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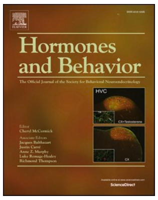

Life-threatening, high-intensity trauma- and context-dependent anxiety in zebrafish and its modulation by epinephrine

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ABSTRACT

Trauma-related psychopathology transpires in some individuals after exposure to a life-threatening event. While aberrant adrenergic processes may contribute to this, a clear understanding of how said processes influence trauma-related conditions, remain inadequate. Here, we aimed to develop and describe a novel zebrafish (*Danio rerio*) model of life-threatening trauma-induced anxiety that may be representative of trauma related anxiety, and to evaluate the impact of stress-paired epinephrine (EPI) exposure in the model system. Four groups of zebrafish were each exposed to different and unique stress-related paradigms, i.e., i) a sham (trauma free), ii) high-intensity trauma (triple hit; THIT), iii) high-intensity trauma in the presence of EPI exposure (EHIT), and iv) EPI exposure on its own, all applied in the presence of a color context. Novel tank anxiety was subsequently assessed at 1, 4, 7 and 14 days after the traumatic event. The present results demonstrate that 1) through day 14, THIT or EPI exposure alone induced persistent anxiety-like behavior, 2) EHIT blunted the delayed anxiety-like sequalae associated with severe trauma, 3) exposure to a trauma-paired color context prior to anxiety testing bolstered the subsequent anxiety-like behavior of THIT, but not EHIT -exposed fish, and 4) despite this, THIT- and EPI-exposed fish showed a lesser degree of contextual avoidance behavior compared to sham- or EHIT-exposed fish. These results indicate that the stressors induced long-lasting anxiety-like behavior reminiscent of post trauma anxiety, while EPI displays complex interactions with the stressor, including a buffering effect to subsequent exposure of a trauma-paired cue.

1. Introduction

Stress and anxiety are intricately linked to adrenergic mechanisms (Adell et al., 1988; Fink, 2016). Rapid stress- or trauma-associated norepinephrine (NE) and epinephrine (EPI) release prepares an organism faced with potential threats to effectively respond to such threats (Björkstrand et al., 2017; Stegmann et al., 2019). While the effects of both peripherally and centrally released NE in mammals are vital at the time of experiencing a traumatic life event, perpetuating dysregulated noradrenergic processes may contribute to the manifestation of chronic post-trauma anxiety, panic attacks, and post-traumatic stress disorder (PTSD) (Goddard et al., 2010). This would explain to some extent the potential therapeutic benefit of sympatholytics, e.g. alpha-1 and beta adrenoceptor antagonists observed in some individuals suffering from PTSD (Koek et al., 2016; North et al., 2018). However, since most trauma-exposed individuals remain resilient against developing later-life trauma-related psychopathologies (with PTSD for example having a one-year prevalence of 2.3–9.1 % in the civilian population; Schein et al. (2021)), our understanding of how central NE and peripheral EPI secretion uniquely contribute to normal and dysfunctional fear-related responses, remains elusive.

To this end, animal models of anxiety-like behavior have revealed several insights into the processes underlying the manifestation of stress-induced psychobiology (Daskalakis, Yehuda, & Diamond, 2013; Richter-Levin, Stork, & Schmidt, 2019). Modeling trauma-related conditions is complex (Daskalakis & Yehuda, 2014; Richter-Levin et al., 2019), likely

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because it is problematic to replicate circumstances of severe trauma that are reminiscent of an actual life-or-death event in mammals (Verbitsky et al., 2020).

Over the past decades, the zebrafish (*Danio rerio*) has emerged as a useful preclinical model species. With respect to trauma and stress, anxiety-like behaviors in zebrafish are motivated by similar environmental influences as in rodents, i.e., risks of predation (or death) and environmental novelty (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Stewart et al., 2010). Further, zebrafish present with evolutionary conserved, mammalian-like neurobiological systems, including noradrenergic, serotonergic, and dopaminergic neurotransmission (Agetsuma et al., 2010; Jesuthasan, 2012), as well as a cortisol-mediated stress axis (Ramsay et al., 2009) where interrenal cells in teleost fish are functionally homologous to the adrenocortical layer (Lee et al., 2018). What remains relatively unknown however, is how peripheral and central noradrenergic processes contribute to the manifestation of anxiety and stress in zebrafish. As alluded to above, in humans and other mammals, the central nervous system plays a pivotal role in the psychobiological stress response, exerting top-down influences on peripheral EPI release which subsequently contributes to fear and anxiety processing. While a similar dynamic might be true for zebrafish (Barcellos et al., 2007), zebrafish also convey stressogenic signals to conspecifics via the release of pheromones (i.e. alarm substance) into water (Quadros et al., 2016). Fish absorb most molecules via the gills, gastrointestinal system and skin (MacRae and Peterson, 2015), where they first reach the systemic circulation (Berghmans et al., 2007); this would suggest a peripheral, though psychobiological, trigger for the stress response in fish. Strikingly, the physiological and behavioral stress response of fish to these molecules alone seems to effectively mimic the responses of fish exposed to visual or other sensory stressors (Fontana et al., 2018). Against this background, it is possible that peripheral EPI secretion, and thus bottom-up mechanisms, might play a unique role in the manifestation of stress in zebrafish. Towards future applications of zebrafish as a translational model system for trauma-related research, an improved understanding of zebrafish stress physiology is vital.

While several attempts have been made at developing a zebrafish model of trauma-related conditions, e.g. PTSD, most employed a single non-life-threatening stressor, e.g. electric shock, which as of yet, has not been shown to yield a reproducible and sustained fear response (Oliveira et al., 2014; Parra, Adrian Jr., & Gerlai, 2009; Quadros et al., 2016). Further, since none of the stressors that are usually employed resemble a life-threatening event, PTSD-like behavioral sequalae (i.e., sustained, and context-dependent fear responses) have not materialized. Therefore, we aimed to develop and explore an ethologically relevant zebrafish model of persistent and context-driven anxiety that is contingent on experiencing a life-threatening event and that demonstrates temporal stability. Further, to investigate the role of peripheral adrenergic processes in the presentation of trauma-related behaviors in zebrafish, we also aimed to study how the administration of EPI (alone or paired with trauma) would modulate the behavioral fear response.

2. Materials and methods

To facilitate a clear understanding of the research process followed in this work, the methods will be stated and explained in the order in which they were applied.

2.1. Animals and housing

Initially, 295 adult shortfin wildtype (WT) zebrafish *(Danio rerio)* of both sexes (±30–40 mm in length) were selected for experimentation from the zebrafish colony housed at the National Aquatic Bioassay Facility (NABF) of the North-West University (NWU), Potchefstroom, South Africa (Ethics Approval No. NWU-00526-20-A5). The progenitor stock was originally obtained from a national South African importing supplier (WCB Imports Ltd., Pretoria, South Africa). All home tanks were kept and maintained in a fully automated system (ZebTec® Zebrafish Housing System, Tecniplast®, Varese, Italy) under the following conditions: water pH: 7, conductivity: 600 μS, temperature: 26 ± 1 °C, aeration: 7.2 mg O_2/L, and light cycle: 12 h (lights on at 06:00 and off at 18:00). Food (ZM-400® fry food, Zebrafish Management Ltd., Twyford, United Kingdom) was provided once daily.

Of the 295 zebrafish, 72 were used as social conspecifics and 96 as conspecific alarm substance (CAS) donors (see *"Preparation of CAS-releasing zebrafish"*). Conspecifics were housed with the experimental zebrafish (see below), while CAS donors were housed in groups of 24 in standard 8 L tanks, except if stated otherwise. The remaining 127 zebrafish were subdivided into four different experimental groups and housed ($n = 6$–8 per tank) in a modified version of the 8 L home tank that allowed for the separation and continuous monitoring, but not complete social isolation, of each of the eight zebrafish in individual cuboid compartments (Fig. 1; volume of at least 450 mL each). At each terminal end of the eight housing compartments, two additional compartments, with a volume of ±450 mL, have been constructed (green enclosures; Fig. 1) to house four conspecifics per home tank that were not used in further experimentation. These zebrafish, i.e., social conspecifics, have only been housed in the home tank so that each of the eight experimental zebrafish could continuously interact with three other zebrafish (one to each side and one directly in front). Compartments between the 12 zebrafish (eight experimental zebrafish and the four conspecifics) were separated by clear Plexiglas® grid walls, so that each zebrafish had sight of the three other surrounding zebrafish, while water and semiochemicals could flow and diffuse freely throughout the entire home tank (Fig. 1).

2.2. Group allocation

Irrespective of color preference (see *"Day -1: Color preference testing"*), the 127 experimental zebrafish were divided into four main groups, i.e. Group A (sham exposure; $n = 25$), Group B (THIT exposure; $n = 45$), Group C (EHIT exposure; $n = 45$), and Group D (EPI; $n = 12$) (Fig. 2). Further, to explore the effect of trauma-paired contextual (color) re-exposure on post-trauma anxiety-like behavior, the zebrafish in groups B and C, were further subdivided as follows: Groups Bi (no re-exposure; $n = 16$) + Bii (re-exposure; $n = 29$) and Groups Ci (no re-exposure; $n = 16$) + Cii (re-exposure; $n = 29$). On experimental day 0, testing commenced according to the different exposure paradigms (see paragraphs *"Day − 1: Color preference testing"* through *"Days 1, 4, 7 and 14: Investigating the immediate and delayed effects of trauma"*).

2.3. Day -1: color preference testing

Since zebrafish show a high degree of variability in terms of color preference (Roy et al., 2019; van Staden et al., 2019), we first established the individual and naturalistic color, that is red, blue, green, and yellow, preference for each of the 127 experimental zebrafish on day -1. To account for the potential aversive properties of color in subsequent post-trauma anxiety tests, the preferred color of each zebrafish has later been cued with the control/trauma-free (sham), triple high-intensity trauma (THIT), high-intensity trauma in the presence of EPI exposure (EHIT), or EPI-alone protocols. This was to ensure as far as possible that any potential color context-related avoidance behaviors observed in post-trauma anxiety assessments were not an artefact of the inherent and naturalistic aversion that some zebrafish may show to specific colors. Zebrafish were subsequently divided into different color preference groups and housed as per their color preference. Importantly, we regarded the number of zebrafish that preferred a specific color as irrelevant if exposure groups could be constituted with at least six to eight zebrafish per home tank that preferred the same color.

To determine the inherent, naturalistic color preference of each of the 127 experimental zebrafish, each zebrafish was assessed in a rectangle Plexiglas® plus maze comprising of four compartments [Fig. 3; 10

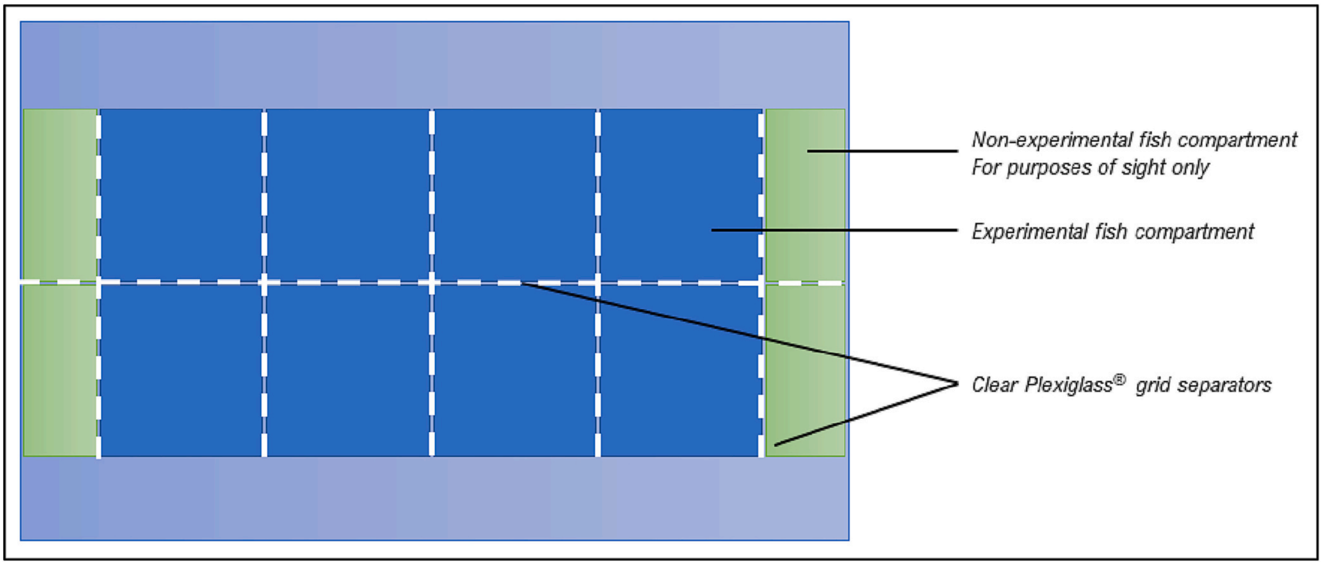

Fig. 1. Schematic representation of the home cage setup, allowing for simultaneous monitoring of individual fish and social interaction between fish Compartment dimensions adjusted to cage dimensions; 450 mL swimming volume per fish (Reed and Jennings, 2011).

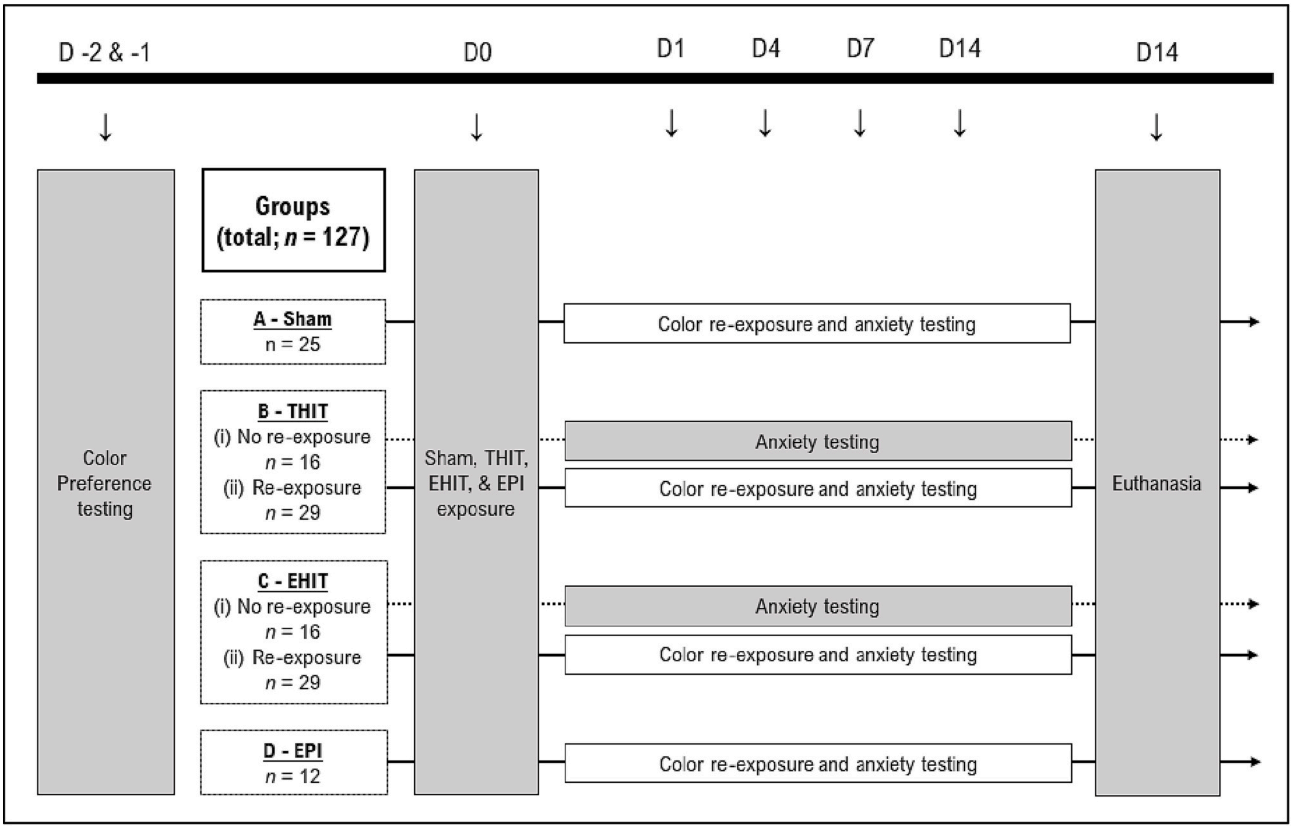

Fig. 2. Experimental layout
Sham: non-stressed group; THIT: triple high-intensity trauma; EHIT: triple high-intensity trauma in the presence of epinephrine; EPI: epinephrine; D in top bar: day.

cm (l) × 5 cm (w) × 15 cm (h) each] that were arranged perpendicularly around an opaque and neutral-colored removable start box (5 × 5 cm). The four arms of the maze were constructed from clear Plexiglas® so that different color sleeves could be attached on the outside of each arm. The maze was filled with system water up to a depth of 8 cm. On experimental day -1, each zebrafish was allowed to habituate in the start box for 1 min, after which the start box was removed. Zebrafish were allowed to explore the maze for a further 1 min in the presence of the four colors, i.e., red, blue, green, and yellow. Scoring then commenced for 5 min. Each zebrafish underwent two sessions on day -1, with the procedure being replicated precisely 3 h later. A digital video-camera (Panasonic® HC-V180) was used to record all experiments which were subsequently analyzed by means of EthoVision® XT 14 (Noldus® Information Technologies, Wageningen, The Netherlands) digital tracking

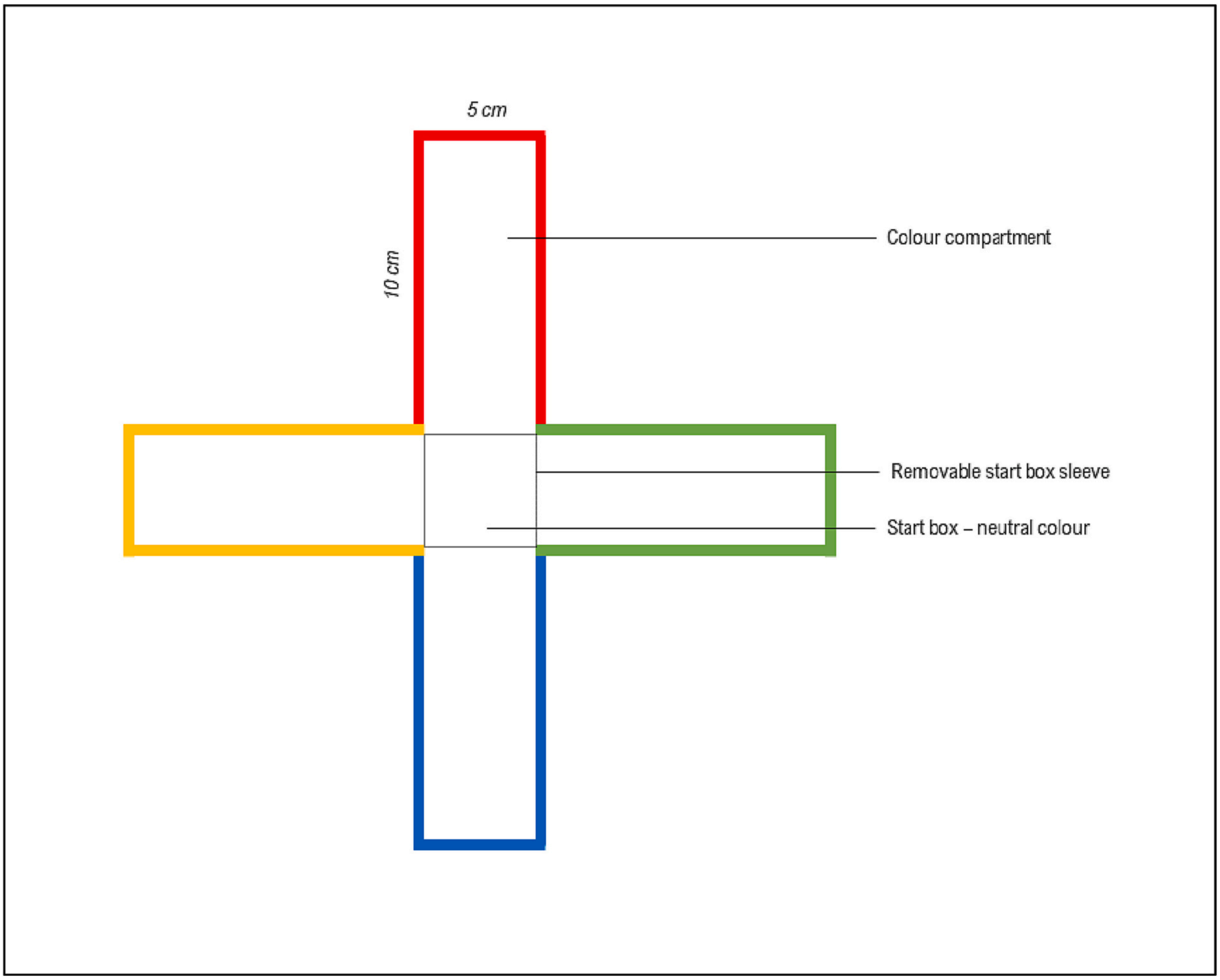

Fig. 3. Schematic representation of the color preference testing maze
Important: Only two arms were used in repeated contextual trigger and avoidance analyses on experimental days 1, 4, 7, and 14.

software.

On day -1, after both sessions were completed, the cumulative time spent in each of the respective color-paired arms were calculated and expressed as a percentage of the total assessment time, i.e., 10 min. The colored arm in which each zebrafish spent most of its time during the cumulative 10-min period, was recorded as the preferred color of each of the tested zebrafish. For each zebrafish, this color was subsequently applied in the sham, THIT, EHIT, or EPI protocols on experimental day 0 (see *"Day 0: THIT, epinephrine and sham exposure"*). The colored arm in which each zebrafish spent the least amount of time was recorded as the 'non-preferred color'. This color was used in combination with the preferred color in subsequent behavioral analyses (see *"Days 1, 4, 7 and 14: Investigating the immediate and delayed effects of trauma"*).

2.4. *Preparation of CAS-releasing zebrafish*

The chemical composition of CAS is still unknown and therefore exact concentrations are not determinable. For this reason, procedures previously described by Speedie and Gerlai (2008), Lima et al. (2016), Maximino et al. (2018) and Quadros et al. (2016), were used as a guideline. Where applicable, fresh CAS was extracted from eight donor zebrafish before each THIT- or EHIT protocol (during which eight experimental zebrafish were in turn simultaneously exposed to trauma) to ensure consistency throughout the protocols. After netting the CAS-donating zebrafish from their respective home tanks, they were euthanized by means of rapid decapitation with a scalpel, and the body of the fish immediately removed and placed on ice to prevent the skin being contaminated with blood as far as possible. Three shallow, long incisions were then made into the epidermis on each side of the zebrafish using a standard razor blade, again ensuring that as little blood as possible was introduced to the sample. All the donor zebrafish were then placed into a single new petri dish to which 10 mL of distilled water was added. After adding the euthanized donors to each of the trauma-paired compartments (see Fig. 4 and supplementary photograph), the remaining liquid in the petri dish was also dispersed in the apparatus.

2.5. *Chemicals and apparatus*

EPI was obtained from Fresenius Kabi® South Africa (Pty) Ltd. (Midrand, South Africa). All experimental and behavioral testing apparatus were custom manufactured according to specification at the NWU, South Africa. Behavioral analyses were performed with Ethovision XT 14 (Noldus® Information Technology, Wageningen, The Netherlands).

2.6. *Day 0: THIT, epinephrine and sham exposure*

2.6.1. *Apparatus*

The exposure apparatus consisted of 8 × 500 mL cuboid compartments [Fig. 4 and supplementary photograph; 7 cm (l) × 7 cm (w) × 10 cm (h)], arranged in a 4 × 2 configuration and constructed from clear Plexiglas®. Individual compartments were separated by clear Plexiglas®

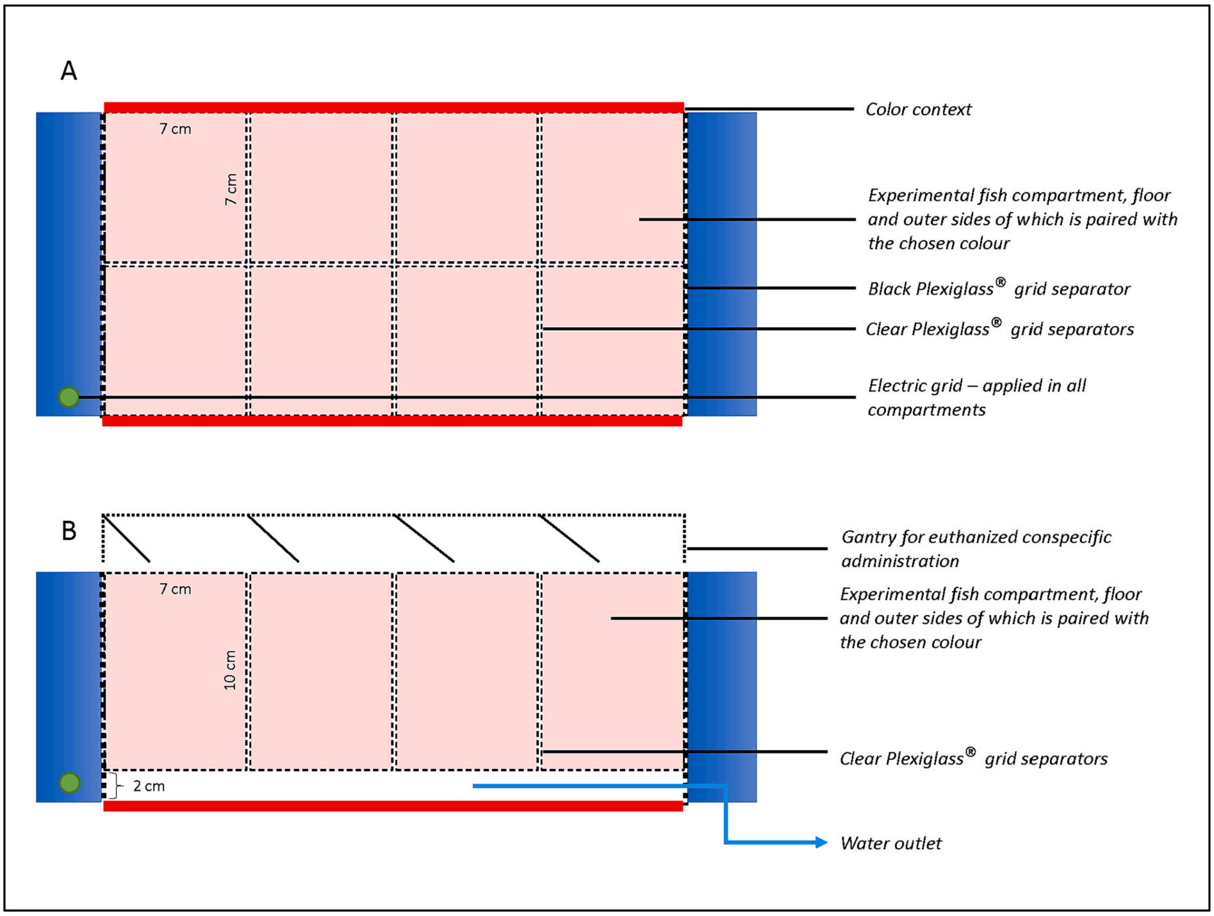

Fig. 4. Schematic representation of the exposure apparatus. (A) - top view; (B) - side view.

grids so that each zebrafish had sight of other fish. Further, water and semiochemical substances flowed and diffused freely, while electricity was conducted throughout the entire apparatus. However, zebrafish were not able to cross over the grids and move from one compartment to another. At the terminal ends of the 4 × 2 arrangement, the apparatus was flanked by two additional compartments, each 14 cm (l) × 3.5 cm (w) × 12 cm (h). These were used to house the electrical peripherals and drain water. A grid of electrical wiring that conducted electrical current (peak voltage: 600 V; peak current: 240 mA or 30 mA per compartment; discharge time: 100 ms; tank resistance: 8 × 20 kΩ parallel connections; Manuel et al. (2014)) uniformly throughout the entire apparatus, was affixed throughout the apparatus (Fig. 4).

The internal floor of the apparatus underneath the zebrafish-containing compartments (false floor) consisted of a Plexiglas® grid to facilitate water drainage. 2 cm below this floor, another solid floor was attached which sealed the apparatus to its walls (Fig. 4B). The space between the false inner floor and the solid floor was used as a conduit for water drainage. Last, during any of the given procedures (sham, THIT, EHIT, or EPI exposure), the outer walls of the compartments, as well as the entire floor of the arena, were covered in the preferred color of the respective exposed groups of zebrafish. In other words, zebrafish were exposed in groups of eight fish that showed the same color preference on day -1. If this color was red for a specific group, the apparatus was covered in red and so forth. This color served as the respective exposure contexts.

2.6.2. THIT exposure

The THIT procedure broadly consisted of a combination of previously applied stress methods used in zebrafish, that is i) water drainage up to a level of dorsal fin air-exposure in the exposure apparatus (Piato et al., 2011), ii) electric shock in the exposure apparatus (Manuel et al., 2014), and iii) CAS-exposure (Maximino et al., 2010; Speedie and Gerlai, 2008), which in this work, was paired with the actual presentation of a euthanized, CAS-releasing zebrafish, introduced into each of the eight compartments.

On any of the specific exposure days, eight zebrafish were gently netted from the home compartments and introduced to the exposure apparatus which was already wrapped in the preferred color (exposure context) of the sham- or trauma-exposed fish under investigation. At this time, the apparatus was filled to a depth of 5 cm (2 L) with home tank system water. Zebrafish were left as such for 1 min. Subsequently, the following sequential steps followed:

a. the water level was slowly drained (over the course of 3 min) up to the level of dorsal fin air-exposure (with 1.1 L remaining in the apparatus). All of the following steps then followed while zebrafish had their dorsal fins (or other parts of the body, while struggling) exposed to air for 2 min (Piato et al., 2011);
b. following water drainage, the apparatus was electrocuted for 100 ms (as described above), the shock having been administered to all fish simultaneously, followed by -
c. dropping euthanized, CAS-releasing zebrafish into each compartment and dispersing the remaining 10 mL of CAS-containing liquid into the entire apparatus to contextualize the low water levels and electric shock as a presumed life-threatening event;
d. the apparatus was then electrocuted again (as described above); and
e. zebrafish were left for 1 min before being transferred back to the home tank. The complete procedure took approximately 6 min to complete.

2.6.3. *EHIT exposure*

For the EHIT protocol, the same procedures as described above were followed. However, zebrafish were exposed to EPI (1 g/L; Iyengar et al. (2015)) in a replica of the home tank (to which 2 L of drug solution, constituted in system water, was added) for 1 h (van Staden et al., 2019) prior to being transferred to the THIT apparatus and subjected to the THIT stressor, as explained above. An exposure period of 1 h was chosen to ensure adequate pre-exposure absorption.

2.6.4. *EPI exposure*

EPI-only-exposed zebrafish were exposed to EPI (1 g/L) for 1 h as explained for the EHIT protocol. Afterward, zebrafish were also transferred to the THIT exposure apparatus, which was prepared as explained for the THIT protocol, albeit left to explore the apparatus in the absence of additional THIT stressors for 6 min. Zebrafish were then transferred back into their home tanks.

2.6.5. *Sham protocol*

For the sham protocol, zebrafish were also introduced to the color wrapped THIT exposure apparatus for 6 min, but in the absence of EPI or traumatic intervention. After each sham session, zebrafish were transferred back into their home tanks.

2.7. *Days 1, 4, 7 and 14: investigating the immediate and delayed effects of trauma*

2.7.1. *Repeated contextual (color) re-exposure vs. no re-exposure*

Where contextual re-exposure was applicable (Groups A, Bii, Cii, & D), zebrafish were gently netted from their home tanks and placed into the color preference testing maze (Fig. 3). However, during these assessments only two color-compartments opposite to one another were used as the other two arms were closed off. The colors applied in these experiments were the most and least preferred colors as determined for each fish on days -2 and -1, respectively (see *"Day -1: Color preference testing"*). To force exposure to the sham, THIT-, EHIT, or EPI -paired color context, zebrafish were left in the sham- or THIT-paired colored arm for 1 min. This was done to force contextual exposure by preventing zebrafish from avoiding the trauma-paired context from the outset of testing. The arm separator was then removed so that zebrafish could freely move between the two colors for 5 min. Color preference (or aversion) was measured by calculating the time spent in each of the color compartments as a percentage of the total time spent in both color compartments. Following completion of the assessment, fish were gently netted into the novel tanks (see below). This procedure was repeated on days 1, 4, 7 and 14 post-trauma-, EPI-, or sham-exposure.

Importantly, two groups of fish, one of the THIT (group Bi)- and EHIT (group Ci)-exposed groups respectively, were not contextually reminded prior to anxiety testing. These fish were only assessed for novel tank behavior.

2.7.2. *Repeated anxiety testing*

Depending on whether zebrafish were exposed to the color context (groups A, Bii, Cii, and D) or not (groups Bi and Ci) as described above, fish were subsequently assessed for anxiety-like novel tank diving behavior. Since four novel tank diving tests were conducted for each zebrafish over days 1, 4, 7, and 14, four different tank designs were used to prevent habituation to the testing arena (Fig. 5). On each testing day, one specific tank design was employed to enable future methodological reproduction. All tanks were constructed of Plexiglas® and consisted of a clear front-facing panel, with opaque panels for the surrounding areas. Tanks were filled up to a level 2 cm below the top end.

On any specific assessment day, zebrafish were moved either from their home tanks (in the case of no-contextual reminding), or from the color preference testing maze and transferred to the novel tanks. The tests were conducted over 6 min. Novel tank anxiety was characterized by calculating the average time spent in the bottom third of the tank, an established indicator of heightened anxiety in zebrafish (Stewart et al., 2011). After each novel tank test, zebrafish were gently netted back into their home tanks.

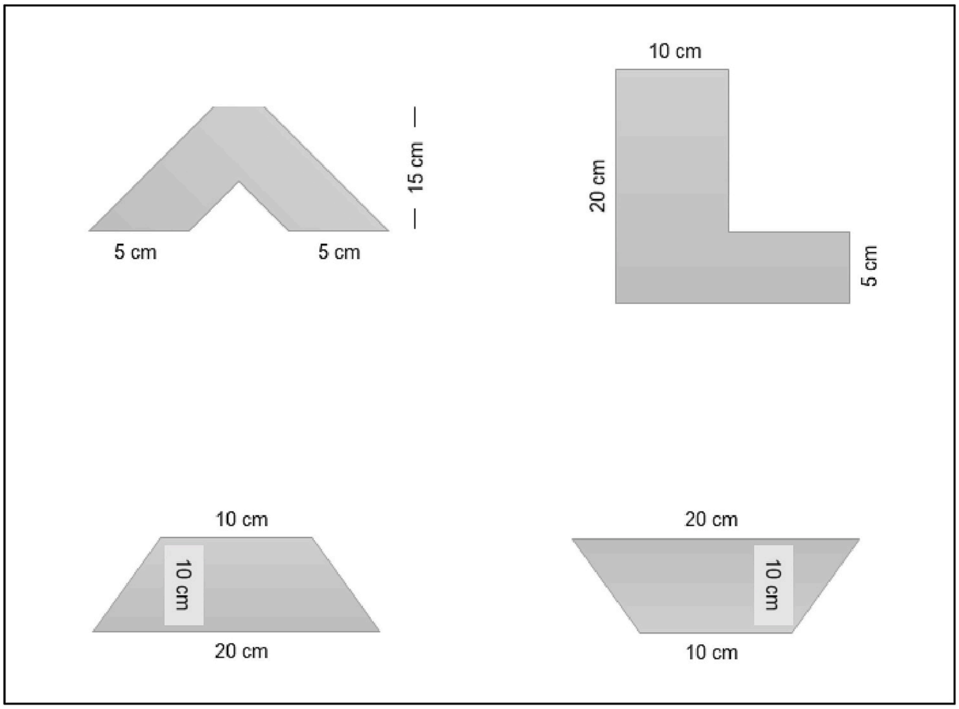

Fig. 5. Schematic representation of the tank shapes and dimensions of the four novel tank anxiety assessments
The front-to-back depth of all tanks was 5 cm. Tanks had a clear front side, but opaque sides, back and floor. Tanks used on the respective testing days are shown from left to right: top, d1, d4; bottom, d7, d14.

2.8. Study endpoint

After the behavioral tests were completed on day 14 zebrafish were immediately euthanized by means of rapid decapitation with a scalpel.

2.9. Statistical analysis

Statistical analyses were performed with IBM® SPSS® version 28, and graphical representations with GraphPad Prism®, version 9.3.0. Two-way mixed analysis of variance (2-way mixed ANOVA) was applied to analyze the effect of exposure (or contextual re-exposure) and time on novel tank bottom-dwelling (or color avoidance) behavior. Time and exposure or contextual re-exposure were set as independent variables, while the percentage time spent bottom-dwelling or the degree of contextual avoidance were set as the respective dependent variables. Data sets were explored for outliers by means of studentized residuals and for normality of distribution applying Shapiro-Wilk's test. Homogeneity of variances were assessed by Levene's test, while Maulchy's test was used to determine if assumptions of sphericity were met. All analyses were followed up by Bonferroni post-hoc tests to determine the significance level of pairwise differences and calculations of Cohen's *d* (with confidence intervals) to establish the magnitude of the effects observed. Descriptive statistics (median, interquartile range) were used to visually explore the distribution of data points and inform the statistical analyses.

3. Results

3.1. General background

The results of color-preference testing are not shown, since this was a categorical experiment performed to expose fish to sham, THIT, EHIT, or EPI in a simulated naturally preferred environment. Irrespective of which colors were chosen, the experimental procedures were the same. Since the assumptions of normality for most data sets and the sphericity with respect to the reported two-way interaction effects were violated, all graphs are expressed in terms of medians and all applicable results are reported based on Greenhouse-Geisser interpretation. Although normally distributed data sets are ideal for ANOVAs, two-way mixed ANOVA, for which no suitable non-parametric equivalent exists, is regarded sufficiently robust to violations of normality (Laerd, 2015). Also, since only some data sets violated the assumption of normality, it was decided not to split each data set into four separate repeated measures analyses. Rather, results from the analysis are reported in full, with resultant conclusions drawn taking statistical violations into mind. Also, given the non-parametric distribution of some data sets, descriptive statistics reflect both means and medians in the interests of transparency. Last, outliers, identified by means of studentized residuals were retained in the analyses, since no justifiable reason for their exclusion exists.

To facilitate reading of the results, group numbers refer to the following exposures:

- **Group A**: sham exposure;
- **Groups Bi & Ci**: THIT- and EHIT-exposed fish respectively, that were assessed for anxiety in the absence of prior contextual re-exposure;
- **Groups Bii & Cii**: THIT- and EHIT-exposed fish respectively, that were assessed for anxiety and contextual avoidance after of prior contextual re-exposure; and
- **Group D**: EPI-exposure.

3.2. Fig. 6, Table 1: novel tank anxiety of context-exposed fish over time

Here, the reported results apply to zebrafish that were exposed to either a sham protocol, THIT, EHIT or EPI on day 0 of the investigation. All fish, in this comparison i.e. groups A, Bii, Cii and D, were exposed in an identical setup, differing only with respect to the stressogenic protocol that was followed (*refer Day 0: THIT, EHIT, EPI, and sham exposure*). The setup was cladded in the color of preference for each of the exposed fish, while fish were contextually re-exposed (i.e. reminded of the trauma-paired color-context) prior to testing on the respective testing days (days 1, 4, 7, and 14; see *"Days 1, 4, 7 and 14: Investigating the immediate and delayed effects of trauma"*).

2-way mixed ANOVA failed to reveal a significant time-exposure interaction with respect to the time spent in the bottom third zone of a novel tank [$F(8.2, 253.9) = 1.32$, $p = 0.233$, partial $\eta^2 = 0.04$]. However, while time also did not significantly impact novel tank bottom-dwelling [$F(2.7, 253.9) = 2.41$, $p = 0.073$, partial $\eta^2 = 0.03$], there was a significant main effect of exposure [$F(3, 93) = 6.28$, $p < 0.001$, partial $\eta^2 = 0.17$] (descriptive statistics are reported in Table 1). Subsequent between-exposure pairwise comparisons made irrespective of time, revealed significant differences between fish exposed to THIT and sham ($p = 0.02$, 95CI [−25.8, −1.4]; $d = 0.6$, dCI [0.4, 0.9]), and THIT and EHIT ($p < 0.001$, 95CI [−29.5, −5.5]; $d = 0.7$, dCI [0.5, 1.0]),

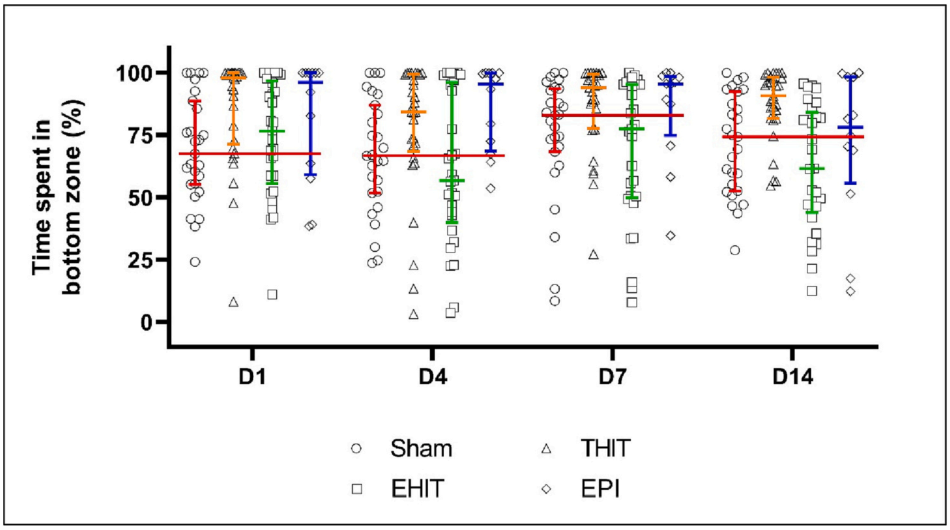

Fig. 6. Anxiety-like bottom-dwelling behavior of context-exposed sham-, THIT-, EHIT and EPI-exposed fish over time
Horizontal lines: median values of sham-exposed fish. Sham: non-stressed control; THIT: triple high-intensity trauma; EHIT: triple high-intensity trauma in presence of epinephrine; EPI: epinephrine.

Table 1
Descriptive statistics with respect to the novel tank bottom-dwelling behavior of fish in the different exposure groups that were contextually re-exposed prior to novel tank testing.

		Median % (interquartile range) ***Mean % ± SD***			
Day		**Sham**	**THIT**	**EHIT**	**EPI**
1	Time spent bottom dwelling (%)	67.5 (55.3, 88.8) *69.3 ± 21.3*	98.1 (71.4, 100) *85.8 ± 21.7*	76.6 (55.6, 96.8) *74.0 ± 23.4*	96.1 (59.2, 100) *81.2 ± 24.7*
4		66.8 (51.8, 87.0) *66.7 ± 22.9*	84.4 (68.5, 99.4) *77.4 ± 26.8*	56.8 (39.9, 96.2) *61.2 ± 30.2*	95.5 (68.7, 100) *85.7 ± 17.2*
7		83.0 (68.4, 93.7) *75.2 ± 24.6*	94.1 (77.7, 99.5) *86.5 ± 17.8*	77.6 (50.0, 95.4) *69.4 ± 28.6*	95.5 (74.9, 98.6) *85.4 ± 20.5*
14		74.3 (52.7, 92.4) *71.4 ± 20.1*	90.9 (81.8, 98.2) *87.4 ± 13.9*	61.7 (44.1, 84.2) *62.5 ± 25.0*	78.2 (55.8, 98.4) *71.4 ± 30.3*

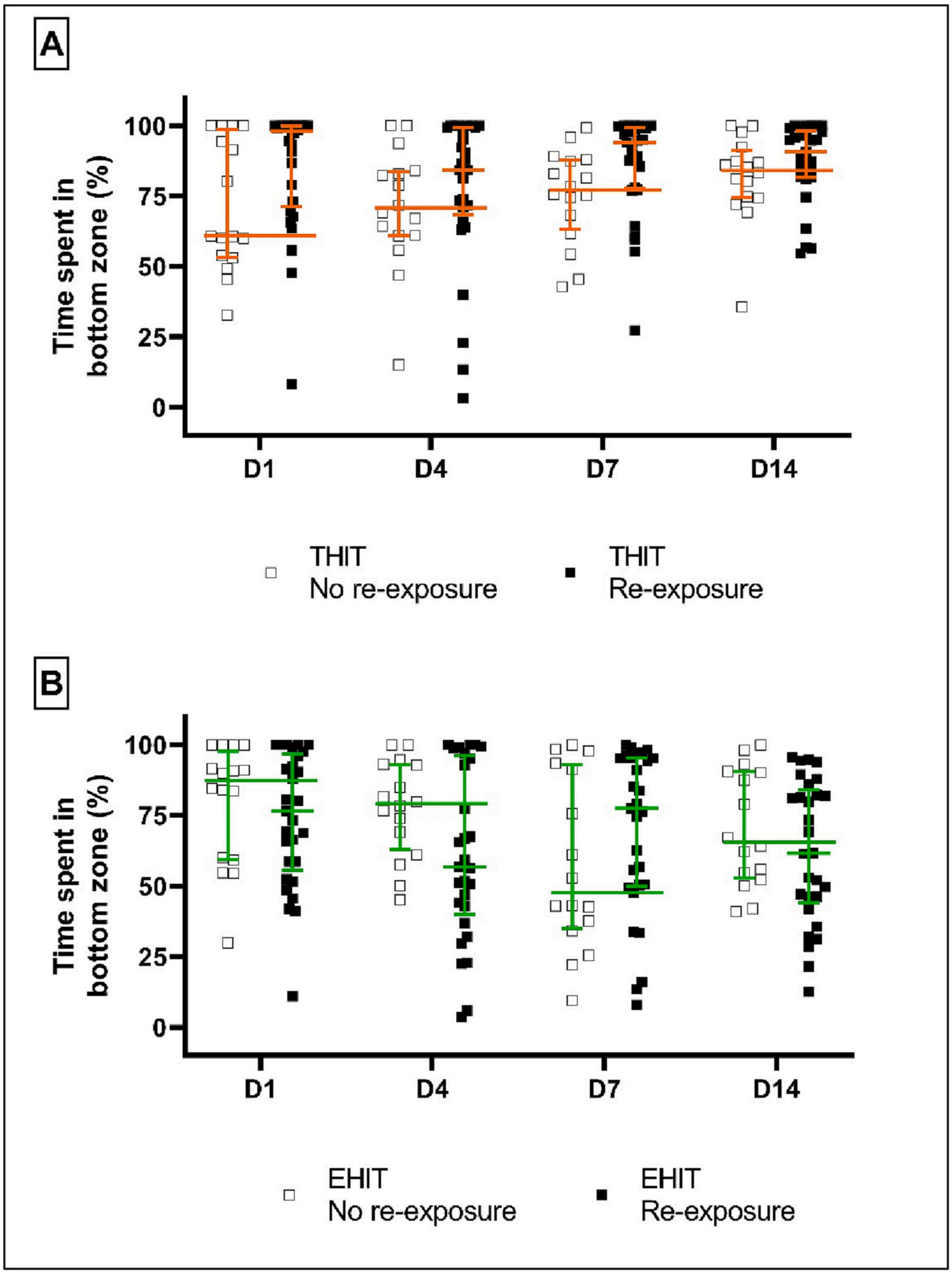

Fig. 7. Anxiety-like bottom-dwelling behavior (A) THIT- and (B) EHIT-exposed fish in the presence or absence of pre-test exposure to the trauma-paired color Horizontal lines: median values of fish that were not contextually reminded; THIT: triple high-intensity trauma; EHIT: triple high-intensity trauma in the presence of epinephrine.

with THIT-exposed fish showing significantly more bottom-dwelling behavior (84.3 ± 20.7 %) compared to sham- (70.7 ± 22.2 %) and EHIT- (66.8 ± 27.1 %) exposed fish.

This result is further informed by differences in the distribution of data points, with the medians and interquartile distributions of sham- and EHIT- exposed fish largely being similar (Table 1). In contrast, the bottom-dwelling behavior of at least 75 % of THIT-exposed zebrafish was consistently more than the median sham and EHIT values (Fig. 6). The same pattern was observed for the EPI-exposed groups over all testing days except day 14.

3.3. *Fig. 7, Table 2: effect of context on novel-tank behavior over time*

To determine whether contextual (THIT and EHIT-paired color) exposure influenced anxiety-like behavior in the novel tank diving test, we compared the percentage time spent bottom-dwelling by separate groups of THIT- (Fig. 7A;) and EHIT-exposed (Fig. 7B) zebrafish that were tested in the absence (groups Bi and Ci, i.e. no re-exposure) or presence (groups Bii and Cii) of said exposure. Thus, apart from data generated from groups Bi and Ci, the results in this section also pertain to that of the color-re-exposed fish that underwent THIT and EHIT, of which the repeated anxiety data are reported above. However, the non-color re-exposed zebrafish were separate groups of THIT and EHIT-exposed fish that were also subjected to the sham or stress protocols in the presence of the preferred color but tested for anxiety in the absence of prior exposure to such color again.

For THIT-exposed fish (Fig. 7**A**, Table 2), no significant interaction was detected between context and time [$F(2.3, 100.8) = 0.55$, $p = 0.605$, partial $\eta^2 = 0.01$]. Instead, there was a main effect of context [$F(1, 43) = 5.47$, $p = 0.024$, partial $\eta^2 = 0.11$] but not of time [$F(2.3,100.8) = 2.34$, $p = 0.082$, partial $\eta^2 = 0.05$] on bottom-dwelling behavior. Post hoc testing revealed a significant difference between the overall bottom-dwelling of no-context- and context-exposed zebrafish across all days of testing ($p = 0.024$, 95CI [−17.5, 1.3]; $d = 0.5$, dCI [0.2, 0.8]), with context-exposed zebrafish, showing significantly more bottom-dwelling behavior over time (84.3 ± 20.7 %), compared to non-re-exposed fish (74.8 ± 19.5 %).

For the EHIT-exposed group (Fig. 7B**,** Table 2), a significant time-context interaction was revealed [$F(2.6, 113.1) = 3.67$, $p = 0.019$, partial $\eta^2 = 0.08$], with time [$F(2.6, 113.1) = 3.45$, $p = 0.024$, partial $\eta^2 = 0.07$], but not context [$F(1, 43) = 0.59$, $p = 0.445$, partial $\eta^2 = 0.01$] having significantly impacted the result. Irrespective of context, EHIT-exposed fish spent significantly less time engaging in bottom-dwelling behavior on day 7 (65.4 ± 29.5 %), compared to day 1 (76.0 ± 22.6 %) ($p = 0.01$, 95CI [−23.8, −2.3]; $d = 0.4$, [0, 0.8]).

Considering visual inspection of the data distribution, we consider two trends. In the THIT-exposed group, most of the contextually re-exposed zebrafish spent more time in the bottom third zone of the novel tanks than the median values generated by no-context-exposed fish on all testing days. In contrast, zebrafish in the EHIT-exposed-groups spent a similar duration of time in the bottom third zone of the respective novel tanks, regardless of their re-exposure status. There was an exception on day 7 however, when 75 % of context-exposed fish generated greater bottom-dwelling scores than the median score generated by no-context exposed fish.

3.4. *Fig. 8, Table 3: contextual avoidance over time*

To establish whether sham-, THIT-, EHIT-, or EPI-exposed fish would avoid a previously preferred, but exposure-paired color upon subsequent exposures to the same color, we compared color avoidance behavior between groups. While no time-exposure interaction was detected [$F(8.6, 266.3) = 0.83$, $p = 0.585$, partial $\eta^2 = 0.03$], both time [$F(2.9, 266.3) = 4.00$, $p = 0.009$, partial $\eta^2 = 0.04$] and exposure [$F(3, 93) = 3.32$, $p = 0.023$, partial $\eta^2 = 0.1$] significantly impacted the color choice behavior of the different groups (Table 3). Overall, the contextual avoidance was most on days 4 and 7 of testing, compared to day 1 (25.8 ± 25.3 % time in the non-preferred color) (**day 4**: 34.8 ± 26.2 %; $p = 0.018$, 95CI [−19.0, −1.15]; $d = 0.3$, dCI [−0.6, −0.1]; **day 7**: 35.5 ± 27.2 %; $p = 0.018$, 95CI [−16.0, −1.0]; $d = 0.4$, dCI [−0.7, −0.1]) with no pairwise differences being significant between any of the exposure groups.

In this experiment, the medians and interquartile distributions of sham- and EHIT -exposed zebrafish again demonstrated similar distributions (Table 3), whereas the majority of THIT- and EPI-exposed zebrafish showed a lesser degree of trauma-paired color avoidance than EHIT-exposed fish on most testing days, except day 4.

4. Discussion

In this work, we aimed to develop a naturalistic zebrafish model of post-traumatic anxiety that is etiologically and translationally relevant by applying a combination of stressors that, together, constitute a life-threatening scenario to the species in question. We also aimed to explore the role of trauma-associated adrenergic processes in the manifestation of post-trauma anxiety following exposure to a contextual reminder of the original trauma. Importantly, we show that 1) THIT- and EPI- exposure induced persistent anxiety-like behavior that lasted through day 14 of this experiment, 2) trauma-paired EPI exposure consistently blunted the delayed anxiety-like sequalae associated with severe trauma (EHIT paradigm), 3) exposure to a trauma-paired color context prior to anxiety testing exacerbated the subsequent anxiety-like behavior of THIT, but not EHIT -exposed fish, and 4) despite this, THIT- and EPI-exposed fish showed a lesser degree of contextual avoidance behavior, compared to sham- or EHIT-exposed fish.

Although several attempts have been made to elucidate the contributions of peripheral adrenergic mechanisms to post-traumatic fear processing and anxiety, our understanding remains inadequate, mostly due to challenging nature of investigating such mechanisms in trauma-exposed individuals (Babson and Feldner, 2010; Malikowska-Racia and

Table 2

Descriptive statistics with respect to the novel tank bottom-dwelling behavior of fish in the THIT and EHIT groups in the absence and presence of contextual (trauma-paired color) exposure.

		Median % (interquartile range) ***Mean % ± SD***			
Day		**THIT No Context**	**THIT Context**	**EHIT No Context**	**EHIT Context**
1	Time spent bottom dwelling (%)	60.7 (53.3, 98.6) *71.4±23.2*	98.0 (71.4, 100) *85.8±21.8*	87.2 (59.5, 97.9) *79.7±21.2*	76.6 (55.6, 96.8) *74.0±23.4*
4		70.4 (61.0, 83.8) *70.9±21.5*	84.4 (68.5, 99.5) *77.4±26.8*	79.2 (63.1, 93.0) *77.5±17.1*	56.8 (39.9, 96.2) *61.2±30.2*
7		77.7 (63.3, 87.9) *75.1±16.8*	94.1 (77.7, 99.5) *86.5±17.8*	48.0 (35.0, 93.1) *58.1±30.6*	77.6 (50.0, 95.4) *69.5±28.7*
14		84.3 (74.5, 91.2) *81.7±15.5*	90.9 (81.8, 98.2) *87.4±13.9*	65.7 (85.9, 90.5) *70.5±20.5*	61.7 (44.1, 84.2) *62.5±25.0*

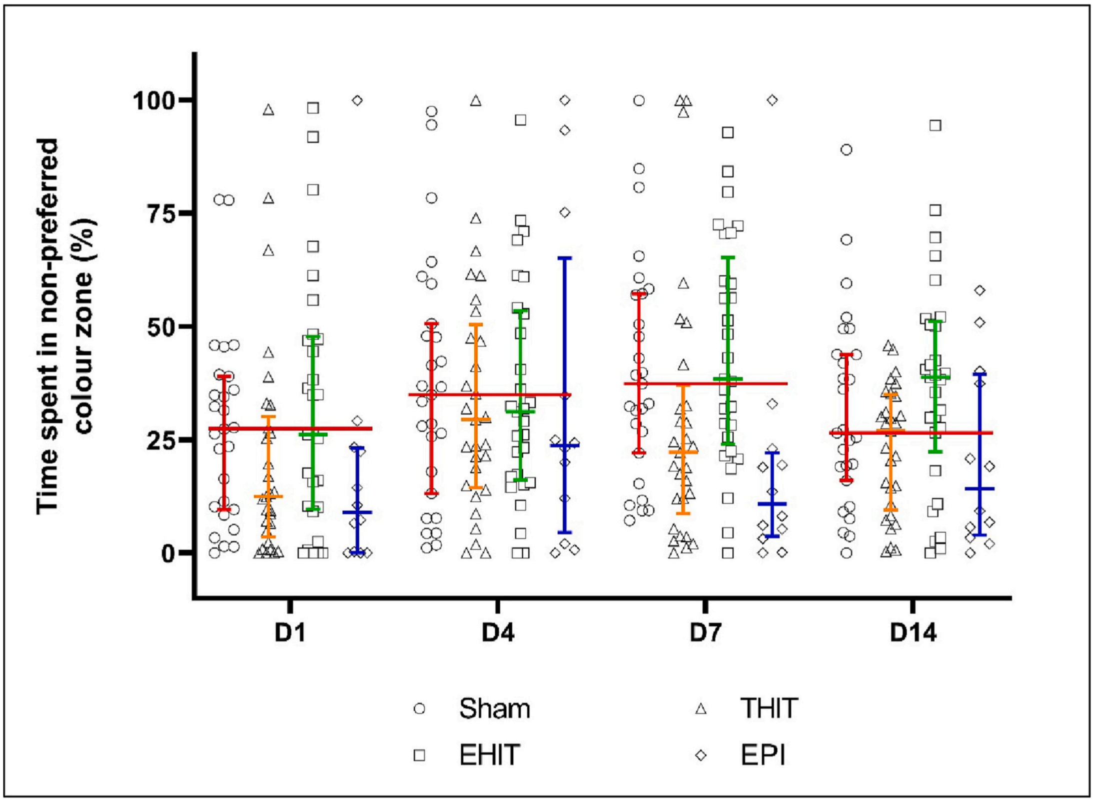

Fig. 8. Time spent by sham-, THIT-, EHIT, and EPI-exposed fish in the naturalistically non-preferred color, i.e. as opposed to the procedure-paired (preferred) color Horizontal lines: median values of sham-exposed fish. Sham: non-stressed control; THIT: triple high-intensity trauma; EHIT: triple high-intensity trauma in presence of epinephrine; EPI: epinephrine.

Table 3
Descriptive statistics with respect to context-avoiding behavior of fish in the different exposure groups.

		Median % (interquartile range) ***Mean % ± SD***			
Day		**Sham**	**THIT**	**EHIT**	**EPI**
1	Time spent in the non-preferred color zone (%)	27.4 (9.6, 39.0) *27.3±20.7*	12.5 (3.6, 30.1) *21.0±24.5*	26.2 (9.7, 47.8) *32.6±28.1*	8.9 (0.1, 23.1) *17.8±27.8*
4		34.9 (13.2, 50.7) *36.8±26.8*	29.5 (14.4, 50.4) *±33.2±24.4*	31.2 (16.2, 53.5) 34.9±*24.1*	23.7 (4.5, 65.1) *34.2±35.5*
7		37.4 (22.1, 57.3) *40.4±24.5*	22.3 (8.7, 37.1) 29.2±28.8	38.4 (24.0, 65.3) *44.0±24.7*	10.8 (3.7, 22.1) *19.2±27.4*
14		26.5 (16.0, 43.8) *31.4±21.5*	26.9 (9.5, 35.0) 23.0±13.9	38.0 (22.4, 51.1) *37.1±23.3*	14.2 (3.9, 39.5) *21.1±20.4*

Salat, 2019; Peleg and Shalev, 2006). Such uncertainties are further exemplified by the fact that two selective serotonin reuptake inhibitors, i.e., sertraline and paroxetine, remain the only Food and Drug Administration approved drugs registered for the treatment of PTSD, while both adrenergic (e.g. imipramine) and antiadrenergic drugs (e.g. prazosin and propranolol) show varying therapeutic benefit. To this end, we aimed to conceptualize a novel zebrafish model with robust translational relevance in which these questions can be explored. The first key finding of this work showed that THIT, as well as EPI, induced a significant degree of anxiety-like behavior in zebrafish that remained stable through days 1–14 of experimentation (Fig. 6). This result is informative for two reasons. First, it shows that, similar to humans and other mammals, life-threatening trauma in zebrafish can be applied as a useful model for the longitudinal study of delayed trauma-associated behavioral sequelae. Second, it confirms that peripheral adrenergic triggers, on their own, are necessary and even sufficient to elicit a similar degree of stress in zebrafish. Considering that zebrafish possess a blood-brain barrier that is functionally homologous to that of humans and other mammals (Eliceiri et al., 2011; Fleming et al., 2013; Ruuskanen et al., 2005), EPI administered via aqueous immersion is restricted to the periphery. While peripherally circulating EPI can modulate central NE release via vagal signaling (McGaugh and Roozendaal, 2002), EPI release in humans and other mammals is mainly triggered by means of top-down mechanisms via the hypothalamus-pituitary axis. However, in contrast to humans and other mammals, fish also convey psychobiological stress to other conspecifics by releasing alarm substances. These substances can trigger an immediate stress response in zebrafish (Canzian et al., 2017) suggesting that the ability of pheromones to evoke stress and fear-related responses in zebrafish is likely founded upon a peripheral, rather than a central mechanism. The results from the current study support such a conclusion, and elegantly highlight an evolutionary conserved mechanism by which zebrafish are also able to communicate and trigger stress and anxiety responses purely by means of peripheral processes. In extension, an intriguing possibility is that our data might suggest that a constituent of CAS may be an EPI-like substance or one that releases EPI in vivo. The latter idea is provocative to future new research.

The second key finding of this work is that simultaneous EPI exposure (i.e. paired with THIT) blunted the expression of trauma-induced anxiety (Fig. 6). This result is striking because for the first time in zebrafish we present evidence of an influence of EPI on fear retention that seems to be similar to the mammalian response originally shown by Gold and colleagues (Gold and van Buskirk, 1978; Gold and Van Buskirk, 1975; McCarty and Gold, 1981; Mcgaugh et al., 1975), as will be explained. Considering the aforementioned effects of THIT and EPI in isolation, this result can potentially be ascribed to the concentration-dependent effect of EPI on fear-related memory retention that broadly follows an inverted 'U-shape' relationship (Baldi and Bucherelli, 2005). Whereas memory retention is augmented in mammals by low to moderate concentrations of EPI, it becomes impaired at high concentrations (Baldi and Bucherelli, 2005). In contrast, concentrations of EPI similar to those that enhance memory retention under conditions of mild trauma, prevent fear memory retention and cause amnesia when administered in combination with severe trauma (Gold and Van Buskirk, 1975). Thus, it is highly plausible that the THIT- and EPI-alone-exposed groups were influenced by low-to-moderate peripheral EPI concentrations necessary for memory retention to occur.

In terms of EPI alone, we propose that its administration to zebrafish (i.e. under novel and thus mildly anxiogenic circumstances), elicited a similar response on memory retention as THIT on its own, replicating the finding of McCarty and Gold (1981) in mammals. In contrast, the post-traumatic behavioral response observed in the combined EHIT-exposed group represents a response that cannot be related to prior fear memory retention. This could potentially have resulted from the additive effects of THIT and EPI administration on peripheral EPI concentrations, resulting in post-traumatic amnesia (Baldi and Bucherelli, 2005; Martinho et al., 2020). This explanation would also support the finding that contextual re-exposure prior to delayed anxiety testing was necessary to elicit anxiety-like behavior in THIT, but not EHIT-exposed zebrafish, compared to fish that underwent the same traumatic experience but that were not reminded of the trauma-paired context prior to testing (Fig. 7). Indeed, showing sensitivity to the traumatic context, the behavior of context-exposed THIT-exposed zebrafish is reminiscent of post-traumatic symptomology (Ehlers et al., 2004) and shows that context-specific memory retention occurred in these animals. Collectively, these findings pave the way forward for continued exploration of a potentially useful zebrafish model system in which to study the impact of ethologically and etiologically relevant stressors within a novel translational framework.

Finally, the finding that trauma-exposed zebrafish did not avoid the context of their traumatic experience even though the same context triggered inflated anxiety-like behavior (Fig. 8) was interesting. Zebrafish display highly individual preferences for or aversion of color (Roy et al., 2019; van Staden et al., 2019); we aimed as far as possible to account for this in the present investigation by first establishing the naturalistic color preference of each fish. Nevertheless, while the purpose of this procedure was to prevent potential color aversion from confounding the meaning of later context-triggered anxiety-like behavior, we cannot exclude the possibility that exposure to naturalistically preferred colors is rewarding. Thus, while zebrafish were able to recognize the contextual value of trauma-paired colors, it is possible that such colors did not necessarily possess trauma-related aversive properties per se. Alternatively viewed, to force contextual re-exposure, zebrafish were first introduced to the area of the maze covered in the preferred, but trauma-paired color, and thus an immediate context-triggered decrease in exploration cannot be excluded. Nevertheless, such a response would also support the translational usefulness of the present research framework since it would nonetheless point to context-related fear memory recall.

Some important limitations of our approach should be noted. First, since we did not employ different adrenergic receptor targeting drugs, detailed conclusions regarding the exact mechanisms underlying the effects of EPI in this model cannot be drawn. That said, since we show that a THIT model of trauma-related anxiety-like behavior is viable in zebrafish, continued endeavors can focus on exploring adrenergic mechanisms in the acquisition, consolidation, and retrieval of fear memory in this paradigm. Secondly, biological analysis of EPI, NE, and cortisol over all days of traumatic exposure and anxiety testing could yield insights into how temporal crosstalk between central and peripheral stress-related biology influence behavioral manifestation. Last, while the data presented are promising, additional behavioral tests that may be relevant for understanding trauma-related behaviors, i.e., tests of sociability, aggression, and anhedonia, would have been informative.

5. Conclusion

Here, we show that zebrafish can contextualize a severe and translationally relevant life-threatening stressor. We further show that the resultant post-trauma anxiety, which is context-sensitive, manifests for at least two weeks after trauma exposure. Because the concepts of persistent and delayed post-trauma anxiety, as well as contextual relevance, are important diagnostic symptoms of trauma-related psychopathologies, the present work supports current research efforts in the field by describing a readily reproducible zebrafish model system in which the psychobiological mechanisms that may underlie traumatic fear processing, can be further explored. Identification of EPI as a modulator of the acquisition and consolidation of fear memory in this model provides a neurochemical anchor on which to build a neural circuit underlying trauma-induced behaviors in zebrafish, a possibility that necessitates continued research in the model system.

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Ethics approval

This study was approved by the Animcare Research Ethics Committee of the NWU (approval number: **NWU-00526-20-A5**). All procedures were in accordance with the rules and guidelines stipulated by the South African National Standard 10,386:2021 *"The care and use of animals for scientific purposes"*.

CRediT authorship contribution statement

All authors contributed equally to this work. This work from the M. Sc. degree project of VT at the NWU.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability

The data and materials for all experiments are available at Mendeley Data (file designation: VT_DWW_Tra_Anx; DOI:10.17632/vsm5xcbds6.1).

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