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TITLE:

Shuttle Box Assay as an Associative Learning Tool for Cognitive Assessment in Learning and Memory Studies using Adult Zebrafish

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zebrafish, regeneration, traumatic brain injury, blunt-force trauma, learning, memory

SUMMARY:

Learning and memory are potent metrics in studying either developmental, disease-dependent, or environmentally induced cognitive impairments. Most cognitive assessments require specialized equipment and extensive time commitments. However, the shuttle box assay is an associative learning tool that utilizes a conventional gel box for rapid and reliable assessment of adult zebrafish cognition.

ABSTRACT:

Cognitive deficits, including impaired learning and memory, are a primary symptom of various developmental and age-related neurodegenerative diseases and traumatic brain injury (TBI). Zebrafish are an important neuroscience model due to their transparency during development and robust regenerative capabilities following neurotrauma. While various cognitive tests exist in zebrafish, most of the cognitive assessments that are rapid examine non-associative learning. At the same time, associative-learning assays often require multiple days or weeks. Here, we describe a rapid associative-learning test that utilizes an adverse stimulus (electric shock) and requires minimal preparation time. The shuttle box assay, presented here, is simple, ideal for novice investigators, and requires minimal equipment. We demonstrate that, following TBI, this shuttle box test reproducibly assesses cognitive deficit and recovery from young to old zebrafish. Additionally, the assay is adaptable to examine either immediate or delayed memory. We demonstrate that both a single TBI and repeated TBI events negatively affect learning and immediate memory but not delayed memory. We, therefore, conclude that the shuttle box assay reproducibly tracks the progression and recovery of cognitive impairment.

INTRODUCTION:

Learning and memory are routinely used as metrics of cognitive impairment, which happens due to aging, neurodegenerative disease, or injury. Traumatic brain injuries (TBIs) are the most common injury that results in cognitive deficits. TBIs are of growing concern because of their association with several neurodegenerative disorders, such as frontotemporal dementia and Parkinson's disease[1,2]. In addition, the increased beta-amyloid aggregations observed in some TBI patients suggest that it may also be associated with the development of Alzheimer's disease[3,4]. TBIs are often the result of blunt-force trauma and span a range of severities[5], with mild brain injuries (miTBI) being the most common. However, miTBIs are often unreported and misdiagnosed because they result in minor cognitive impairments for only a short period, and the injured individuals usually recover fully[6]. In contrast, repeated miTBI events have been a growing concern because it is highly prevalent in young and middle-aged adults, can accumulate over time[7], can impair cognitive development, and exacerbate neurodegenerative diseases[1-5], similar to individuals who experience either a moderate or severe TBI[8].

Zebrafish (*Danio rerio*) is a useful model for exploring a variety of topics in neuroscience, including the ability to regenerate lost or damaged neurons throughout the central nervous system[9-13]. Neural regeneration was also demonstrated in the telencephalon, which contains the archipallium in the dorsal-inner region. This neuroanatomical region is analogous to the hippocampus and is likely required for cognition in fish and for the short-time memory in humans[14-16]. Furthermore, zebrafish behavior has been extensively characterized and cataloged[17]. Learning has been studied through various techniques, including habituation to the startle response[18], which can represent a rapid form of non-associative learning when performed in short blocks and with attention to the rapid decay time[19]. More complex tests of associative learning, such as T-boxes, plus-mazes, and visual discrimination[20,21] are used but often are time-consuming, require days or weeks of preparation, and rely on shoaling or positive reinforcement. Here, we describe a rapid paradigm to assess both associative learning and either immediate or delayed memory. This shuttle box assay uses an aversive stimulus and negative reinforcement conditioning to assess cognitive deficits and recovery following blunt-force TBI. We demonstrate that undamaged control adult zebrafish (8-24 months) reproducibly learn to avoid the red light within 20 trials (<20 min of assessment) in the shuttle box, with a high degree of consistency across observers. Additionally, using the shuttle box we demonstrate that learning and memory abilities across adult (8-24 months old) are consistent and are useful for assaying cognition with significant impairments between either different TBI severities or repeated TBI. Furthermore, this method could be rapidly employed as a metric to track a wide range of disease progressions or efficacy of drug interventions impacting maintenance or recovery of cognition in adult zebrafish.

Here, we provide an instructional overview of a rapid cognitive assessment that can examine both complex associative learning (section 1) and memory in terms of both immediate and delayed memory. This paradigm provides an assessment of the short and long-term memory of a learned associative cognitive task (section 2).

PROTOCOL:

Zebrafish were raised and maintained in the Notre Dame Zebrafish facility in the Freimann Life Sciences Center. The methods described in this manuscript were approved by the University of Notre Dame Animal Care and Use Committee (Animal Welfare Assurance Number A3093-01).

1. Shuttle box learning paradigm (**Figure 1A**)

NOTE: The learning paradigm provides a rapid assessment of cognition regarding associative learning.

1.1 Prepare the shuttle box by modifying a 30.5 x 19 x 7.5 cm gel box with a 5 x 19 cm piece of aquarium grade plexiglass added to each side at a 45° angle. Make a line marking the halfway point of the tank to assess when fish have crossed the middle of the tank (**Figure 1B**).

1.2 Add 800 mL of system water to the shuttle box. Make this water by dissolving 60 mg of Instant Ocean in 1 L of deionized RO water. Fill the water to the middle of the tank to a depth of 5 cm.

NOTE: Replace with fresh system water at 28 °C every h or after testing 3 fish.

1.3 Place 2-3 fish into a holding tank containing system water, located in a dark room where the shuttle box assay will be performed.

1.3.1 In the dark room, place 1 fish in the center of the shuttle box, secure the lid, and attach the electrodes to a power supply.

NOTE: The room should remain as dark as possible during acclimation and testing.

1.4 Acclimate the fish in the shuttle box for 15 min.

NOTE: The investigator should remain in the room during the acclimation period or return to the testing room quietly with ample time before the testing to allow fish to adjust to the investigator's presence. Successful acclimation can be considered when the fish freely explores the tank.

1.4.1 If the fish fails to explore, continue acclimation for an additional 15 min. If the fish still fails to acclimate to the shuttle box, remove the fish. Do not use this fish for testing.

1.5 Manually shine an 800-lumen red lens flashlight ~2 cm from the gel box wall on the side occupied by the fish, following acclimation.

NOTE: Do not start a trial if the fish is resting next to the platinum wire against the wall near the deep ends of the shuttle box.

1.6 Shine the light stimulus directly on the fish and manually follow any lateral movement of the fish with the light to ensure continual visualization of the stimulus (**Figure 1C**). Continue to provide the light stimulus until either of the following conditions are met.

1.6.1. Consider the trail successful if the fish crosses over the halfway point of the tank within the 15 s of light exposure. Once the fish crosses the halfway point, stop the light stimulus immediately (**Figure 1D**).

1.6.2. Consider the trail as failed if the fish does not cross over the halfway point of the box in 15 s. In this case, use an electrophoresis power supply to apply a negative shock stimulus (20 mV:1 A) alternating 2 s of On, 2 s of Off for a 15 s period (maximum of 4 shocks), or until the fish passes the halfway point of the box, at which point terminate both the light and negative stimulus.

1.7 Let the fish rest for 30 s and repeat step(s) 1.5-1.6.2. Keep a detailed record of the order of successful trials (1.6.1) and failed trials (1.6.2).

NOTE: Here, we defined learning as the completion of 5 consecutive successful trials. Once the learning has been demonstrated, the fish should be removed from the shuttle box and humanely euthanized.

2. Memory paradigm (Figure 1A)

NOTE: This paradigm provides an assessment of the short and long-term memory of a learned associative cognitive task.

2.1 Training Period

2.1.1 Add 800 mL of system water to the shuttle box. Make this water by dissolving 60 mg of Instant Ocean in 1 L of deionized RO water. Fill the water to the middle of the tank to a depth of 5 cm.

NOTE: Water should be replaced with fresh system water at 28 °C every h or after testing 3 fish.

2.1.2 Place 2-3 fish into a holding tank that contains system water, located in a dark room where the shuttle box assay will be performed.

2.1.3 In the dark room, place 1 fish in the center of the shuttle box, secure the lid, and attach the electrodes to a power supply.

NOTE: The room should remain as dark as possible during acclimation and testing.

2.1.4 Acclimate fish in the shuttle box for 15 min.

NOTE: The investigator should remain in the room during acclimation period or return to the

testing room quietly with ample time prior to testing to allow fish to adjust to the investigator's presence. Determine successful acclimation when the fish is freely exploring the tank.

2.1.5. If the fish fails to explore, continue acclimation for an additional 15 min. If the fish still fails to acclimate to the shuttle box, remove the fish and do not use it for testing.

2.1.6 After the successful acclimation, manually shine an 800-lumen red lens flashlight ~2 cm from the gel box side wall, on the side of the shuttle box that is occupied by the fish.

2.1.7. Shine the light stimulus directly on the fish and follow any lateral movement of the fish with the light to ensure continual visualization of the stimulus by the fish.

2.1.8. While the light is shining on the fish, simultaneously apply the adverse shock stimulus (20 mV:1 A) alternating 2 s On, 2 s Off for 15 s (maximum of 4 shocks), or until the fish passes the halfway point of the box. Once this is achieved, terminate both the light and the adverse stimulus.

NOTE: Allow the fish to rest for 30 s then repeat step 2.1.6-2.1.8 for 25 iterations (**Figure 1A**).

2.2 Initial testing

2.2.1. Allow 15 min of rest to the fish following the training period. Do not remove them from the shuttle box. Test initial memory retention by recording each trial as strictly pass/fail, immediately following this rest period.

2.2.2 Apply only the light stimulus for up to 15 s and record the responses as follows.

2.2.2.1 Consider the trial successful if the fish crosses over the halfway point of the shuttle box within 15 s after starting the light stimulus. Stop the light stimulus immediately when the fish crosses the halfway point.

2.2.2.2 Consider the trial as failed if the fish does not cross over the halfway point of the shuttle box 15 s after starting the light stimulus. Stop the light stimulus after 15 s.

NOTE: During the initial testing, an adverse stimulus is not applied following a failed attempt.

2.2.3 Repeat step 2.2.2, with a 30 s rest period between trials, and record successful trials (2.2.2.1) and failed trials (2.2.2.2) across 25 trials. This value will serve as an individual reference for each fish.

2.3 Immediate memory

2.3.1 Induce injury immediately following the initial testing period by preferred damage paradigm (e.g., a blunt-force trauma using the modified Marmarou weight drop). House fish individually for an easy identification. Record their initial testing values and return fish to the

animal facility.

NOTE: Fish were injured by blunt-force TBI as previously described[22].

2.3.2 Gather 2-3 undamaged or TBI fish 4 h after initial testing and/or 4 h post-injury (or at the experimental timeframe in question) from the animal facility. Keep all fish in the dark room in individual tanks containing system water.

2.3.3. Place fish in the center of the shuttle box (prepared with system water as described in 1.1), one fish at a time, and secure the lid. Attach the power supply and allow the fish to acclimate for 15 min.

2.3.4 Following acclimation, assess immediate memory (strictly pass/fail) by applying only the light stimulus for up to 15 s and record the responses as follows.

2.3.4.1 Consider the trial successful if the fish crosses over the halfway point of the box within the 15 s test period. Terminate the light stimulus upon crossing the halfway point.

2.3.4.2 Consider the trial as failed if the fish does not cross over the halfway point of the box within 15 s of starting the light stimulus. Terminate the light stimulus after 15 s period is over.

NOTE: During this post-injury testing, adverse shock stimulus is not applied following a failed attempt.

2.3.5 Repeat step 2.3.4, with a 30 s rest period between trials, and record the number of successful trials (2.3.4.1) and failed trials (2.3.4.2) across 25 trials.

2.3.6 Calculate the percent difference in successful trials post-injury to the initial testing period using the equation:

$$\%\ Diff\ of\ Successful\ Trials = \frac{successful\ post\ injury\ trials - successful\ inital\ testing\ trials}{successful\ post\ injury\ trials} * 100\%$$

2.4 Delayed memory

2.4.1 Return fish, housed individually for easy identification and recording of their initial testing values, to the animal facility immediately following the initial testing period.

2.4.2 Allow fish 4 days (or the experimental timeframe in question) between the initial testing and injury and/or delayed memory testing.

2.4.3 Induce injury by the preferred damage paradigm (such as the modified Marmarou weight drop to induce a blunt-force trauma). House fish individually for easy identification of initial

testing values, and return fish to the animal facility.

NOTE: Fish were injured by blunt-force TBI as previously described[22].

2.4.4 Gather 2-3 undamaged or TBI fish 4 h after initial testing and/or 4 h post-injury (or at the experimental timeframe in question) from the animal facility.

2.4.5 Keep all fish in the dark room in individual tanks containing system water, and place one at a time in the center of the shuttle box (prepared with system water as described in 1.1), secure the lid, attach the power supply, and allow fish 15 min to acclimate.

2.4.6 Following acclimation, assess immediate memory (strictly pass/fail) by applying only the light stimulus for up to 15 s and record the following responses:

2.4.6.1 Consider the trail successful if the fish crosses over the halfway point of the box within the 15 s testing period. Terminate the light stimulus upon crossing the halfway point.

2.4.6.2 Consider the trail as failed if the fish does not cross over the halfway point of the box within 15 s of starting the light stimulus, terminate the light stimulus.

NOTE: During this post-injury testing, an adverse shock stimulus is not applied following a failed attempt.

2.4.7 Repeat step 2.4.6, with a 30 s rest period between trials, and record the number of successful trials (2.4.6.1) and failed trials (2.4.6.2) across 25 trials.

2.4.8 Calculate the percent difference in successful trials of post-injury to the initial testing period with the equation:

$$\%\ Diff\ of\ Successful\ Trials = \frac{successful\ post\ injury\ trials - successful\ inital\ testing\ trials}{successful\ post\ injury\ trials} * 100\%$$

REPRESENTATIVE RESULTS:

The learning paradigm, outlined in the protocol and schematic (**Figure 1**), provides a rapid assessment of cognition with respect to associative learning. In addition, this paradigm has a high level of stringency, by defining learning as a repeated and consistent display of 5 consecutive positive trials. This paradigm is also applicable to a range of ages and injuries. Undamaged fish at 8 months (young adult), 18 months (middle-aged adult), and 24 months (elderly adult) required a similar number of trials to learn the behavior of avoiding the red light (Undamaged 8 m: 15.28 ± 4.92 trials, 18 m: 17.66 ± 5.5 trials, 24 m: 16.2 ± 4.79 trials, 8 m vs. 18 m p=0.92, 8 m vs. 24 m p=0.98, 18 m vs. 24 m p=0.97, **Figure 2A**). We also utilized a severe blunt-force traumatic brain injury (sTBI) model[22] and observed that fish at different ages required similar number of trials to master the assay across 1-5 days post-injury (dpi; 8 m vs 18 m, p=0.09, 8 m vs 24 m, p=0.96, 18

m vs 24 m, p=0.12, **Figure 2A**). At Day 1 following sTBI, fish of all ages (8, 18, and 24 m) required a similar number of trials to learn the behavior (8 m: 73.3 ± 9.45 trials, 18 m: 79.33 ± 6.35 trials, 24 m: 68.25 ± 6.65 trials, 8 m vs. 18 m p=0.71, 8 m vs. 24 m p=0.76, 18 m vs. 24 m p=0.28, **Figure 2A**) and they were all significantly greater than the undamaged controls (p<0.01). Collectively, these data demonstrate that the shuttle box can be utilized to examine injury-induced cognitive deficits across age ranges and suggest that adult zebrafish can recover cognitively following blunt-force injury.

Because repeated miTBI events can increasingly impair cognitive function, we used the shuttle box assay as a metric to track dose-dependent progression using repetitive TBI. We employed this assay to assess learning following a miTBI blunt force injury[22] that is repeated daily for the different lengths of time. As previously observed, undamaged fish rapidly mastered the shuttle-box achieving 5 consecutive positive trials in 16.4 ± 3.5 trials (**Figure 2B**). One day following a single miTBI, fish display a significant increase in the number of trials to learn the behavior (40.25 ± 12.65 trials, p<0.05, **Figure 2B**). This deficit increased after 2 miTBI events (48 ± 14.9 trials) and was further elevated after 3 miTBI injuries (56.63 ± 12.75 trials, **Figure 2B**). Additionally, we observed a significant increase in cognitive impairment between miTBI fish which received a singular injury and 3 injuries (p<0.05).

We also examined how memory was affected following repeated miTBI events using the protocol for immediate and delayed memory paradigms (**Figure 1A**). Naïve undamaged fish were given a training period and an initial testing period, after which a portion of fish were injured for immediate memory and others were returned to the fish facility for 4 days to access delayed memory (**Figure 2C**). Undamaged fish exhibit a slight increase in the percent difference of successful trials in both immediate memory (6.22% ± 4.7%) and delayed memory (6.13% ± 5.57%) relative to the initial testing period. We, then examined the effect of multiple blunt-force TBI events had on memory. Significant deficits were observed following miTBI in immediate memory, but not in delayed memory. Following a single miTBI, fish displayed significant immediate memory deficits (-26.77% ± 8.93%) compared to undamaged fish (p<0.0001, **Figure 2C**). This trend continued with repeated injury with increasing deficits following both 2x miTBI (-37.42% ± 10.01%) and 3x miTBI (-39.71% ± 11.39%). Furthermore, we observed a similar dose-effect between fish treated with a single (1x) miTBI and 3x miTBI (p<0.05, **Figure 2C).** These data suggest that learning and memory is reduced in miTBI fish with the increasing number of injuries, significantly increasing the deficit and the shuttle box assay and protocols described above are sensitive enough to detect these differences.

FIGURE AND TABLE LEGENDS:

Figure 1: The Shuttle Box Assay. (**A**) Instructional overview of the learning and memory paradigms for cognitive assessment. (**B**) Schematic of a converted large DNA gel box for the shuttle box assay. (**C,D**) Graphical representation of stimuli application during trials.

Figure 2: Zebrafish display cognitive deficits following blunt-force TBI. (**A**) Following sTBI, zebrafish at 8, 18, and 24-months of age exhibit learning deficits that are not significantly different between age groups. Significant increases in the number of trials to learn the shuttle

box paradigm compared to age-matched controls were observed at 1 dpi returning to undamaged levels by 4-5 dpi. (**B,C**) Repeated miTBI fish displayed both learning (**B**) and memory (**C**) deficits in a dose-dependent manner. The mean ± SEM is plotted in **A** and **B**, while the mean ± Standard deviation is plotted in **C**. Each data point on all three graphs represents a single adult zebrafish. Statistical analyses were performed with either a One-Way or Two-Way ANOVA followed by a Tukey post-hoc test. # $p<0.05$, ## $p<0.01$.

DISCUSSION:

Cognitive impairment can significantly and negatively impact the quality of life. Because of the increased visibility and occurrence of concussions and traumatic brain injuries throughout the population, it is important to understand how they cause cognitive impairment and how the damage can be minimized or reversed. For these reasons, model organisms that can be tested for cognitive decline play a critical role in these studies. Rodents have long been the primary model to investigate neurobehavior and cognition, however, zebrafish have emerged as a useful model with numerous distinct behaviors to investigate a range of developmental, age-related, and acquired cognitive deficits[17,20,23-26]. Various methods to assess cognition have been utilized from one-dimensional learning in the form of habituation, to complex learning and spatial memory, novel object and location recognition, and decision making[18-21,27,28]. However, these cognitive tests are limited to testing non-associative cognition or require a complex set-up, financial investment in equipment, or an extensive time commitment before tests can be performed. In contrast, the shuttle box and the learning and memory paradigms described here utilize a complex associative learning assay that is cost-effective, a rapidly assessed, and easily employed by a novice investigator. Most importantly, consistent with the other cognitive tests, our assay demonstrates that undamaged fish rapidly learn the associative task and can memory the task days later without intermittent training[29].

The adaptability of the assay provides avenues to investigate various time points of learning and memory as a metric of disease progression or mechanistic interventions. There are two primary features of the assay. First, the method is simple. The assay is quickly set up and has clear and distinct end points with respect to successful and failed trials, making it accessible to a range of investigators. We found that because of the simplicity of this assay, there is very little troubleshooting needed to use the shuttle box successfully. Second, the assay is extremely quick in comparison to other cognitive exams, which provides flexibility or the ability to examine a large number of fish rapidly in a single day. The time to assess learning is at a minimum 19.75 min (**Figure 1**), with the fish requiring 15 minutes to acclimate to the shuttle box (determined by tank exploration), followed by a single failed trial (15 s light stimulus, 15 s aversion stimulus, 30 s between trials) and 5 immediate and consecutive positive trials (<15 s light stimulus). In practice, we observed that undamaged fish require 6-30 trials (19.75 min-43.75 min), while in extreme cases (following a severe blunt-force trauma), the most severe deficits can require 100 trials (113.75 min). Memory studies are also rapidly performed. Following the protocol outline, the minimum time necessary for acclimation, training, and initial testing is 67.5 min (15 min acclimation, 25 iterations of light and shock for 15 s, 30 s rest between trials, and repeat for initial testing without the adverse stimuli). While retesting either immediate or delayed memory requires only 33.75 min (15 min acclimation, 25 iterations of only light stimulus for 15 s, and 30

s rest between trials), regardless of injury, treatment, or cognitive deficit.

When assessing neurobehavior, various paradigms utilize either positive or adverse stimuli. Positive stimuli in the form of food or social interaction, often used in classical T-box mazes, can aid in a strong response of a learned task. However, assays utilizing positive association do so at the expense of time. In contrast, while conditioning in response to an adverse stimulus provides a rapid association and strong behavioral response, it is at the expense of the adverse stimulus. Undamaged fish often learn the shuttle box assay quickly and are therefore subjected to a minimal number of shocks, and as a result seem to have no adverse events. However, neurologically compromised fish (TBI), with severe cognitive deficits, require a significant number of trials and electrical shocks. These multiple shocks have been observed to occasionally result in tonic-clonic seizures. Any fish experiencing a tonic-clonic seizure while within the shuttle box should be immediately removed and ethically euthanized. All trials for the euthanized fish, up to and including the seizure event, should be excluded in any statistical analysis. Furthermore, it is worth noting that electrical shock to a neurologically damaged subject could impose unintended differences between damaged fish that are and are not resulting from the shuttle box. For that reason, we suggest all fish subjected for neurobehavior assessment should not be used for any other quantitative metric (serum biomarker, IHC, etc.). It is also important to understand that this method of learning is based on a visual stimulus and is not appropriate for damage that may compromise visual circuits, as it will confound the results.

Our results demonstrate that following blunt-force TBI, zebrafish exhibit a rapid cognitive deficit that results in increased trials to master an associative task in the shuttle box assay. Similar immediate deficits are seen in rodent models of TBI, however these deficits can diminish, they often persist and remain significant[30]. In contrast, zebrafish display cognitive recovery within 7 days following injury. The regenerative capacity of the adult zebrafish is well documented[9-15], with known neurogenic niches in the ventricular/subventricular zones of the telencephalon[31,32]. The cognitive recovery observed in our assay following TBI provides insight into needed exams to identify if these neurogenic niches are stimulated and play a role in tissue and cognitive recovery.

In conclusion, the shuttle box provides a rapid assessment of cognition in regard to associative learning and memory. The assay utilizes minimal and conventual equipment and is technically simple. Future applications could be utilized to assess genetic and pharmacological interventions to neurologically insulted fish in regard to neuroprotection as well as other injury paradigms or neurodegenerative models.

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DISCLOSURES:

The authors have nothing to disclose.

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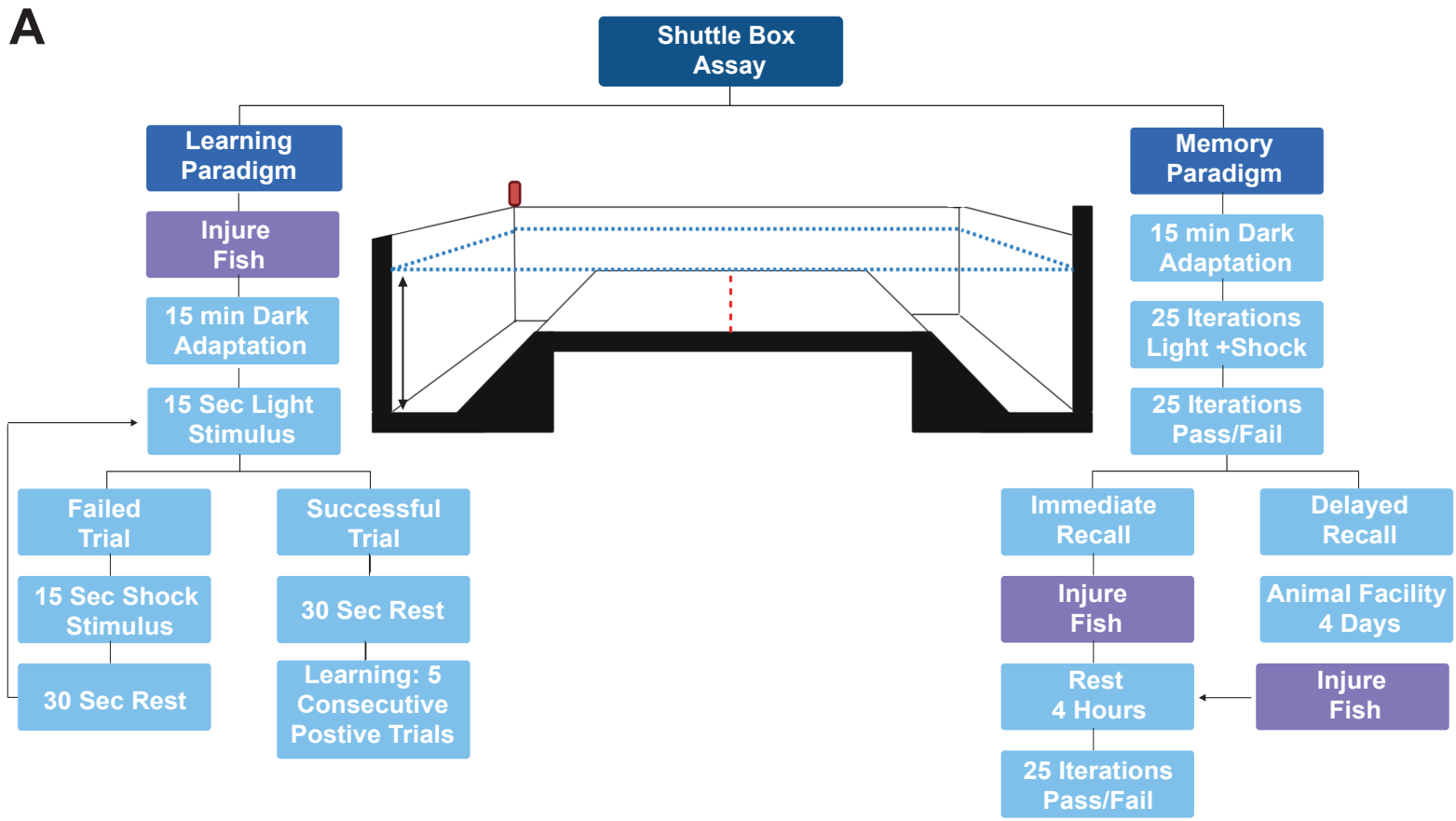
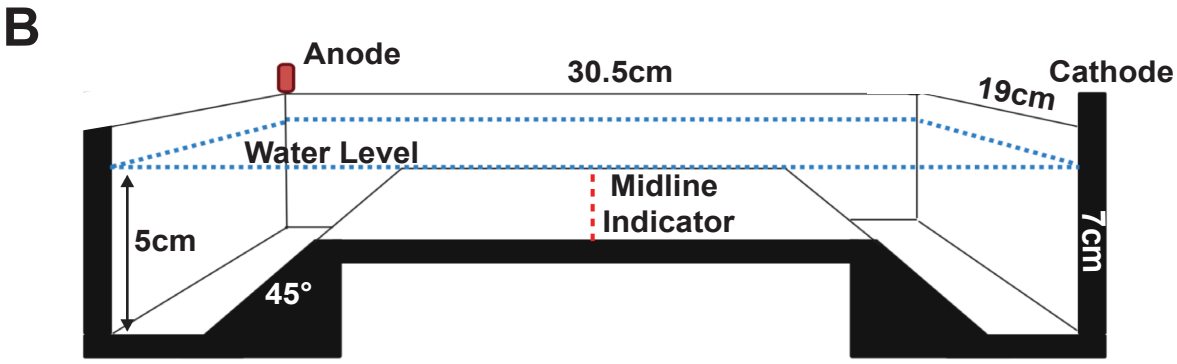
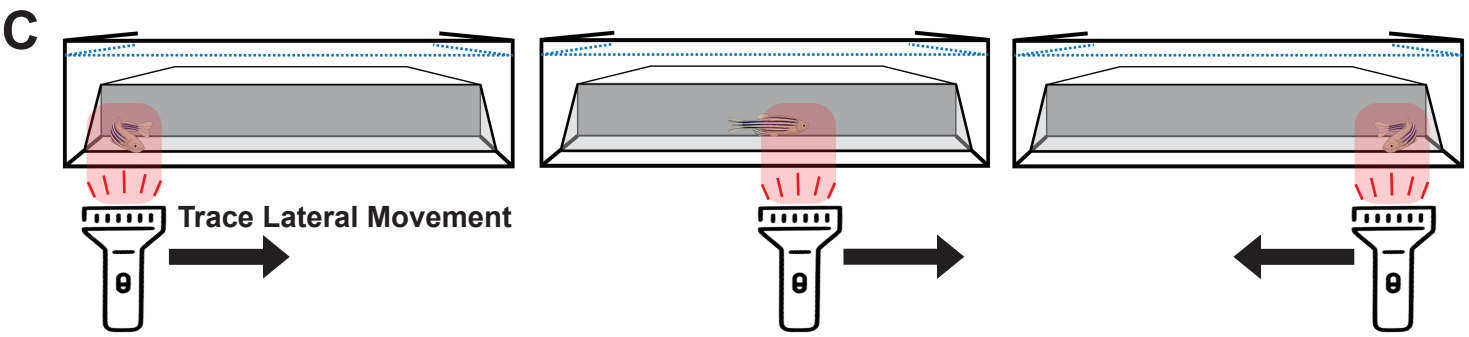
Figurer 1

Click here to access/download;Figure;Figure 1. The Shuttle Box.pdf







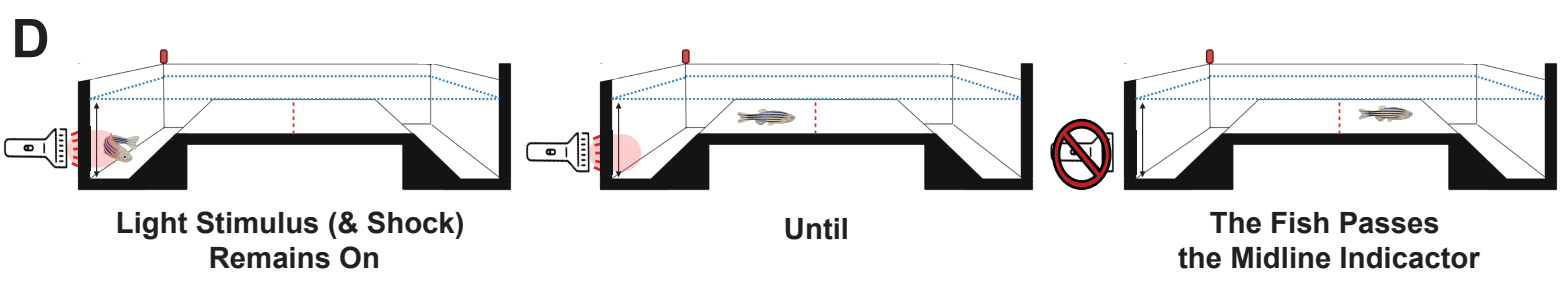


Figure 2

Click here to access/download;Figure;Figure 2. Zebrafish display cognitive deficits .pdf

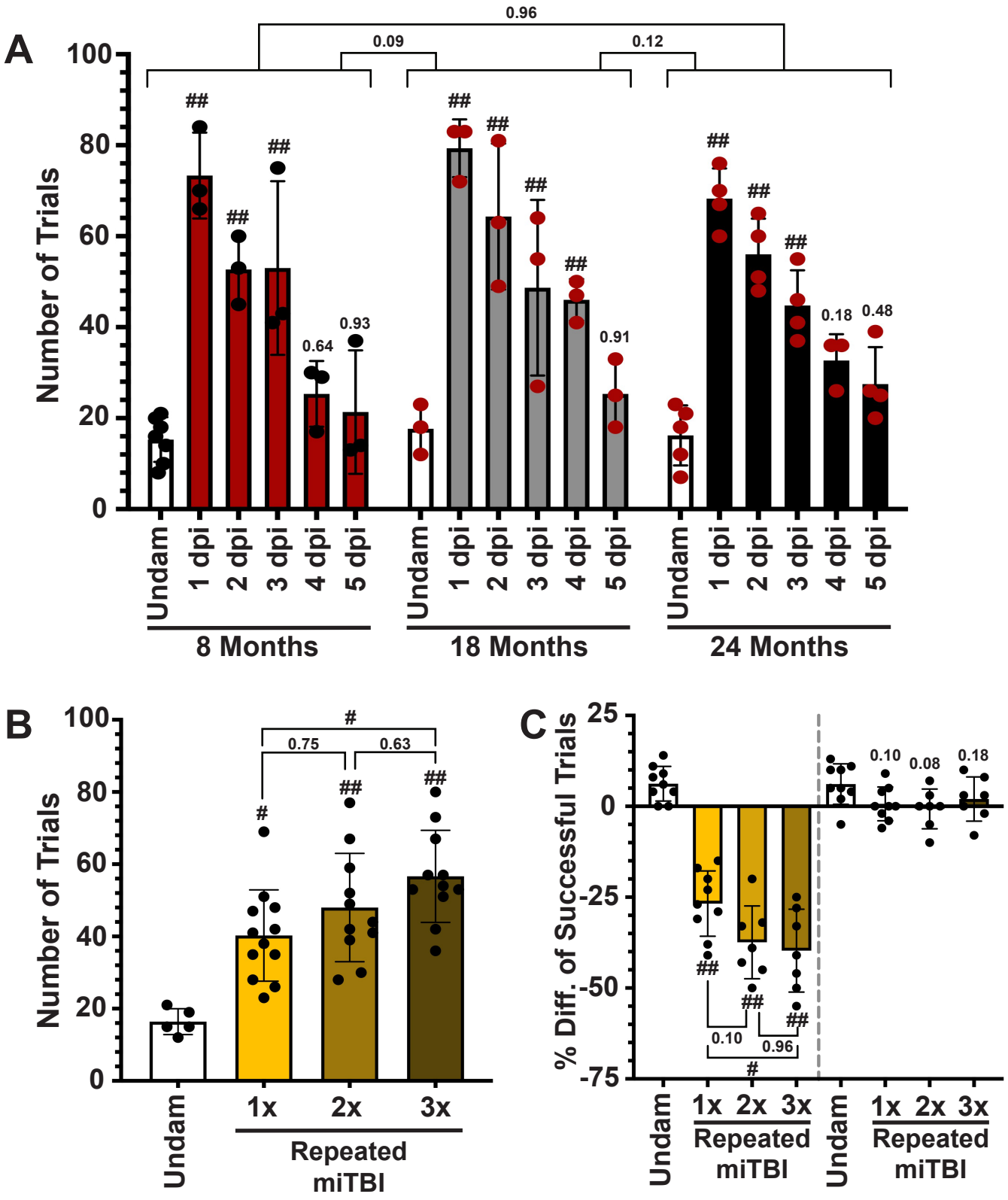


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Flashlight	Ultrafire	9145	
Instant Ocean	Instant Ocean	SS15-10	
Large DNA Gel Box	Fisher Scientific	FB-SB-1316	Shuttle Box
Power Supply	Fisher Scientific	FB-105	

Below are the comments by the editor and reviewers (in black) and our responses to each comment (in red). We appreciate the time and effort of the reviewers in helping to clarify specific points in this methods paper and making it a stronger contribution to the field.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Done

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Instant Ocean (Cat#: SS15-10, Instant Ocean); Cat: FB-105, Fisher Scientific; Cat: 9145, Ultrafire etc

All commercial language was removed

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

No personal pronouns are present within the protocol.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Done

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

2.1.4 is the largest step. It contains three actions and 3 sentences covering 7 lines.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. PLEASE LEAVE A ONE-LINE SPACE BETWEEN THE STEPS AND NOTES. AFTER ENSURING YOU HAVE ONLY 2-3 ACTIONS PER STEP AND NOTES ARE SEPARATED FROM STEPS, HIGHLIGHT ONLY STEPS (NOT NOTES) TO ENSURE YOU DO NOT EXCEED 3 PAGES OF HIGHLIGHTED TEXT.

We have a total 2 pages of highlighted text.

8. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names.

Done

9. Please sort the Materials Table alphabetically by the name of the material.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, Hentig and colleagues describe the development of an assay to assess the cognitive capacity of the adult zebrafish.

The described method is very straightforward, easy and comparably inexpensive.

The manuscript is very well- written and the experimental procedures are thoroughly explained.

Major Concerns:

non

Minor Concerns:

At multiple occasions, the authors refer to a yet unpublished method by Hentig et al.

(reference number 19). I would suggest to briefly describe this method when it is first mentioned to help the reader understand the background.

This manuscript has already been accepted and will be published in the same special edition.

Reviewer #2:
Dear authors,

This article can make a valid methodological scientific contribution. However, I would like to invite you to consider the following (1-18) points, divided per section (Abstract, Introd. etc), which in my opinion would improve the clarity and the potential outreach of your article.

My comments follow the ">>"

----------Abstract----------

1. "Here, we describe a rapid associative-learning test that utilizes a negative stimulus and requires minimal preparation time." >> please add in brackets which property defines a stimulus as being 'negative': negative (i.e., unpleasant/adverse)

lines 53-55: The sentence was changed to read: "Here, we describe a rapid associative-learning test that utilizes an adverse stimulus (electric shock) and requires minimal preparation time."

----------Introduction----------

2. Passage in lines 68-70: "TBIs are of growing concern because they have been linked to other neurodegenerative disorders, such as Alzheimer's, Frontotemporal dementia, and Parkinson's disease1-3" >> The statement requires a little information on how TBIs are related to neurodegenerative disorders. The authors may want to mention some evidence of linked structural and functional pathologies, which can make their argument stronger. For example, the accumulation of amyloid-beta plaques, well-known to be a hallmark of dementias (Alzheimer's) have also been found in more severe TBI cases (Johnson et al., 2010, Nat Rev Neurosci, doi: 10.1038/nrn2808)

lines 67-68: As suggested by the reviewer, we modified the text: "such as Alzheimer's, with protein aggregations of beta-amyloid being identified in human TBI patients,…" and added the appropriate reference.

3. Passage in lines 83-84: "including habituation to the startle response, which represents a rapid and simple form of non-associative learning" >> Startle habituation should not be generalised as being a 'rapid'. form of learning. Indeed, it can form and decay at different time-rates, depending on the duration of the stimulation: rapid habituations decays within 15 mins and is quantified with a single block of 15-20 stimuli,

short-term habituation lasts a few to 24 hours and is quantified with a few blocks of stimuli. Long-term habituation, lasting days, is quantified with multiple sessions. For a review see López-Schier, 2019, Current opinion in neurobiology, 54, pp.134-139)

lines 83-85: We modified the text: "… which can represent a rapid form of non-associative learning when performed in short blocks and with attention to the rapid decay time." And provided the appropriate reference.

4. Passage in line 90: "Undamaged control fish reproducibly learn to avoid the red light within 20 trials" >> Please add the age-range (adults from A to B months), and specify whether it is a finding of your own study or of a past study (if this is the case, the paper should be cited).

*lines 90-93: We modified the text: "*We demonstrate that undamaged control adult zebrafish (8-24 months) reproducibly learn to avoid the red light within 20 trials (<20 minutes of assessment) in the shuttle box, with a high degree of consistency across observers."

5. Passage in line 91: "with a high degree of fidelity across investigators" >> The term "fidelity" might not be appropriate scientifically speaking. The authors might want to rephrase as follows: "consistently observed in different studies (at least 3 original research studies, or 2 reviews, are needed)."

See modifications to the text in the previous reply.

6. Passage in line 92: "the shuttle box is reproducible across ages" >> An assessment tool cannot be described as being 'reproducible'. The authors perhaps meant to say "our analyses demonstrate that learning and memory abilities, as measured by the shuttle box, are consistent across adult zebrafish of [specify age range]" (as the shuttle box is not usable in larval zebrafish, it is important to specify the adult age)

*lines 93-94: We modified the text: "*Additionally, we demonstrate that learning and memory abilities, across adult age ranges of 8-24 months, are consistent using the shuttle box,..."
----------PROTOCOL----------

7. Shuttle Box Learning Paradigm, section 1.4.2: "alternating 2 seconds on, 2 seconds off for 15 seconds" >> Please, for simplicity and clarity, add the total number of shock stimulations (tot = 4)

*lines 152-155: We modified the text: "*Failed trial: If the fish fails to cross over the half-way point of the box in 15 seconds, apply the negative shock stimulus (20 mV:1 A) alternating 2 seconds on, 2 seconds off for 15 seconds (maximum of 4 shocks), or until the fish passes the half-way point of the box, at which point both the light and negative stimulus should be terminated."

8. Shuttle Box Learning Paradigm, section 2.1.4. >> same as point 7

*lines 187-191: We modified the text: "*While the light is shining on the fish, simultaneously

apply the adverse shock stimulus (20 mV:1 A) alternating 2 seconds on, 2 seconds off for 15 seconds (maximum of 4 shocks), or until the fish passes the half-way point of the box, at which point both the light and the adverse stimulus should be terminated."

9. Memory Paradigm, Training Period >> The fact that the Memory Paradigm and the Shuttle Box Learning Paradigm are two different paradigms should be explained early on. Please introduce these two paradigms, in a few sentences, at the opening of the "protocol" section to guide a reader through the sections. For example, "The learning paradigm provides a rapid assessment of cognition in regard to associative learning, while the memory paradigm ……. "

*lines 109-111: We inserted the following text at the beginning for the protocol, "*The learning paradigm provides a rapid assessment of cognition in regard to associative learning (section 1), while the memory paradigm provides assessment of short and long-term recall of a learned associative cognitive task (section 2). "

10. Memory Paradigm, Initial Testing, Section 2.2.7: "Repeat step 2.6, with a 30 sec rest period between trials, and record successful trials (2.6.1) and failed trials (2.6.2) across 25 trials" >> Steps 2.6, 2.6.1 and 2.6.2 are missing. The authors perhaps meant 2.2.6, 2.2.6.1, 2.2.6.2)

lines 211-213: We corrected these errors as noted by the reviewer.

----------REPRESENTATIVE RESULTS----------

11. Passage in lines 276-277: "In addition, this paradigm has a high level of stringency, by defining learning as 5 consecutive positive trials" >> Please clarify what is meant by stringent, and why 5 trials are supposed to be enough to make the paradigm stringent.

*lines 293-295: we clarified this explanation in the text be rewriting: "*In addition, this paradigm has a high level of stringency, by defining learning as a repeated and consistent display of 5 consecutive positive trials."

12. >> Generally, when giving numbers, one should specify the type of descriptive mean measure (mean, median or mode) and variance (Std, SErr, variance). Please add these anytime numbers are mentioned in the results section, specifying if the numbers are results of the learning or memory paradigm.

We felt that inserting this descriptor in the text every time we mention a quantitative measure would make the text more choppy. However, we realize the importance of specifying that we are describing the mean ± standard error of the mean.
Therefore, we explicitly state in the Figure 2 legend (lines 466-468) "(B,C) Repeated miTBI fish displayed both learning (B) and memory (C) deficits in a dose-dependent manner. The mean ± SEM are plotted in A and B, while the mean ± Standard deviation is plotted in C."

13. Passage in lines 334-335 "Statistical analyses were performed with either a One-

Way or Two-Way ANOVA followed by a Tukey post-hoc test. #p<0.05, ## p<0.01">> For unequivocal understanding and for a researcher to be able to replicate your work, for each analysis performed, please detail the independent variables (IVs) and the dependent variable (mean/median/mode of the total amount of trials). For example, if the results in Figure 2A refer to a mixed 2-way ANOVA, you would need to state that: "A mixed 2-factor ANOVA was run. The within-group IV was time, with 6 levels: undamaged, 1dpi, 2dpi, 3 dpi, 4 dpi, 5 dpi,6 dpi). The between-group IV was age, with 3 groups (8,18 or 24 months)." Please do the same for the analyses in Figures 2B and 2C (A 1-way ANOVA….)

This is not typically the way we have represented our data in numerous journals within the field of cell and molecular neuroscience. Furthermore, the number of timepoints and groups analyzed for these learning and memory assays can vary depending on the needs of the scientists. Because we clearly state the number of timepoints and groups analyzed in our experimental example, we retained our description.

14. >> The results for each (un)significant main effects and interaction effect would need to be reported in full according to standard formats, including effect size (very important), not just p-values. Some helpful guidelines are in the following link https://www.open.ac.uk/socialsciences/spsstutorial/files/tutorials/two-way-mixed-ANOVA.pdf

We respectfully disagree with the reviewer. While it may be important if the data that we are describing are critical for a research manuscript, is not critical for this methods manuscript. Furthermore, we have never provided this information in any of our research manuscripts and feel that including it in this manuscript would make the flow of the text more choppy without providing further clarity.

15. Passage in lines 292-296: "A single moderate or severe blunt-force trauma can lead to significant cognitive impairments, while a single mild TBI (miTBI) may not result in a significant cognitive deficit20. However, repeated miTBI events has been a growing concern because it is highly prevalent in young and middle-aged adults, can accumulate over time20, can impair cognitive development and exacerbate neurodegenerative diseases1-4." >> This background information (with citations) should be moved to the introduction. In the results section, one should simply report their own findings. In the discussion one will have the chance to interpret their findings and relate them to the background studies (discussed in the introduction).

*As suggested by the reviewer, we moved this text to the Introduction (lines 78-81). The paragraph in the Results now begins with (lines 333-334): "*Because repeated miTBI events can increasingly impair cognitive function, we used the shuttle box assay as a metric to track dose-dependent progression using repetitive TBI.*"*

----------Discussion----------

16. Passage in lines 348-353: "These cognitive tests are limited by testing simplistic

cognition, complexity of set up the assay, financial investment in equipment, or the extensive time required to run the tests" >> Basic forms of cognition (i.e., non-associative learning e.g., startle habituation) should not be intended as a limitation, in that they are not less valid or less reliable than more complex forms of learning (i.e., associative). In fact, startle habituation is one of the most established cognitive measures in larval zebrafish. Please rephrase, or simply remove, "simplistic cognition".

*lines 361-364: As suggested by the reviewer, we rewrote this sentence to: "*However, these cognitive tests are limited to testing non-associative cognition or require a complex set up, financial investment in equipment, or an extensive time commitment before tests can be performed.*"*

17. Passage in line 355: "high degree of of fidelity" >> Please rephrase with appropriate scientific terminology. Perhaps the authors meant "reproducibility"? If so, please add 2-3 references that demonstrate reproducibility.

We removed this text.

18. >> The discussion lacks a section where the learning paradigm and memory paradigm (described in the methods and results sections) are confronted in terms of sensitivity and specificity in TBI contexts (based on your findings), but also, in terms of their different use and applicability (in this regard, the authors might want to offer suggestions as to when to choose one or the other).

We respectfully disagree with the reviewer on this point. We felt that we provided an appropriate discussion of our data, the learning and memory protocol, and its relationship to the other published paradigms in the field.

Reviewer #3:
Manuscript Summary:
The manuscript entitled "Shuttle Box Assay: An associative-learning assay for cognitive assessment in learning and memory
4 studies using adult zebrafish" is highly interesting and well presented. I support the publication of this study in the present form.

We appreciate the reviewer sees the value of this manuscript for the journal.

Reviewer #4:
Manuscript Summary:
The manuscript JoVE62745 deals with a description of an associative-learning test that utilizes a negative stimulus in order to obtain a possible translational model to study the recovery of Traumatic Brain Injury (TBI). The authors declare to obtain results from a single TBI and repeated TBI events that negatively affected immediate recall, but not delayed recall. The authors suggest that the proposed shuttle box assay can be applicable to reproducibly track progression and recovery of cognitive impairment study

also in other models such as mammals.

Major concerns:
Introduction:
Lines 78-80. The authors write: "Zebrafish (Danio rerio) is a useful model for exploring a variety of topics in neuroscience, including the ability to regenerate lost or damaged neurons throughout the central nervous system. Neuronal regeneration was also demonstrated in the telencephalon, which contains the pallium, the neuroanatomical region analogous to the hippocampus and likely required for cognition".
The author should be marked that the hippocampal region in fish is called "archipallium" that in mammals evolved in the hippocampus The correct detail is: Neural regeneration was also demonstrated in the telencephalon, which contains in the dorsal-inner region the archipallium, the neuroanatomical region analogous to the hippocampus and likely required for cognition in fish and for the short-time memory in humans.
The authors should report also review manuscripts about the comparative study of CNS regeneration and cognition (Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease Volume 1812, Issue 3, March 2011, Pages 364-380; Rev Neurosci. 2018 Dec 19;30(1):45-66. doi: 10.1515/revneuro-2018-0020).

*lines 79-81: We modified the text as suggested by the reviewer. It now reads: "*Neural regeneration was also demonstrated in the telencephalon, which contains in the dorsal-inner region the archipallium, the neuroanatomical region analogous to the hippocampus and likely required for cognition in fish and for the short-time memory in humans.[13-15]."

PROTOCOL
Lines 102-104 "Zebrafish were raised and maintained in the Notre Dame Zebrafish facility in the Freimann Life Sciences Center. The methods described in this manuscript are approved by the University of Notre Dame Animal Care and Use Committee".
The authors should indicate the number of protocol approbation here or at the end of the manuscript.

lines 105-107: We modified the sentence so that it now reads: "The methods described in this manuscript are approved by the University of Notre Dame Animal Care and Use Committee (Animal Welfare Assurance Number A3093-01)."

The temperature of the acclimation water should be indicated or at least is important remarked the indication to maintain the water temperature all the time. A temperature shock could be determinant to change the results of the experiment!.

*lines 123-124: We included the temperature as suggested by the reviewer. It now reads: "*Note: Water should be replaced with fresh system water at 28° C every hour or after testing 3 fish."

Discussion
Lines 342-347 Next to the cognitive recovery of fish and mice, the authors should

mention the time of recovery of the damaged area in fish as in mammals (read and cited the manuscripts that I have suggested below) in order to correlate the tissue recovery from neuro-niche cells and cognitive capacity. This part in the discussion, that lack completely, could be fundamental to appreciate the authors-work.

No manuscripts were listed below or with this comment. Furthermore, while the tissue recovery from neurogenic niches and their role to cognitive recovery is something we are actively working on, the current manuscript, which is focused on a method to analyze learning and memory, does not provide any tissue or region-specific regeneration that could be correlated to the cognitive recovery observed. For this reason, we addressed the reviewer's comments by including the topic as a future direction or application.