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The Tapping Assay: A Simple Method to Induce Fear Responses in Zebrafish

Yoojin Ro[1] · Marigrace Noronha[2] · Bashir Mirza[2] · Rida Ansari[2] · Robert Gerlai[2]

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Abstract
The zebrafish is increasingly employed in behavioral neuroscience as a translationally relevant model organism for human central nervous system disorders. One of the most prevalent CNS disorders representing an unmet medical need is the disorder cluster defined under the umbrella term anxiety disorders. Zebrafish have been shown to respond to a variety of anxiety and fear inducing stimuli and have been suggested for modeling human anxiety. Here, we describe a simple method with which we intend to induce fear/anxiety responses in this species. The method allows us to deliver a visual and lateral line stimulus (vibration or "tapping") to the fish with the use of a moving object, a ball colliding with the side glass of the experimental tank. We describe the hardware construction of the apparatus and the procedure of the behavioral paradigm. We also present data on how zebrafish respond to the tapping. Our results demonstrate that the method induces significant fear/anxiety responses. We argue that the simplicity of the method and the efficiency of the paradigm should make it popular among those who plan to use zebrafish as a tool in anxiety research.

Keywords anxiety · fear · lateral line · zebrafish · 3D-printing

Introduction

Anxiety disorders are a cluster of human central nervous system disorders with distinct etiologies (Craske & Stein, 2016). Although numerous anxiolytic drugs have been approved and employed in the human clinic, anxiety disorders still represent a substantial unmet medical need as this disease cluster is one of the largest in terms of prevalence compared to other CNS disorders (Murrough et al., 2015). Given the complexities of human clinical research of anxiety, numerous investigators have turned to animal models. The zebrafish is perhaps the newest species used in this field (Gerlai, 2010b; Stewart et al., 2012; Jesuthasan, 2012; Facciol et al., 2019). Nevertheless, this species is considered highly promising because it represents a reasonable compromise between system complexity and practical simplicity (Gerlai, 2012; 2010a). Although numerous features of the zebrafish are complex, evolutionarily conserved and thus mechanistically similar to those of mammals, it is a small vertebrate that is easy and cheap to breed and keep in large numbers in the laboratory (Gerlai, 2020a; Kalueff et al., 2014).

Several anxiety and fear inducing paradigms have already been developed for the zebrafish (Bass & Gerlai, 2008; Speedie & Gerlai, 2008; Parra et al., 2009; Gerlai et al., 2009; Gerlai, 2010b; Maximino et al., 2010; Ahmed et al., 2011; Luca & Gerlai, 2012; Stewart et al., 2012; Jesuthasan, 2012). Here we adopt a simple definition of these two behavioral responses/states. Fear represents a set of behavioral responses that are elicited by a clearly present aversive stimulus, e.g., a predator or a painful stimulus. Anxiety represents the set of behavioral responses that are not induced by the presence of a specific aversive stimulus but rather by a more diffuse environmental condition, e.g., novelty. Fear responses tend to be immediate and short, whereas anxiety responses tend to be prolonged. However, we note that the border between, and in fact the distinctive nature of, fear versus anxiety is still a matter of debate (Perusini & Fanselow, 2015). From here onward, we refer to the set of behavioral

✉ Robert Gerlai
robert_gerlai@yahoo.com; robert.gerlai@utoronto.ca

[1] Department of Chemical & Physical Sciences, University of Toronto Mississauga, Mississauga, Ontario, Canada

[2] Department of Psychology, Rm CCT4004, University of Toronto Mississauga, 3359 Mississauga Road, Mississauga, Ontario L5L 1C6, Canada

responses induced in zebrafish by aversive stimuli as fear responses.

Fear responses have been successfully induced in zebrafish by either one of three distinct types of aversive cues: electric shocks, visual or olfactory stimuli. Electric shocks we will not discuss here, because we argue it is inappropriate as an aversive stimulus for studies aimed at understanding neurobiological or behavioral mechanisms in fish. In the aquatic environment, experimentally delivered electric currents running through the brain of the fish can have direct effects on neuronal communication and plasticity.

Visual stimuli, including sight of live predators, or presentation of animated or video-recorded predators have turned out to be effective aversive cues (Bass & Gerlai, 2008, also see Gerlai, 2017 and Chouinard-Thuly et al., 2017). Mimicking some aspects of predators using computer animated stimuli, e.g., the expanding dot, bouncing ball, or moving bird silhouette, has been shown to induce robust fear responses in the zebrafish (Luca & Gerlai, 2012; Pelkowski et al., 2011). The advantage of live stimuli is that they can provide cues of all modalities, i.e., they can be ethologically and ecologically valid. Their disadvantage, however, is that stimulus intensity and specificity depend upon the behavioral activity or status of the live predator. Thus, live stimuli are rather difficult to standardize within and across experiments. The advantage of computer animated or video-recorded stimuli is that they are consistent, precisely controlled and can be manipulated, turned on or off or altered at will (Gerlai, 2017). However, their disadvantage is that they may be somewhat artificial. Furthermore, they require expensive video-screens and computers, electronic equipment that is not suited to the wet/humid environment of the zebrafish facility.

Olfactory cues, including the natural alarm "pheromone" (a mixture of a number of substances) (Speedie & Gerlai, 2008) and chemically synthesized components of such substances, e.g., H3NO have also been employed in zebrafish fear research (Parra et al., 2009). The natural alarm pheromone needs to be extracted fresh by cutting the skin of euthanized zebrafish and washing the cuts. Subsequently, a dilution sequence is created, which thus allows a dose response analysis within the experiment. However, the doses are only relative to each other within the sequence, and thus specific concentrations across experiments are difficult to ascertain (Speedie & Gerlai, 2008). The synthetic alarm substance H3NO does not suffer from this problem because its chemical identity, and thus molecular weight, is known. However, the problem with this substance is that it deteriorates with time (Parra et al., 2009). Thus, unless it is freshly synthesized, it loses its potency. Another complication with using alarm substances is that, similarly to other olfactory cues, the onset and even more so the offset of the stimulus is difficult to control. For example, after the delivery of the alarm substance, water in the test tank must be completely removed, the tank washed and new water added for the next subject/trial, as even trace amounts of the substance may alter behavioral responses. For these reasons, alarm substances may not be the ideal fear inducing stimuli from the perspectives of practicality, reproducibility and replicability in zebrafish research (Gerlai., 2010b).

Stimuli that have enjoyed less attention in zebrafish fear research, but ones which may not suffer from the above disadvantages, are cues that may be perceived by the lateral line of zebrafish. The lateral line is essentially a long-range tactile sensory system that allows the fish to detect low frequency (below the frequency of audible sound) vibrations in the water (for review see, e.g., Mogdans, 2019). Lateral line responses have been shown to play roles in a variety of behavioral contexts in fish, including rheotaxis (Suli et al., 2012; Olszewski et al., 2012) and predator detection and escape responses (McHenry et al., 2009; Eaton & Didomenico. 1986) in larval zebrafish. Other studies with zebrafish focused on a non-behavioral feature of the lateral line system: the similarity between zebrafish lateral line hair cell and mammalian inner ear hair cells (Lush & Piotrowski, 2014). Nevertheless, lateral line-dependent behavioral responses of adult zebrafish have not been well utilized in behavioral neuroscience research.

In nature, there are a large number of piscivorous fish as well as birds that pray upon small fish like the zebrafish (Engeszer et al., 2007; Spence et al., 2008; Parichy & Postlethwait, 2020). Zebrafish live in a variety of aquatic habitats including shallow rivers, streams, irrigation canals, rice paddies, small ponds and flood plains (Engeszer et al., 2007; Spence et al., 2008; Parichy & Postlethwait, 2020). In these habitats, water flow and turbulence induced vibrations represent a rich set of stimuli that the zebrafish may perceive using their lateral-line. The visibility in these waters also varies due to the presence of submerged or aquatic vegetation and/or to the turbidity of the water (Engeszer et al., 2007; Spence et al., 2008; Parichy & Postlethwait, 2020). Thus, visual stimuli are not always available for zebrafish to perceive danger, i.e., to detect an approaching predator. However, vibrations induced by a fishing bird splashing into the water or the leap of a piscivorous fish may be detected by the lateral line of zebrafish even in such environments. Thus, we argue that vibration-based fear induction may have ethological-ecological relevance.

In the current paper we describe a simple method that utilizes vibration to induce fear responses in zebrafish. The idea to employ vibration or tapping as an aversive stimulus for the adult zebrafish is not new (e.g., Pittman & Lott, 2014; Eddins et al., 2010). However, pioneering studies using this concept employed manual methods, e.g. tapping with a hand, which are often not described properly, and more important, are inherently prone to error variation. Here we

provide details on the construction and manufacturing of the hardware, the tapping apparatus, that allows us to induce vibration in a consistent manner. The hardware is designed to retrofit a popular and cheap fish tank sold by most pet stores in North America, the 40-liter glass aquarium. In addition to providing hardware design and manufacturing details, we also present data on how zebrafish respond to the vibration stimulus, and we demonstrate effective induction of fear responses. We argue that the cheap, simple, yet effective apparatus with which robust fear responses may be induced will be a good alternative to currently existing methods in zebrafish fear and anxiety research.

Materials and Methods

Simple Pendulum System

Our hardware is designed with a simple idea in mind: An object (a rubberized metal ball) attached to a string is released to hit the side of the glass of the experimental tank (Fig. 1A). As the position and the weight of the ball are pre-determined, the force with which the ball hits the glass, and thus the vibration it generates, is consistent within and across studies every time this stimulus is delivered. The rod acts as the pivot point for the ball. Gravity acts on the weight of the ball as it is released, generating a set force with which the ball hits the side glass.

The calculation of the force generated is as follows:

$F_x = F_g * tan(\theta)$; where $F_g = weight\ of\ the\ ball * 9.81 m/s^2$

The metal ball employed in our study was covered with a thin rubber layer. When this ball hits the glass wall of the tank, the resulting force creates vibration within the water. The simple pendulum system results in multiple elastic collisions until the ball reaches its resting state. The repeated motion of the ball thus results in multiple and attenuating vibrations transmitted through the glass to the water of the experimental tank.

Bridge Design

The vibration-inducing bridge was designed using AutoCAD design and drafting software to create a 3D file format. The hardware was designed to fit on the top of a standard 40-liter glass tank (50 x 26 x 31 cm, length x width x height). The outside dimensions of the hardware were 51.0 x 27 x 32 cm (length x width x height) and included a bridge holder (2.5 x 2.5 x 15.0 cm, length x width x height) with triangular anchors (3.0 x 3.0 x 5.0 cm, length x width x height) at the bottom. The bridge holder's height of 15.0 cm ensured that it was tall enough to account for space needed to accommodate different lengths of strings used to fasten the weights (balls) on to the apparatus. Furthermore, the presence of the triangular anchors (Fig. 1B), with a thickness of 1.5cm and right-angle notches on the anchor interior, aligned the bridge with the corners of the testing tank and increased the grip of bridge to the testing tank. The entire holder was designed to hold a rod (length = 27cm, radius= 1.0cm) that would support a weight at either side of the testing tank. The rod's radius was determined with the material used in mind considering the weights it was expected to hold. Finally, two side rails (48.5 x 2.5 x 2.0 cm, length x width x height) were designed to support and connect both bridge holders.

The AutoCAD file was input into a 3D printer and printed using a high-quality 3D polymer filament called 'nGen' (coloFabb). This material is considered safe as it is expected to withstand humidity and the higher temperature in zebrafish facilities. After each bridge section was printed, they were securely attached together using superglue.

3D printer

The 'TAZ6' 3D-printer designed by the company 'LuluBot' was employed to print the vibration-inducing hardware. The accompanying program 'Cura LuluzBot' was used with 'Basic' settings. The 'Basic' setting has five different options that control how the object is printed, including Quality, Fill, Printing process, Support, and Filament. For Quality, layer height was set at 0.1 millimeters and the shell thickness was 1.0 millimeters. For Fill, bottom and top thickness were set as 0.1 millimeters and its fill density was set between 30 to 35%. The fill density may be adjusted depending on the weight of the metal ball. Printing speed of the vibration-inducing hardware was 50mm/s. Furthermore, the printing temperature, known as nozzle temperature, was set as 230°C. Its Bed temperature, where the object is printed, was set at 80°C. 'Support' was set as follows: the platform adhesion type was set as Brim, and the support type was set as touching the build plate. The filament diameter was set at 0.112 inches, and its flow at 100%.

Weight

A metal weight (m = 70.0 g, F = 0.70 N), a ball, was used to create vibrations within the water. This weight was chosen based upon preliminary studies that evaluated both the effect of the weight on fish behavior and on safety (potential damaging effect on the glass wall of the tank). To protect the glass, the metal ball was rubberized by immersing the ball into Plasti-Dip liquid (Plasti Dip International, Blaine, MN, USA) that created a 0.5 mm thick layer of rubber on the surface of the ball. The metal weight was then attached to the bridge system using a nylon fishing line (length = 32.5 cm) so that the weight could hit around the center of the side glass of the experimental tank.

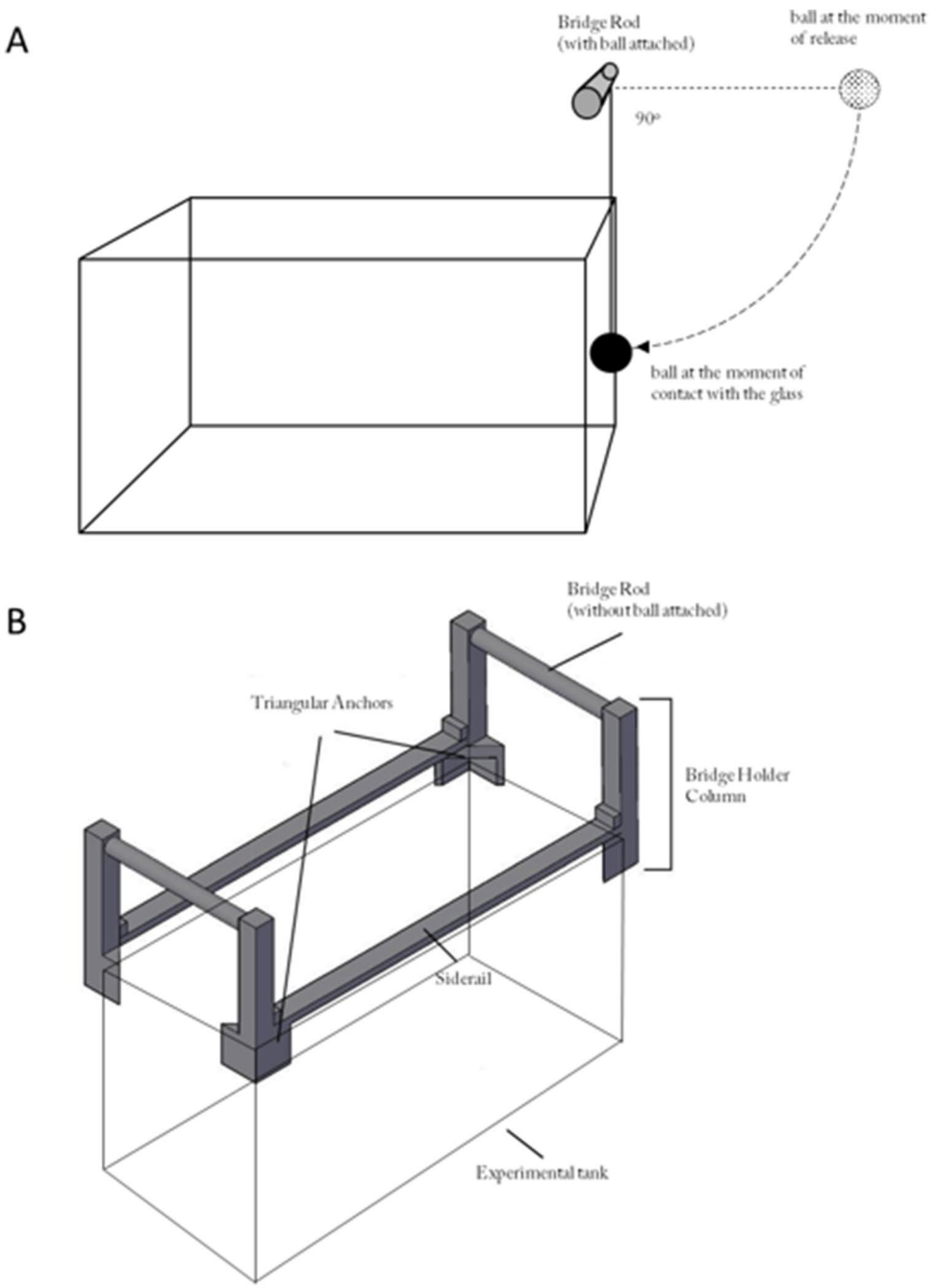

Fig. 1 Schematic diagram showing the concept of the tapping apparatus (**A**) and the design of the tapping bridge that was manufactured using a 3D printer (**B**). For further details, please see methods

Height/Angle of Drop

The height from which the ball is released determines the terminal velocity and thus the force with which the ball hits the glass. The vibration inducing hardware and the weight of the ball were established so that the ideal height of drop would correspond to the height at which the bridge rod was (Fig. 1A). That is, if the vertical line of the string, resting position of the ball, is considered 0°, the angle of drop was 90°. This bridge system requires the experimenter to manually raise the ball and release it at the desired time from the predetermined height. However, the system is easily modifiable with the addition of servo-motors rolling back the ball, and an electromagnet holding and releasing the ball at desired times, all controlled remotely, hardware and software solutions we are currently developing.

Animals and Housing

To experimentally test the effectiveness of the vibration inducing apparatus we exposed adult zebrafish to a single ball drop event. Twenty-four adult zebrafish (6 months of age, 50-50 male-female) of the AB strain bred and raised in University of Toronto Mississauga were used for behavioral testing. Zebrafish were housed in 40 L tanks (20 fish per tank) in system water. System water was made by deionizing municipal water via reverse osmosis, and subsequently reconstituted by supplementing with 100 mg/L Instant Ocean Sea Salt. System water was maintained at optimal water parameters: pH: 6.5–7.5; conductivity: 200-600 μS; temperature: 27-30°C. The water was filtered using overhang filters (Penguin 150B Bio Wheel Power Filter, Marineland Spectrum Brands Pet, LLC, Blacksburg, VA, USA). The fish were held and tested in the same room. The rooms operated on a 14:10 light-dark cycle using fluorescent light tubes that turned on at 07:00 and off at 21:00. Zebrafish were fed on an alternating diet of commercial flake food (Spirulina and Omega Flakes mix at 1:1 ratio) and freshly hatched brine shrimp (*Artemia salina*) nauplii. Zebrafish were fed three times a day. Feeding took place at least an hour before behavioral testing. Behavioral testing was conducted between 09:00 and 19:00h.

Behavioral testing

The test tank in which the experiments were conducted was a standard 40-liter aquarium (50 x 26 x 31 cm, length x width x height) available from a variety of vendors, including most pet stores in North America. We chose this tank because we have been using it in most of our anxiety/fear research studies with the zebrafish (Gerlai, 2020b), and because its larger volume allows zebrafish to exhibit a broad spectrum of passive and active avoidance reactions as well as other behavioral responses (Gerlai , 2020b, also see Levin, 2011). This experimental tank was filled with system water matching the water of the home tanks of the experimental fish. The back and bottom of experimental tank was covered with white corrugated plastic sheets for improved visibility and contrast necessary for tracking of the movements of zebrafish and to provide a consistent background. The vibration inducing apparatus was placed on top of the experimental tank.

Zebrafish were individually placed into the experimental tank for a period of 60-minutes. At the 50-minute mark, the ball was released at 90°, creating an attenuating series of "taps" that ended with a ball staying at its resting and motionless position within about 3 seconds after its release. Zebrafish behavior in response to the taps was recorded for an additional 10-minutes. We chose the lengths of these recording periods for the following reasons. The first 50 min long no-tap period allowed us to observe temporal changes in the behavior of zebrafish due to habituation to novelty. This long (pre-stimulus) habituation period also allowed zebrafish to reach a stable behavioral state in which anxiety and fear responses were expected to be absent by the time of tapping stimulus delivery. In other words, this long period allowed us to contrast the behavior of fully habituated zebrafish to the behavior induced by the delivery of the aversive stimulus. The length of the post-stimulus monitoring period was chosen to be 10 minutes because our preliminary studies suggested return of all behaviors to the fully habituated baseline within this length of time. In fact, we found most behaviors returned to baseline within 2-3 minutes post-stimulus, a temporal change that is also well documented by the line graphs shown.

All zebrafish were tested only once. The side from which the weight was released was randomized across subjects. The behavior of the zebrafish was recorded using a JVC GZ-MG50U digital video camera viewing the front glass of the tank. All fish remained healthy and unharmed and were returned to their holding tank after experimentation for future breeding.

Quantification of behavior

Video-recordings were replayed, and swim paths of the experimental fish were quantified with the video-tracking software application Ethovision XT 12.1 (Noldus Info Tech, Wageningen, The Netherlands). We have extracted and analyzed seven swim path parameters that have been employed for the quantification of fear and anxiety or antipredatory responses in zebrafish (for reviews see Gerlai, 2020b; 2013; 2010b; Kalueff et al., 2013; Stewart et al., 2012; Levin, 2011; Maximino et al., 2010).

Velocity (cm/sec) measures the speed with which fish swam. In previous studies, general activity of zebrafish has also been measured by equivalent parameters such as total distance travelled, "ambulation score" or "shuttling frequency" during a set observation period (e.g. Blaser & Gerlai, 2006). Velocity, and values of these other related measures too, has been found to decrease under aversive conditions in zebrafish, including during the first few minutes of being exposed to a novel test tank as well as in response to the delivery of aversive stimuli (Ahmed et al., 2012; Luca & Gerlai, 2012a, 2012b; Seguin et al., 2016; Gerlai et al., 2009).

Absolute turn angle (degree) quantifies the amount of turning irrespective of direction. Turning may be associated with a variety of behavioral responses, but it has been found to strongly correlate with erratic movement (or zig-zagging) (Gerlai et al., 2009), a direct response to the delivery of aversive stimuli (Ahmed et al., 2012; Luca & Gerlai, 2012a, 2012b; Seguin et al., 2016; Gerlai et al., 2009).

Duration of time on the tapping side (sec) is the time fish spent in the third of the segment (demarcated by an imaginary vertical border) closest to the side of tank where the tapping occurred. We expected zebrafish to be able to localize the tapping stimulus using both their lateral-line as well as their visual systems. Thus, we hypothesized zebrafish would avoid the tapping side and spend more time away from this side after the delivery of the tapping stimulus, as such an avoidance reaction has been successfully quantified using this measure in response to a variety of aversive stimuli in zebrafish (Ahmed et al., 2012, Luca & Gerlai, 2012; Blaser & Gerlai, 2006). We also chose this parameter because duration of time can easily be measured using a stop watch, and thus can be a useful measure in laboratories where expensive video-tracking systems are unavailable.

Distance to the tapping side (cm) is the average distance the fish was from the vertical line representing the side glass of the tank where the tapping occurred. This measure can be precisely quantified only with video-tracking systems. Although it is somewhat redundant with duration of time on the tapping side, it is expected to be a more precise measure of how much the fish tried to avoid the tapping side, and it has been successfully used to quantify predator avoidance reactions in zebrafish (Ahmed et al., 2011; Ahmed et al., 2012; Seguin et al., 2016).

Intra-individual variance of distance to the tapping side (cm^2) quantifies how much each fish changed their horizontal position relative to the vertical line representing the side glass of the tank where the tapping occurred. We emphasize that this measure represents temporal variance of where the individual experimental fish swam relative to the tapping side, i.e., it quantifies horizontal exploration of the tapping versus the other side of the tank. Increased intra-individual temporal variance of distance has been found as a direct and immediate response to the appearance of aversive stimuli, while reduction in such measures has been shown in case of consistent avoidance lasting several minutes following the delivery of such stimuli (Gerlai et al., 2009).

Distance from bottom (cm) measures how far the fish were from the horizontal line representing the bottom glass of the tank. Although somewhat controversial and stimulus or context dependent (e.g. Luca & Gerlai, 2012), distance from bottom has been found to decrease under aversive conditions in zebrafish (Levin, 2011; Luca & Gerlai, 2012a, 2012b; but see Ahmed et al., 2011; Ahmed et al., 2012; Gerlai et al., 2009).

Intra-individual variance of distance from bottom (cm^2) measures how much each fish changed their vertical position relative to the horizontal line representing the bottom glass of the tank. This parameter quantifies temporal variance of the location of the fish in terms of vertical position, i.e., it is a measure of vertical exploration. Vertical exploration has been shown to diminish under certain aversive conditions or in response to the delivery of an aversive stimulus (Ahmed et al., 2011; Ahmed et al., 2012; Gerlai et al., 2009).

Statistical analysis

All behavioral measures are expressed for 1-minute intervals of the 60 min recording sessions, and the data were analyzed using SPSS (version 24 written for the PC). Sex differences were not found in any behavior, and thus data for the two sexes were pooled for all behaviors. Univariate repeated measures ANOVA (with Interval as a 60-level repeated measure factor) was used to investigate whether there was any temporal change in the behavior of the fish. In order to investigate the effect of tapping, we also graphed and analyzed the pre-tap and post-tap 1-minute interval performance of the fish. To statistically compare the pre- and post-tap performance, we employed two-tailed paired t-test. Last, in some behaviors the tapping induced change was transient lasting only a minute or two, while in others the change appeared to last a few more minutes. Because the repeated measure design (intervals) cannot be analyzed using multiple range post-hoc methods like the Tukey HSD test, to avoid committing type 1 error, instead of comparing several 1-minute intervals with each other, we averaged three intervals right after the tap and the subsequent three 1-minute intervals close to or at the end of the recording session, and compared these averages using paired t-test. Furthermore, because we had seven behavioral variables, to reduce the experiment-wide error rate, i.e., to minimize type 1 error, we used the Bonferroni-Holm sequential correction method and report the corrected p-values. Significance was accepted for all statistical tests when the probability (p) of the null hypothesis (no effect) was found not larger than 5%, i.e., when $p =< 0.05$.

Results

The vibration inducing apparatus worked as expected. The rubberized metal ball created consistent vibrations as it was hitting the side glass of the experimental tank. The first collision had the largest force and was followed by 3 attenuating collisions occurring within a 3 second interval. As glass does not insulate well against vibrations or sound, we expected this method to generate waves in the water likely detectable as lateral line cues, and perhaps also as auditory cues, by zebrafish. We have not quantified the specific frequencies the collision between the ball and the glass wall generated in the water. However, the behavioral responses of the experimental fish suggested that they perceived these stimuli and also that they responded to them as one would expect in case of aversive, fear inducing, stimulation.

Upon placing the single experimental fish into the test tank, they started to swim relatively slowly and increased their swim speed gradually during the first half hour of the recording session, after which their speed appeared to plateau, with some fluctuation, until the tapping episode. In response to tapping, velocity immediately plummeted and subsequently slowly recovered (Fig. 2).

These observations were supported by our statistical analyses. ANOVA found the effect of Interval significant ($F(59, 1357) = 3.827$, $p < 0.001$) suggesting time dependent change in velocity. Paired t-test confirmed that during the 1-minute interval that followed the tap, zebrafish significantly reduced their velocity, i.e., swam more slowly, compared to the minute that preceded the tap (Fig. 2B, $t = 4.275$, $df = 23$, $p < 0.001$). Following the tapping episode, the reduced velocity slowly recovered by the end of the recording session. To statistically analyze whether this latter temporal trajectory represented a significant change, i.e., whether the low swimming speed induced by the tapping recovered and increased with time, we averaged the three 1-minute intervals right after the tapping episode, and the last three 1-minute intervals of the session and compared these averages using a paired t -test. The result showed a significant return from the reduced swim speed to a higher baseline level speed ($t = 3.818$, $df = 23$, $p < 0.001$).

Absolute turn angle appeared to be somewhat elevated at the beginning of the 60-minute recording session declining with some fluctuation by the second half of the session (Fig. 3). Delivery of the tapping stimulus appeared to induce a robust and immediate elevation of turn angle and a subsequent reduction within 4 minutes. These observations were supported by our statistical analyses. ANOVA found the effect of Interval significant ($F(59, 1357) = 1.889$, $p < 0.001$) suggesting time dependent change in Turn angle. Paired t-test confirmed that during the 1-minute interval that followed the tap zebrafish significantly elevated their turn angle, i.e., turned more, as compared to the minute that preceded the tap (Fig. 3B, $t = 2.587$, $df = 23$, $p = 0.016$). The increase of absolute turn angle appeared to last for only 3 minutes and subsided subsequently. To statistically analyze whether this temporal trajectory represented a significant change, we averaged the three 1-minute intervals right after the tapping episode, and the subsequent three 1-minute intervals and compared these averages using a paired t-test. The result showed a significant return from the elevated turn angle to a lower level within this 6-minute period ($t = 2.167$, $df = 23$, $p = 0.041$).

The duration of time spent on the tapping side, although with some variation across intervals, appeared to be stable until the tapping event. Tapping appeared to robustly reduce the value of this measure (Fig. 4). ANOVA found the effect of Interval significant ($F(59, 1357) = 2.319$, $p < 0.001$), and paired t-test demonstrated a significant reduction of time spent on the tapping side between the 1minute intervals pre- and post-tapping ($t = 4.906$, $df = 23$, $p < 0.001$). After the tapping event, the time on the tapping side gradually increased till the end of the behavioral recording session, a change whose significance is demonstrated by the comparison of the average of three 1-minute intervals right

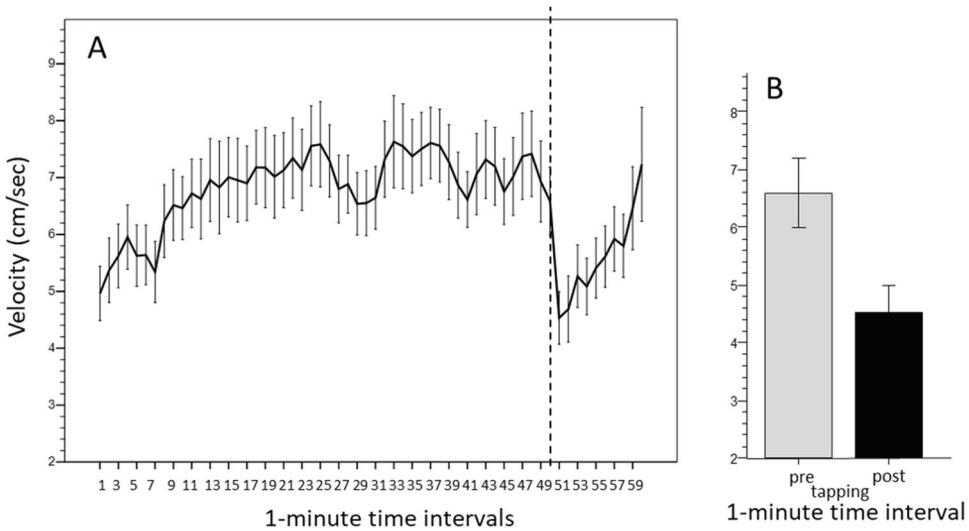

Fig. 2 Velocity (swim speed) expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the gradual increase of velocity with time up to the point of tapping. Also note the immediate and robust reduction of velocity in response to tapping, a change in behavior that gradually recovered by the end of the behavioral recording session. For further details and results of statistical analyses, see Results

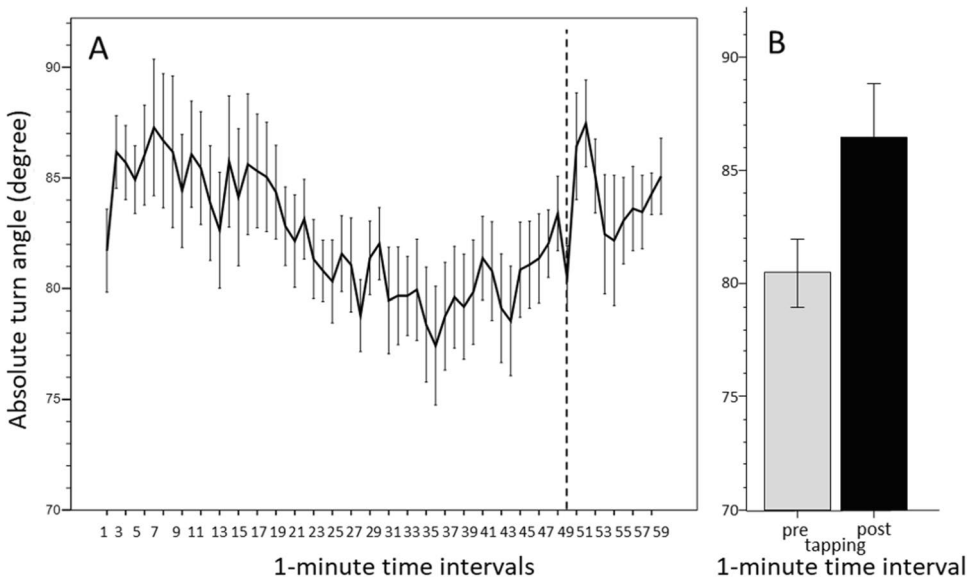

Fig. 3 Absolute turn angle expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the gradual decrease of turn angle with time up to the point of tapping. Also note the immediate and robust increase of turn angle in response to tapping, a change in behavior that rapidly recovered within a few minutes. For further details and results of statistical analyses, see Results

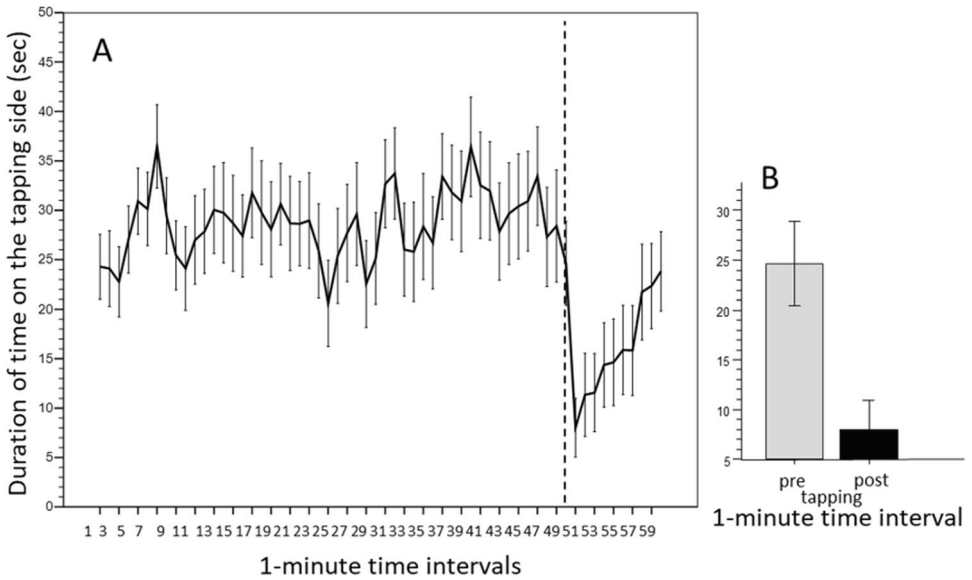

Fig. 4 Duration of time on the tapping side expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the immediate and robust reduction of time spent on the tapping side in response to tapping, a change of behavior that gradually recovered by the end of the behavioral recording session. For further details and results of statistical analyses, see Results.

after tapping and the average of three 1-minute intervals at the end of the session ($t = -2.689$, $df = 23$, $p = 0.013$).

Distance to tapping side appeared to remain approximately at around 20-25 cm throughout the session before tapping occurred (Fig. 5), suggesting that before tapping the fish showed no preference for either side and were on average in the middle of the 50 cm long tank. However, in response to the tap the distance of the experimental zebrafish rapidly increased to about 35 cm, and remained elevated above 25 cm for several minutes. ANOVA found a

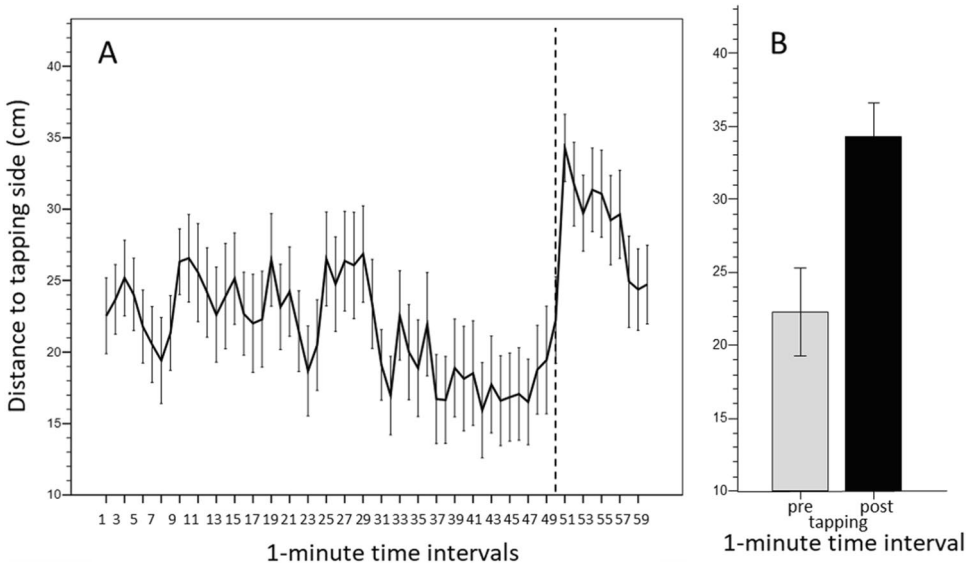

Fig. 5 Distance to tapping side expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the immediate and robust increase of distance to the tapping side in response to tapping, a change of behavior that gradually recovered by the end of the behavioral recording session. For further details and results of statistical analyses, see Results

significant Interval effect ($F(59, 1357) = 2.399$, $p < 0.001$). Paired t-test confirmed that during the minute after the tap zebrafish were significantly further away from the tapping side compared to where they were during the minute preceding the tap ($t = 4.458$, $df = 23$, $p < 0.001$). Similarly to what we found for the duration on the tapping side, the distance to tapping side also showed a gradual change (decrease) from the tapping induced elevation of distance till the end of the behavioral recording session, a change whose significance is demonstrated by the comparison of the average of three 1-minute intervals right after tapping and the average of three 1-minute intervals at the end of the session ($t = -2.345$, $df = 23$, $p = 0.028$).

The intra-individual variance of distance to tapping side tended to diminish with time, but also showed a robust increase in response to the tap, a response that was rapid and transient (Fig. 6). ANOVA found the Interval effect significant ($F(59, 1357) = 4.914$, $p < 0.001$), and paired t-test demonstrated that the fish varied their distance to the tapping side significantly more during the 1-minute interval right after as compared to just before the tapping event ($t = 2.201$, $df = 23$, $p = 0.038$).

Distance from bottom appeared to show some temporal variation across the intervals with a slight trend towards increase as the test session progressed (Fig. 7). However, tapping did not appear to have an effect on this measure. ANOVA found the Interval effect significant ($F(59, 1357) = 1.752$, $p < 0.001$). However, the difference between the pre- and post-tap 1-minute intervals was found non-significant ($t = 0.866$, $df = 23$, $p = 0.395$).

The intra-individual variance of distance to bottom, a measure of vertical exploration, however, did appear to respond to tapping (Fig. 8). Fish robustly decreased their vertical exploration after the tapping event for a couple of minutes. ANOVA confirmed significant temporal changes (Interval $F(59, 1357) = 3.578$, $p < 0.001$) and paired t-test demonstrated a significant difference between the 1-minute intervals pre- and post-tap ($t = 4.761$, $df = 23$, $p < 0.001$).

Discussion

Here we presented design and construction details of a novel apparatus, the tapping hardware, and discussed the procedure of how to use this apparatus to induce vibrations in the water of zebrafish with the goal to elicit fear responses in the adult fish. We emphasize that the apparatus and procedure are simple, yet it can deliver consistent aversive stimuli. The simplicity of the design and procedure and the consistent nature of the stimulus it delivers should make results obtained with this paradigm replicable and thus the method useful in zebrafish research aimed at the analysis of fear and/or anxiety.

Using this apparatus, we have found the tapping stimulus to induce numerous changes in the behavior of zebrafish that we could detect by quantifying swim path parameters of the fish using video-tracking analysis. Notably, these changes occurred immediately following the tapping episode and included reduction of swim speed, increased turning, enhanced distance from the side where tapping

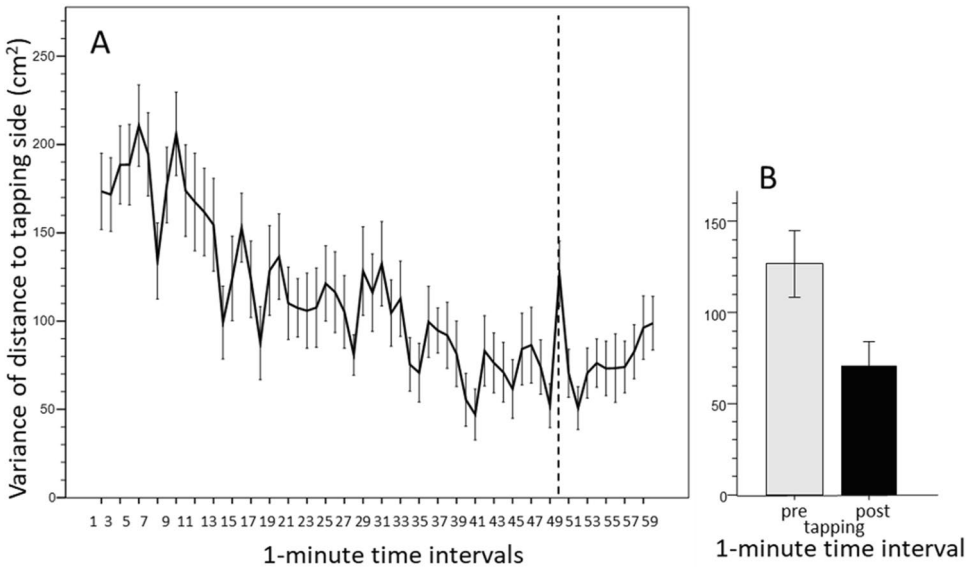

Fig 6 Intra-individual temporal variance of distance to tapping side expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the gradual decrease of intra-individual temporal variance of distance to tapping side as the behavioral recording session progressed to the point of tapping. Also note the immediate increase of the variance of distance to tapping side in response to tapping, a small but significant change that rapidly recovered. For further details and results of statistical analyses, see Results.

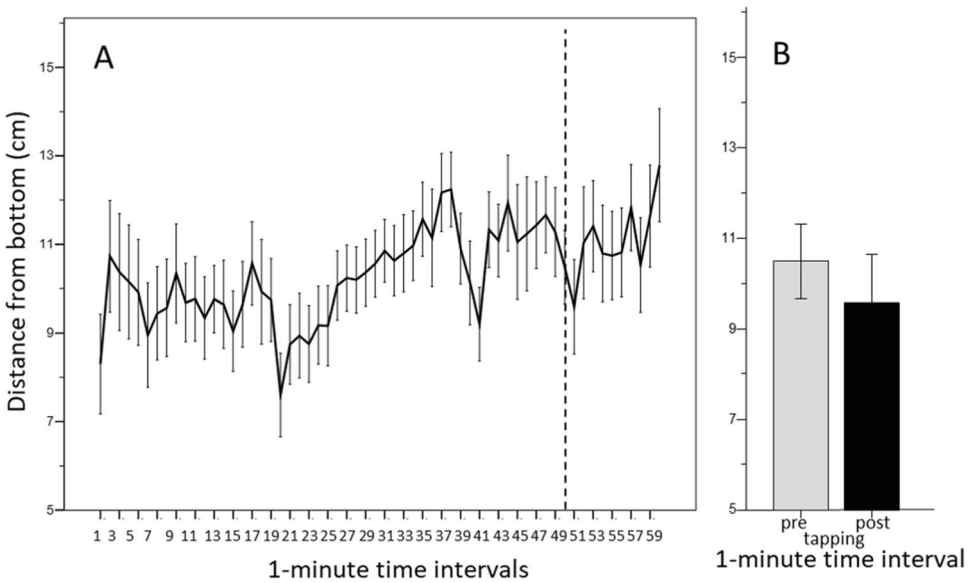

Fig. 7 Distance to bottom expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the temporal fluctuation of distance to bottom and the lack of significant response in this behavior to tapping. For further details and results of statistical analyses, see Results.

occurred, increased intra-individual temporal variance of distance to tapping side and decreased vertical exploration. Reduction of swim speed and reduced vertical exploration (i.e., increased passivity), increase of turning (e.g., erratic movement), and avoidance of the location where aversive stimuli are present are all signs that have been found to be associated with fear or antipredatory behavior in zebrafish (Ahmed et al., 2011; Bass & Gerlai, 2008; Gerlai, 2010b;

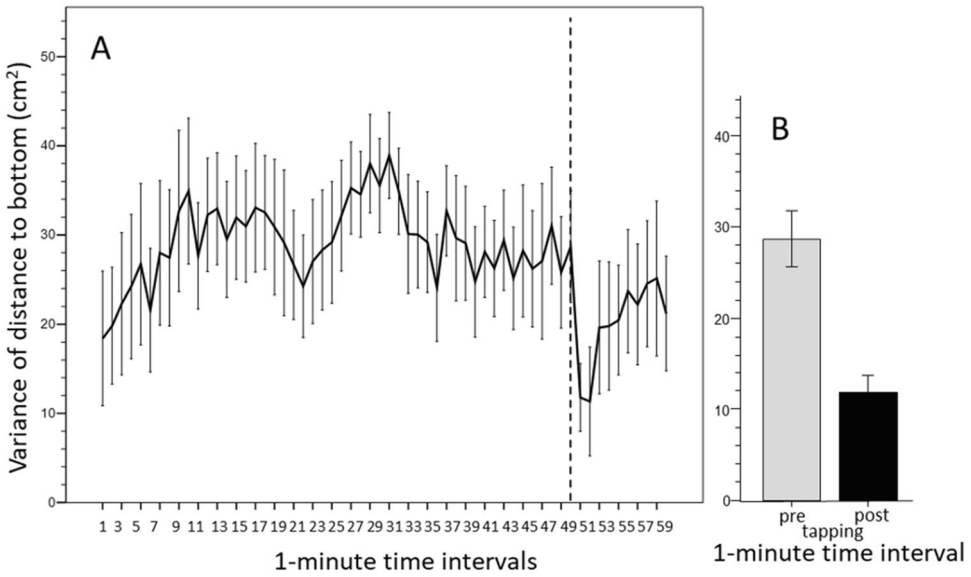

Fig. 8 Intra-individual temporal variance of distance to bottom expressed as a function of time (1 min intervals) (Panel **A**) and the same behavioral measure shown for the pre- and the post-tapping one minute interval (panel **B**). Mean ± S.E.M. are shown. The time point of tapping is indicated by the dashed vertical line. Note the immediate decrease of the variance of distance to bottom in response to tapping, a significant change that gradually recovered by the end of the recording session. For further details and results of statistical analyses, see Results

Luca & Gerlai, 2012; Maximino et al., 2010; Parra et al., 2009; Speedie & Gerlai, 2008; Stewart et al., 2012). The immediate and temporary increase of intra-individual variance of distance to tapping side is also consistent with fear (Gerlai, 2020b). This response means that for a short period following the tapping, the fish swam close to and then moved away from the tapping location, i.e., they increased their variance of distance relative to the tapping side, a behavior reminiscent of predator inspection described in a variety of fish species (Pitcher, 1992; Dugatkin & Godin, 1992; Gerlai 1993; Maximino et al., 2010). In summary, the behavioral changes induced by the delivery of the tapping stimulus are consistent with elevation of fear. This conclusion is also supported by the temporal changes observed in several behaviors between the time of introduction of the experimental fish to the novel test tank and the start of delivery of the tap. Briefly these habituation trajectories suggest that reduced velocity, increased turn angle, increased variance of distance to the side and reduced variance of distance to bottom are all associated with fear (novelty and/or human handling induced fear in this case). Thus, although pharmacological validation of the tapping paradigm with anxiogenic and anxiolytic drugs has not been performed, the above cited published results on the behavioral responses of zebrafish to aversive stimuli suggest that the tapping stimulus is an aversive, fear inducing cue and that the tapping paradigm is effective. We also note that psychopharmacological validation of a novel fear paradigm is a complex effort in all species including the zebrafish (de Abreu et al., 2021), especially if the task is expected to detect novel anxiolytic drug candidates with previously unidentified modes of action, a long term goal we hope the tapping paradigm will fulfill.

Interestingly some, but not all, of the behavioral changes elicited by the tap took several minutes to recover. For example, swim speed only gradually increased from the tapping induced low velocity level over the entire 10 minutes post-tapping period. Distance to tapping side also showed a similar change during the post-tapping period, a gradual reduction from the highest value of the post-tapping 1-minute interval. Tapping induced changes in other behaviors recovered faster. For example, absolute turn angle and intra-individual variance of distance to bottom (vertical exploration) returned to pre-tapping levels within 3 minutes, and intra-individual distance to tapping side within a minute. Interestingly, distance from bottom, a behavior often considered an index of anxiety (Levin et al., 2007), was not significantly affected by tapping, a result that supports previously reported negative findings (Blaser & Gerlai, 2006; Gerlai et al., 2009). The behavior specific temporal trajectories we report here confirm a potentially complex behavioral repertoire associated with fear in zebrafish. In other words, the fear response of zebrafish is likely multidimensional, and depending on the nature of threat, the type and strength of the aversive stimulus and how long ago it was perceived, zebrafish may exhibit a variety of species-specific responses, a conclusion that is in accordance with studies investigating the effects of different aversive stimuli (e.g., Luca & Gerlai, 2012; Gerlai, 2010b). One may also

argue that perhaps the rapidly recovering behaviors after tapping represent fear responses, whereas the slowly recovering ones anxiety responses (Perusini & Fanselow, 2015). The complex, and potentially context and stimulus dependent fear responses, as well as the distinct behavior specific habituation temporal trajectories we view as an opportunity that may, in the future, allow investigators to identify novel anxiolytic compounds with a variety of underlying biochemical mechanisms.

Although robust fear responses could be elicited with the novel tapping paradigm, numerous aspects of the tapping stimulus and procedure will need to be explored to fully understand and optimize the method. For example, although the most salient aspect of the tapping stimulus is likely the vibration it induces in the water, we do not yet know what frequencies the zebrafish may perceive. They could use their hearing to detect higher frequency vibrations, i.e., sound waves, or could use their lateral line to detect lower frequency waves. It is also possible, although less likely, that the experimental fish did not respond to vibration at all and instead reacted to the visual stimulus, the sight of the moving ball. Irrespective of these basic unanswered questions, however, the apparatus and procedure worked and did induce robust fear responses.

Other aspects of the tapping procedure may also need to be explored. We note that tapping was delivered after 50 minutes by which time the experimental zebrafish have been well habituated to their test tank. We decided on this long habituation procedure as we wanted to obtain the potentially biggest contrast between a well habituated fish and one that is afraid. However, whether tapping may have had a different effect if it had been delivered during the first few minutes of the session, i.e., before the fish were habituated and before novelty induced anxiety levels got reduced, is not known. It is also unknown whether multiple tapping episodes could lead to sensitization or habituation to the tapping stimulus. Last, optimization of the tapping stimulus has not been attempted. For example, the number of times tapping is delivered, the inter-delivery interval, the strength of the vibration (weight of the ball), whether it should be used in conjunction with other aversive stimuli, e.g., sight of predators, could all be investigated in the future to enhance the utility and efficiency of the tapping paradigm.

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Authors' contributions RG conceptualized and supervised the study, analyzed the data, interpreted the results and wrote the paper. YR designed the hardware, programmed/set up the 3D printer, manufactured the hardware. MN, BM, RA conducted the behavioral experiments, recorded and extracted the behavioral data. All authors read the paper and contributed to finalizing the publication.

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Data Availability Data are available from the corresponding author upon request.

Code Availability Not applicable

Declarations

Ethics approval Research described in this study has been approved by the Local Animal Care Committee (University of Toronto Mississauga, LACC) and is in accordance with University of Toronto, Provinical (Ontario) and Federal (Canada) laws and guidelines for the ethical and humane use of animals in research.

Consent to participate Not applicable

Consent for publication All authors have read the paper and have agreed to publish it.

Conflicts of interest/Competing interests Authors declare no conflict of interest or competing interests.

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