manuscript. Did the author verify the distribution of the data before choosing the statistical test? The authors should improve the description of the results. Statistical data must be provided (F, degrees of freedom, p values). Moreover, the number of animals in each test is lacking.

Thank you for this point. We have added a section to the protocols on statistical methods to use, and state that tests should be performed for normality. In the case that data are not Gaussian, non-parametric statistics should be used. A Gaussian distribution of the data from both Fig. 2 and Fig. 3 (previously Fig 1 and 2) was confirmed using a Shapiro Test, and thus parametric paired t-tests and analysis of variance (ANOVA) were used. We have also added a statistics section, in which we describe testing for normal distribution. In this section, we specifically state the tests use, and, depending on the results, which statistical measure we suggest. In cases were data are not normally distributed, we suggest a Wilcoxon sign rank and a Kruskal-Wallis non-parametric ANOVA.

Reviewer #2:

Manuscript Summary:
This manuscript provides a method to measure stress behaviorally in adult zebrafish. In innate state, zebrafish prefer the bottom half of a tank in a stressful state. Novel tank test is a wildly used method to study the stress/anxiety in zebrafish. This study described the use of the novel tank test as a method to study innate stress, and provided a detailed procedure for stress-related research.

We thank this reviewer for their comments and suggestions on how we may improve the manuscript. We have addressed all reviewer concerns.

Major Concerns:
After reading this paper, I think the following should be addressed:

1. In line 83, "Place a board to cover the sides of the to minimize extraneous moving stimuli", the expression is not clear.

Thank you for this. We meant to say that we wish to visually restrict the fish so that it cannot respond to movements in the room, such as movements by the experimenter or other factors. We have tried to clarify this point. We have also not added a cartoon diagram of the set-up.

2. In line 90, what is the size of 1.8 L plastic tank used here, the length, width, depth? If the plastic tank have short width are preferred to reduce movement, how to certain the tank width you use is adequate.

We have added the dimensions of the tank used in the manuscript and have also added an annotated diagram of the tank on Fig 1.

3. In order to conduct novel tank test, how to choose a suitable tank size?

We have tried a number of tanks, including 1 L trapezoidal tanks, 1.8 L trapezoidal tanks (as the one described in the protocol), and rectangular tanks. In our hands, these options work, and thus any tank roughly 1-2 L in volume will suffice. We have added these details in the manuscript in the note of section 1.2.

4. Is there any influence about the initial location where the zebrafish was put into the tank on the results? Is there any evidence?

In our hands, there is no difference. Regardless of where the adult initially is, they reposition themselves to the bottom of the tank immediately.

5. The manuscript was not well written. The author need to carefully check the article.

We have proof-read the manuscript and fixed typographical and grammatical errors.

Reviewer #3:

Manuscript Summary:
The manuscript perfectly describes the novel tank test. Despite this test is widely used in the zebrafish research, at least at my knowledge, no detailed methodological videos are published. Since the editorial politics of JoVE clearly states that "...publish expanded descriptions of techniques that have previously appeared in results-based journals", my reviewer report is for acceptance of this manuscript.

We thank the reviewer for their time in providing feedback of the manuscript. We have added a section of the ecological relevance of the assay. This is an important advantage of the novel tank test, since the behavior is an innate one. We have added these details, and believe it helps the manuscript.

Minor Concerns:
As a suggestion, in the discussion, author can discuss some points related to ecology approach of these innate behavior.

A short comment on the ecological context has now been added to the discussion. We have compared the ecological relevance of this assay with others that we have used, such as electric shock, and made the point that since this is an innate behavior, it is likely more similar to what behavior would be in the wild.

Reviewer #4:

Manuscript Summary:
The manuscript describes a simple method to measure stress behaviorally in adult zebrafish.

Assessment of the stress was coupled with pharmalogical drug, buspirone.

Major Concerns:
However, the current stage of the manuscript is not suitable for publication. The authors need to improve the manuscript so that the behavioral protocol for measurement of stress in adult zebrafish is reliable. The following are some of the suggestions for improvement:

1) Introduction was not really relevant to the protocol. The introduction can be improved if the authors describe the importance of measurement of stress behavior for different field. Therefore development for standardize and reliable behavior protocol is important.

> We believe that the sentence "the importance of measurement of stress behavior for different field" means that we should explain to an unfamiliar reader why assessing stress in fish models is important. If so, we have addressed this by including the sentence, "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." We believe this provide a reader unfamiliar with stress research sufficient rationale for examining stress in model systems.
>
> We also acknowledge that the description of the HPI axis in zebrafish is not directly relevant to the protocol in this manuscript, but we believe highlighting the conserved physiology between fish and mammals is important in validating the zebrafish as an animal model. We vacillated on whether to remove it, but in the end, we've decided to keep it. We hope this is acceptable and that this addressed this reviewer's concerns.

2) Method was not clearly stated. There are several questions should be addressed by the authors such as how the stress was induced in the zebrafish, what is the minimum and maximum number of zebrafish can be practically use to achieve statistical power. Measurement of the stress in the zebrafish was conducted in plastic tank, however, Cachat and Levin used trapezoid for the measurement of anxiety-like behavior. What is the author's justification of changing the type of the tanks? Since you are using a plastic tank, the behavioral test should be open field test which only allow you assess the locomotors activity. For drugs treatment, you should have both positive and negative control for further validation of stress response toward buspirone treatment.

> - We now have added the sentence, "A power analysis should be performed before experimentation, yet in our hands, an n=10 is usually sufficient to detect statistical significance."
> - The earlier manuscript depicted cubed tanks, though in fact the tanks we use are trapezoid in shape. The error in our drawing has been corrected, and we thank this reviewer for pointing this out.
> - We are not sure what this reviewer means by, "Since you are using a plastic tank, the behavioral test should be open field test which only allow you assess the locomotors activity." It seems this reviewer is suggesting that the material used to generate the tank determines whether the test is an open field test or a novel tank. In our set-up, we record locomotor activity in the y-z direction (i.e., we examine depth). By contrast, open

field tests typically examine x-y displacement. We therefore respectfully disagree that because our tanks are plastic, this constitutes an open field test.

3) Representative results: You should clearly state the parameters that can be measured using your protocol to assess stress.

We thank the reviewer for this point. We have added the following sentence to the representative results: "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."

4) Discussion is not relevant as it does not thoroughly discussing the protocols in terms of troubleshooting, limitations, precautions etc.

We have added these details to the discussion.

Minor Concerns:
In general, the manuscript needs to be proofread by native speaker.

We have proof read the manuscript to correct for the very few grammatical errors in the initial version.

Reviewer #5:

Manuscript Summary:
This methodology paper describes a protocol to behaviorally and pharmacologically assess stress related behaviors in zebrafish using the common Novel Tank Diving Test. It describes the behavioral, pharmacological, and analytical steps involved.

Overall, this manuscript reads well and the protocols are clear. While similar papers describing the behavioral and/or pharmacological methodology for this assay in zebrafish exist in the literature (DOI: 10.1038/nprot.2010.140, ISBNs: 978-1-60761-953-6, 978-1-4939-6002-6, to name just a few), I suppose the most useful contribution would be the video representation of the process.

We thank this reviewer for their positive assessment of our manuscript, and for their thoughtful critique of the work. We have addressed the comments and concerns below, and believe this has improved the manuscript.

Major Concerns:
- I am surprised at the rather limited acknowledgement (or citations) for the many studies in the field (behavioral and/or behavioral pharmacology) that use this assay in zebrafish. I suggest the authors include a broader coverage of the literature.

We agree that the manuscript could benefit from a brief discussion of the widespread use and important findings that have emerged from this assay. While we believe that a detailed list or discussion is out of the scope of the manuscript,

we have added a brief review of relevant findings. These details have been added to the beginning of the discussion.

Minor Concerns:

- Line 24: What are genetic lesions? Perhaps the authors meant genetic mutants or knockouts?

This was meant to refer to any genetic aberration (mutation, transgenic insertion, etc.) We have replaced the word lesion with, 'mutation.'

- In the "Pretreatment with drug", I suggest adding a "note" stating that appropriate behavioral pharmacological procedures be followed (e.g. ensure proper dosage, administration length/frequency, drug crosses blood-brain barrier, and so on). The 10 minute treatment the authors describe may only apply for a specific drug at a specific concentration.

We have added that in a note in step 4.5.

- In the "Video analysis" section, I recommend the authors also note that this protocol is just one of several ways to do the analysis. I use a slightly different procedure in Ethovision XT in my research.

The section on tracking had to be mostly removed, since *JoVE* does not allow mentioning the word Ethovision, since the software is proprietary. In its place, we have highlighted the typical main steps that one would do to proceed with tracking the animals, and have also included some considerations. We also have a note in this section stating explicitly that other labs may have their own variations to this general approach.

- Line 211-213: Change "stress response" to "behavioral stress response". This paper is looking at behavioral measures and not physiological/glucocorticoid stress response.

Done.

- I am not sure if this is common in JoVE articles but a "troubleshooting" section with some common problems and potential solutions could be useful for first-time adopters.

This is an excellent idea. To see if this was an appropriate section, we reviewed several *JovE* papers, but did not find any (at least that we examined) that had a troubleshooting section. Instead, we have added more general notes to the protocol section, with some cases of troubleshooting advice. We hope that the precautions included in notes and the discussion should suffice in helping solve some of the simple complications.

- Figure 1C & 1D: I suggest the authors use a different color font to represent the individual data points. The gray data points on the patterned background (last minute) are difficult to distinguish.

We agree that the individual points in Fig 2 do not contrast well either. We have changed the dots in both figures so that they are more easily visualized when overlaid on the bar graph (i.e, we made them red)..

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