

Exploring quadruple stress in zebrafish (*Danio rerio*) as a novel model of posttraumatic stress disorder

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* * *

“Well, that did ***not*** go as planned”

How to give a masters student PTSD: a memoir

* * *

* * *

With God, you can be:

Unflinching in the face of disappointment

Unshakeable in the face of your past

Unafraid of the darkness

Unwilling to hold back

Undaunted in your faith

Unashamed by your calling

Unhindered by your limitations

Unstoppable in the face of difficulty

* * *

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Congress Proceedings

- THERON, V., HARVEY, B., and WOLMARANS, D. (2021). *“Exploring quadruple stress in zebrafish (Danio rerio) as a novel model of posttraumatic stress disorder (PTSD)”*. Presented at the 54th Annual South African Society for Basic and Clinical Pharmacology (SASBCP) Conference (online), 22nd October 2021.

Abstract

Posttraumatic stress disorder (PTSD)¹ is a severe and incapacitating mental disorder that manifests in some individuals following a personal encounter with or experiencing a perceived threat and/or witnessing/observing others encountering a traumatic life-threatening event, e.g. sexual and/or physical assault, war-related incidents, and natural disasters. What makes PTSD unique from other anxiety-related neuropsychiatric disorders is that its diagnosis is contingent on a specific traumatic experience.

Two principal components contribute to the development of PTSD, namely 1) encountering a severe, life-threatening and traumatizing event and 2) having a genetic, clinical or familial predisposition to the disorder. PTSD is characterized by flashbacks, emotional outbursts (mainly anger), avoidance of contextual reminders of the original traumatic event, and sleep disturbances. Patients suffering from PTSD are often subject to disrupted personal and familial relationships which place further strain on their social, medical, and financial stability. Moreover, up to 75 % of all individuals will at some point in time be exposed to a traumatic life event that fits the prodromal picture of PTSD. However, only a select few of those, i.e. roughly 12 %, will progress to develop PTSD. This points to individual variances in the risk for and resilience against the development of PTSD.

The Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5)² describes the symptoms of PTSD under four main categories, i.e. i) intrusion (reliving the traumatic event in flashbacks); ii) avoidance (suppression or attempted suppression of distressing internal reminders); iii) negative mood and cognition (being incapable of remembering important details of the event); and iv) arousal and reactivity (i.e. sleep disturbances, hypervigilance, and lack of concentration). Suffice to say that the diagnosis of PTSD is complex, in that symptom manifestation varies considerably between individuals. The symptomatic manifestation of PTSD varies in each individual and many patients experience only one of the four main symptoms.

Our knowledge and understanding of the pathophysiology of PTSD is less than optimal. This may likely be due to the complicated nature of its aetiology, as this is intricately linked to events that are mostly unforeseen. However, there are some hypotheses that provide insight into the pathophysiological basis of the condition. These all point to dysfunctional processes underlying the manner in which the human body processes and mitigates the immediate and long-lasting psychobiological effects of acute fear-induced stress following exposure to life-threatening situations.

¹ posttraumatic stress disorder

² The Diagnostic and Statistical Manual of Mental Disorders, 5th edition

Norepinephrine (NE)¹, being a key stress hormone that guide the mammalian and human response to both implicit and external stressors, has been the focus of many PTSD² and other anxiety disorder studies, since it is intricately involved in the processing of emotional memories. Specifically, it has been shown that the adrenergic alpha (α)_{1/2} receptors influence processes of working memory, spatial learning, and fear appraisal, while beta (β)_{1/2} receptors play a role in the processing of auditory fear, spatial reference, and fear memory retrieval. Evidence points to PTSD patients often presenting with an increased level of sympathetic functioning, compared to healthy controls, indicating a clear role for inflated noradrenergic signaling in PTSD. Regardless, given the suboptimal response of PTSD to drugs that target processes, our understanding of the actions of NE in PTSD remains unclear.

Across all fields of neuropsychiatric research, animal models of human illness have historically proven to be invaluable tools to divulge the etiopathological mechanisms underlying human disorders and to identify novel targets for pharmacological therapy. Most rodent models of PTSD, although well studied, deliver varying and incongruent results. Zebrafish recently gained interest in preclinical research since the species presents with a variety of attributes that allow for experimental manipulation and the accurate characterization of specific behavioral traits that provide a valuable background for the study of PTSD-like phenomena. Despite this, further validation of the species as an animal model system of relevance for human disorders is still required.

Here, we attempted to develop a standardized preclinical zebrafish model of PTSD by investigating whether a life-threatening, severe stress paradigm, i.e., a high intensity trauma, that is aimed at combining various but replicable constructs of *life-threatening* trauma, will be adequate to induce sustained PTSD-like fear in a zebrafish model as defined at both 1-, 4-, 7- and 14-days post-trauma. Further, considering the current uncertainty regarding the role of epinephrine (EPI³) in the development and manifestation of trauma-related anxiety, we will further seek to divulge its effect on trauma-related behaviors which will tested against the behavior of subjects exposed to either a sham⁴ (trauma-free) protocol, trauma inflicted in the absence of EPI administration, i.e. triple high intensity trauma (THIT⁵), or EPI administration only.

¹ norepinephrine

² posttraumatic stress disorder

³ epinephrine

⁴ trauma free

⁵ triple high intensity trauma

Importantly, we show that 1) trauma-paired EPI¹ exposure consistently blunted the delayed anxiety-like sequelae associated with severe trauma, but bolstered persistent neophobia-related anxiety when paired with novelty (exposure to a novel circumstance) in the absence of additional stressors, 2) exposure to a trauma-paired context prior to testing strengthened the subsequent anxiety-like behavior trauma-exposed fish in the absence of additional EPI, and 3) despite this, trauma exposed- and EPI-only fish showed a lesser degree of contextual avoidance behavior, compared to sham²- or EPI-free, trauma-exposed fish

In conclusion, we show that zebrafish are able to contextualize a severe and realistic life-threatening stressor. We further show that the resultant post-traumatic anxiety, which is context-sensitive, manifests for at least two weeks after trauma exposure. Since the concepts of persistent and delayed post-trauma anxiety, as well as contextual relevance are pivotal diagnostic symptoms of clinical PTSD³, the present work extends current efforts in preclinical PTSD research by describing a readily reproducible zebrafish model framework in which the psychobiological mechanisms that may underlie the clinical condition, can be explored. Further, the present investigation also highlighted a dichotomous role for adrenergic processes in the acquisition and consolidation of fear memory, which needs continued investigation.

¹ epinephrine

² trauma free

³ posttraumatic stress disorder

Table of contents

Acknowledgements	iii
Congress Proceedings	viii
Abstract	ix
Table of Figures.....	xv
Table of Tables	xvi
1 Introduction	1
1.1 Dissertation approach and Layout.....	1
1.2 Problem statement.....	2
1.3 Study hypothesis.....	5
1.4 Research aims and objectives	6
1.4.1 Aim	6
1.4.2 Objectives.....	6
1.5 Research Methodology.....	8
1.5.1 Study layout	8
1.5.2 Breakdown of experimental fish and fish used as conspecifics and CAS donors	9
1.6 Ethical considerations.....	9
1.7 Summary.....	9
1.8 References	10
2 Literature Review	14
2.1 Posttraumatic disorder (PTSD) in the clinic	14
2.1.1 Description and epidemiology	14
2.1.2 Symptomology and differential diagnosis	15
2.2 The psychobiology of PTSD	19
2.2.1 The human fear response and PTSD	19
2.3 The pharmacotherapy of PTSD	24
2.3.1 Drugs targeting serotonergic and adrenergic mechanisms.....	25
2.3.2 Diverse drugs.....	27

2.4	Pre-clinical modelling of PTSD	28
2.4.1	A brief overview of concepts and validity	28
2.5	The zebrafish (<i>Danio rerio</i>).....	29
2.5.1	Background and ecology	29
2.5.2	Zebrafish as a novel pre-clinical research platform.....	32
2.5.3	Zebrafish behavior within the context of PTSD	33
2.5.4	The zebrafish brain.....	34
2.6	In summary: key concepts of relevance for the current investigation.....	35
2.6.1	Towards the development of a PTSD model of accurate translational relevance.....	35
2.6.2	General anxiety vs. the fear of death.....	36
2.6.3	A new direction forward: quadruple high intensity trauma (QHIT) – the conceptualization of a bolstered fear response	37
2.7	Conclusion.....	37
2.8	References	39
3	Research article	51
	Abstract.....	52
	Keywords	52
3.1	Introduction.....	53
3.2	Materials and methods.....	57
3.2.1	Experimental layout	57
3.2.2	Animals and housing	58
3.2.3	Preparation of CAS-releasing zebrafish	60
3.2.4	Chemicals and apparatus	60
3.2.5	Days -2 and -1: Color preference testing	60
3.2.6	Day 0: QHIT, THIT, epinephrine and sham exposure	62
3.2.7	Days 1, 4, 7 and 14: Investigating the immediate and delayed effects of trauma	65
3.2.8	Study endpoint	67
3.2.9	Statistical analysis	67
3.3	Results	67

3.3.1	Figure 3-6, Table 3-1: Novel tank anxiety of context-exposed fish over time	67
3.3.2	Figure 3-7, Table 3-2: Effect of context on novel-tank behavior over time	69
3.3.3	Figure 3-8, Table 3-3: Contextual avoidance over time	70
3.4	Discussion.....	73
3.5	Conclusion.....	77
3.6	References	78
4	Conclusion	84
4.1	Summary of expected vs actual outcomes	86
4.2	Study shortcomings and future directions	87
4.3	References	88
	Addendum A - Description of Supplementary Methods	89
	Layout of Groups for the Behavioral Investigation	90
	Routines During the Behavioral Investigation.....	91
	Detailed methodology of behavioral tests	93
	Color- baseline determination / aversion test.....	93
	Novel anxiety test.....	94
	QHIT Protocol.....	95
	THIT Protocol	96
	Adrenaline Protocol	97
	Sham Protocol.....	98
	Additional Information.....	99

Table of Figures

Figure 1-1 – Graphical study layout.....	8
Figure 2-1 - Algorithm for assessing the DSM-5 criteria of PTSD (North et al., 2018)	18
Figure 2-2 – Psychological and pharmacological strategies for treatment of PTSD	25
Figure 2-3 - The natural geographical range of zebrafish.	58
Figure 2-4 – Developmental stages of zebrafish.	32
Figure 2-5 – Zebrafish phenotypes.	32
Figure 3-1 - Experimental layout.	58
Figure 3-2 - Schematic (above) and photographic (below) view of home cage setup and separators, respectively, allowing for simultaneous fish identification and social interaction.....	59
Figure 3-3 – Schematic (above) and photographic representation of color preference testing maze.	61
Figure 3-4 – Schematic (above) and photographic (below) representation of the exposure apparatus.	63
Figure 3-5 – Schematic (above) and photographic (below) representation of the tank shapes and dimensions of the 4 novel tank anxiety assessments.	66
Figure 3-6 – Anxiety-like bottom-dwelling behavior or context-exposed sham-, trauma-, and EPI-exposed fish over time.....	68
Figure 3-7 – Anxiety-like bottom-dwelling behavior (A) QHIT- and (B) THIT-exposed fish in the presence or absence of pre-test exposure to the trauma-paired color.....	72
Figure 3-8 – Time spent by sham-, trauma-, and EPI-exposed fish in the naturalistically least-preferred color, i.e. as opposed to the procedure-paired (preferred) color.	73

List of Tables

Table 3-1 - Commonly used rodent models for the study of PTSD.	55
Table 3-2 – Descriptive statistics and pairwise comparisons with respect to the novel tank bottom-dwelling behavior of fish in the different exposure groups.	69
Table 3-3 - Descriptive statistics and pairwise comparisons with respect to the novel tank bottom-dwelling behavior of fish in the QHIT and THIT groups in the absence and presence of contextual (trauma-paired color) exposure.	70
Table 3-4 - Descriptive statistics and pairwise comparisons with respect to context-avoiding behavior of fish in the different exposure groups.	71
Table 4-1 – Final outcomes matched with a summary of the hypothesis study objectives as provided in Chapter 1.	86

1 Introduction

1.1 Dissertation approach and layout

This dissertation is compiled in article format, as detailed, and approved by the North-West University (NWU¹), South Africa. The main body of findings is presented as a single scientific manuscript in Chapter 3. This comprises details of the experimental work conducted, the methods followed, results obtained, as well as a discussion of the main findings presented in the form of a journal article that will be submitted, following input from the examiners, for publication in an accredited international, peer-reviewed neuroscience journal, i.e. “Journal of Neuroscience Research”.

The complete dissertation will, however, consist of four chapters.

- *Chapter 1* offers a concise description of the project problem statement, study hypothesis, research aims and objectives, an overview of research methodology and ethical considerations.
- *Chapter 2* comprises the applicable literature background to support the dissertation as a whole.
- *Chapter 3* will, as alluded to above, report the key findings of the investigation in the form of a scientific manuscript, which will be prepared according to the ‘Instructions to Authors’ provided by “*Journal of Neuroscience Research*”.
- *Chapter 4* concludes the dissertation with a summary of outcomes. Potential shortcomings of the current research are also discussed, alongside the implications of the study for future research which may aim to build upon the current work.

The addendum will contain:

- *Addendum A*: Supplementary methods, data, and background information on the related work performed by the candidate to conceptualize and execute this investigation.

The reference style used throughout this dissertation is that prescribed by ‘*Journal of Neuroscience Research*’. The dissertation is presented in US² English, since this is the language prescribed by ‘*Journal of Neuroscience Research*’.

¹ North-West University

² United States

1.2 Problem statement

Posttraumatic stress disorder (PTSD¹) is a severe and incapacitating mental disorder that manifests in some individuals following a personal encounter with a life-threatening event (Miller, Lin, Wolf, & Miller, 2018; NIH, 2019; Vieweg et al., 2006). PTSD is distinct from other anxiety-related neuropsychiatric disorders in that its diagnosis is contingent on a specific traumatic experience which causes the occurrence of symptoms (Sharma & Ressler, 2019).

There are two principal components contributing to the development of PTSD, namely 1) encountering a severe, life-threatening and traumatizing event and 2) having a genetic, clinical or familial predisposition to the disorder (Sharma & Ressler, 2019). PTSD is characterized by flashbacks, emotional outbursts (mainly anger), avoidance of contextual reminders of the original traumatic event, and sleep disturbances (Bisson, Cosgrove, Lewis, & Roberts, 2015; NIH, 2019). Patients suffering from PTSD are often subject to disrupted personal and familial relationships which places further strain on their social, medical, and financial stability (APA, 2013; Miao, Chen, Wei, Tao, & Lu, 2018). Moreover, up to 75 % of all individuals will at some point in time be exposed to a traumatic life event that fits the prodromal picture of PTSD (APA, 2013). However, only a select few of those, i.e. roughly 12 % (Breslau & Kessler, 2001), will progress to develop PTSD (Skelton, Ressler, Norrholm, Jovanovic, & Bradley-Davino, 2012). This points to individual variances in the risk for and resilience against the development of PTSD (Nemeroff et al., 2006; NIH, 2019).

Symptoms generally manifest from the third month after exposure to a traumatic event (APA, 2013; Schnurr, Friedman, & Bernardy, 2002). The Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5²) describes the symptoms of PTSD under four primary categories, i.e. i) intrusion (reliving the traumatic event in flashbacks); ii) avoidance (suppression or attempted suppression of distressing internal reminders); iii) negative mood and cognition (being incapable of remembering important details of the event); and iv) arousal and reactivity (i.e. sleep disturbances, hypervigilance, and lack of concentration) (APA, 2013). The symptomatic manifestation of PTSD varies in each individual and many patients experience only one of the four main symptoms (APA, 2013).

Our knowledge and understanding of the pathophysiology of PTSD is still largely incomplete. This may likely be due to the complicated nature of its etiology and the intricate ties thereof to unforeseen stressors which are required to develop the disorder. However, several hypotheses point to dysfunctional processes underlying the manner in which the human body mitigates the immediate and long-lasting psychobiological effects of acute fear-induced stress following exposure to life-threatening situations (Vieweg et al., 2006). Primarily, PTSD is believed to result from dysfunctional fear consolidation and stress mitigation mechanisms which may not only

¹ posttraumatic stress disorder

² The Diagnostic and Statistical Manual of Mental Disorders, 5th edition

undermine the acute fear response, but also prevent adequate fear desensitization from occurring (Garcia, Walker, & Zoellner, 2018; Kreutzmann et al., 2021; Van der Kolk, 2001). Norepinephrine (NE)¹ is a key stress hormone (Cahill, Gorski, & Le, 2003). It has been a focus in many PTSD² studies, as it is intricately involved in the processing of emotional memories (Cahill et al., 1996). Specifically, it has been shown that the adrenergic alpha (α)_{1/2} receptors influence processes of working memory, spatial learning and fear appraisal, while beta (β)_{1/2} receptors play a role in the processing of auditory fear, spatial reference and fear memory retrieval (Cahill et al., 1996; Hussain, Reddy, & Maani, 2020). Evidence points to PTSD patients often presenting with an increased level of sympathetic functioning, compared to healthy controls (O'donnell, Hegadoren, & Coupland, 2004), indicating a clear role for inflated noradrenergic signaling in PTSD. Regardless, given the suboptimal response of PTSD to drugs that target noradrenergic processes, our understanding of the actions of NE in PTSD remains unclear.

In fact, the current pharmacotherapeutic treatment regimens for PTSD remain overly ineffective, likely due to the lack of reliable and convincing scientific treatment data in addition to an incomplete understanding of the condition (Galea et al., 2012; Hauger et al., 2012). Only 60 % of patients demonstrate mild clinical response, while symptom remission is achieved in 20 – 30 % of sufferers only (Bailey, Cordell, Sobin, & Neumeister, 2013). Currently, a combination of pharmacological and non-pharmacological interventions are used to treat PTSD (Department of Veterans Affairs, 2020). Specifically, selective serotonin reuptake inhibitors (SSRIs³), and not agents that target noradrenergic signaling, are used as the first line intervention (Bailey et al., 2013; Charney, Hellberg, Bui, & Simon, 2018); these are the only United States (US⁴) Food and Drug Administration (FDA⁵)-approved drugs for the treatment of PTSD (Brunet et al., 2018; Charney et al., 2018; MacNamara et al., 2016; Vieweg et al., 2006), the same being true in South Africa. Additionally, SSRIs are favored by their relative low cost and favorable side-effect profile (Albucher & Liberzon, 2002; MacNamara et al., 2016; Vaswani, Linda, & Ramesh, 2003).

Across all fields of neuropsychiatric research, animal models of human illness have historically proven to be invaluable tools to divulge the etiopathological mechanisms underlying human disorders and to identify novel targets for pharmacological therapy (van der Staay, 2006; Zabegalov et al., 2019). Most rodent models of PTSD, although well studied, deliver varying and incongruent results (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Stewart, Yang, Nguyen, & Kalueff, 2014), which can likely be ascribed to differences in the various rodent strains and the trauma-inducing methods used (Champagne et al., 2010; Stewart et al., 2010).

¹ norepinephrine

² posttraumatic stress disorder

³ selective serotonin reuptake inhibitors

⁴ United States

⁵ Food and Drug Administration

With respect to this investigation, in which we will attempt to develop and characterize a standardized, novel zebrafish (*Danio rerio*) model of PTSD¹, some important aspects of this species require highlighting.

Zebrafish recently gained interest in preclinical research since the species presents with a variety of attributes that allow for experimental manipulation (Khan & Alhewairini, 2018; Spence, Fatema, Ellis, Ahmed, & Smith, 2007) and the accurate characterization of specific behavioral traits (see below) that provide a valuable background for the study of PTSD-like phenomena (Stewart et al., 2011). Despite this, further validation of the species as an animal model system of relevance for human disorders is still required (Khan et al., 2017). Zebrafish present preclinical research with several potential benefits, i.e. a relatively quick reproduction rate (Lucon-Xiccato, De Russi, & Bertolucci, 2020; Vaz, Hofmeister, & Lindstrand, 2019), low maintenance costs (Lucon-Xiccato et al., 2020), and rapid development and maturation (Vaz et al., 2019). Zebrafish also express many of the same neurotransmitter systems and neuroanatomical targets as in mammals (Maximino et al., 2011). Of relevance to PTSD, zebrafish present with a wide range of anxiety-like, quantifiable behaviors (Kiyokawa, Kikusui, Takeuchi, & Mori, 2005; Maximino et al., 2010; Stewart et al., 2011), including bottom dwelling in a novel tank, freezing, shoaling cohesion, and thigmotaxis² (near or close dwelling to a tangible surface) (Maximino et al., 2018; Stewart et al., 2011). These stress-induced behavioral changes can easily be compared to the instinctive behaviors of zebrafish, so as to provide accurate distinction between stress-related and normal behavior (Stewart et al., 2011). Further, zebrafish can be studied throughout the lifespan, from hatching until at least two years of age, which makes it an ideal species for longer-term study. Here, we will attempt to develop a standardized preclinical zebrafish model of PTSD by investigating whether a life-threatening, severe stress paradigm, i.e., a quadruple high intensity trauma (QHIT³), that is aimed at combining various but replicable constructs of *life-threatening* trauma, will be adequate to induce sustained PTSD-like fear in a zebrafish model as defined at days 1, 4, 7,- and 14-days post-trauma. Further, considering the current uncertainty regarding the role of epinephrine (EPI⁴) in the development and manifestation of trauma-related anxiety, we will further seek to divulge its effect on QHIT-related behaviors.

¹ posttraumatic stress disorder

² near or close dwelling to a tangible surface

³ quadruple high intensity trauma

⁴ epinephrine

1.3 Study hypothesis

We hypothesize that exposure to contextual (where the preferred color of each zebrafish is introduced as context) QHIT¹, consisting of, i) electric shocks, ii) water drainage down to the level of dorsal fin air exposure, iii) proximal exposure to conspecific alarm substance (CAS²)-releasing pre-euthanized conspecifics, all of which are iv) administered in the presence of exogenous EPI³ administration, will mimic a traumatic, life-threatening event for zebrafish to such an extent that it will result in the manifestation of acute anxiety and long-term PTSD⁴-like behavioral sequelae at 1, 4, 7 and 14 days after exposure to QHIT. To this end, we hypothesize that such sequelae would involve i) anxiety-like responses in a novel environment; and ii) aversion for a previously preferred, but trauma-paired color. Importantly, considering the well-known, but still elusive influence of noradrenergic signaling on acute and delayed trauma-related neurobiological processes, we hypothesize that NE⁵ will have a notable influence on said behavioral outcomes of QHIT. This hypothesis will be tested against the behavior of subjects exposed to either a sham⁶ (trauma-free) protocol, or the same trauma as inflicted by QHIT, but in the absence of EPI administration, i.e. triple high intensity trauma (THIT⁷), or EPI administration only. Since PTSD is a highly complicated condition to model from a statistical point of view (Cohen & Zohar, 2004), i.e. with reference to the limited number of subjects that will go on to develop PTSD after experiencing trauma, the group sizes chosen for this investigation are based on prior studies where only 25 – 35% of rodents developed PTSD-like behaviors following traumatic exposure (Cohen & Zohar, 2004). As such, we expect to see a near similar incidence in trauma-exposed zebrafish.

¹ quadruple high intensity trauma

² conspecific alarm substance

³ epinephrine

⁴ posttraumatic stress disorder

⁵ norepinephrine

⁶ trauma free

⁷ triple high intensity trauma

1.4 Research aims and objectives

1.4.1 Aim

The central aim of the current investigation is to characterize and validate a novel zebrafish model of PTSD¹ based on its symptomological resemblance of the human condition. As such, we hope to establish a model with robust face validity for further exploration in terms of neurobiology and treatment response in later studies.

1.4.2 Objectives

To realize the aim of this investigation, we will address the following objectives (read with **section 1.5** and **Figure 1-1**):

- i. Randomly select 295 experimental short-fin wild type (WT²) zebrafish of both sexes from the zebrafish colony housed in the National Aquatic Bioassay Facility (NABF)³ of the North-West University (NWU)⁴;
- ii. Determining the natural color preference of each fish (days -2 and -1). This is necessary since zebrafish display highly inter-individual differences in color preference (Stewart et al., 2011). In order to establish whether contextual appraisal is important in the manifestation of trauma-associated acute and delayed anxiety, the preferred colors of each subject will be cued during the sham⁵, QHIT⁶, THIT⁷ and EPI⁸ protocols. To enable optimal housing, zebrafish will subsequently be divided into different groups as determined by their color preference viz. green, yellow, red, and blue (for a minimum number of 48 fish per any color group). Please refer to **paragraph 1.5.2** for details of fish that were not included in experimental groups.
- iii. Fish will then, according to their color preference, be further divided into different groups with respect to experimental objective, i.e. Groups A, B, C and D (see below). Fish in Groups A and B, irrespective of which color they preferred, will be subdivided into two subgroups each, e.g. Ai ($n = 13$) + Aii ($n = 29$) and Bi ($n = 12$) + Bii ($n = 29$). Fish in Groups Ci ($n = 16$) + Cii ($n = 16$) will be used to establish whether a contextual cue influences the manifestation of anxiety-like responses later in life. Fish in

¹ posttraumatic stress disorder

² Short-fin wild type

³ National Aquatic Bioassay Facility

⁴ North-West University

⁵ trauma free

⁶ quadruple high intensity trauma

⁷ triple high intensity trauma

⁸ epinephrine

group D ($n = 12$) will be used to isolate the role of trauma-paired and non-trauma-paired EPI¹ administration on potential later-life PTSD²-like behavior. Specifically:

- a. Groups Ai and Bi will be exposed to a sham³ protocol on day 0;
 - b. Groups Aii and Ci will be exposed to the QHIT⁴ protocol on day 0;
 - c. Groups Bii and Cii will be exposed to the THIT⁵ protocol on day 0;
 - d. Group D will be exposed to a protocol where only EPI is administered on day 0;
- iv. Fish from Groups A, B, and D were retested on days 1, 4, 7, and 14 for contextual color aversion and anxiety; and
- v. Fish from Group C were retested on days 1, 4, 7, and 14 for anxiety in the absence of color preference testing.

¹ epinephrine

² posttraumatic stress disorder

³ trauma free

⁴ quadruple high intensity trauma

⁵ triple high intensity trauma

1.5 Research methodology

1.5.1 Study layout

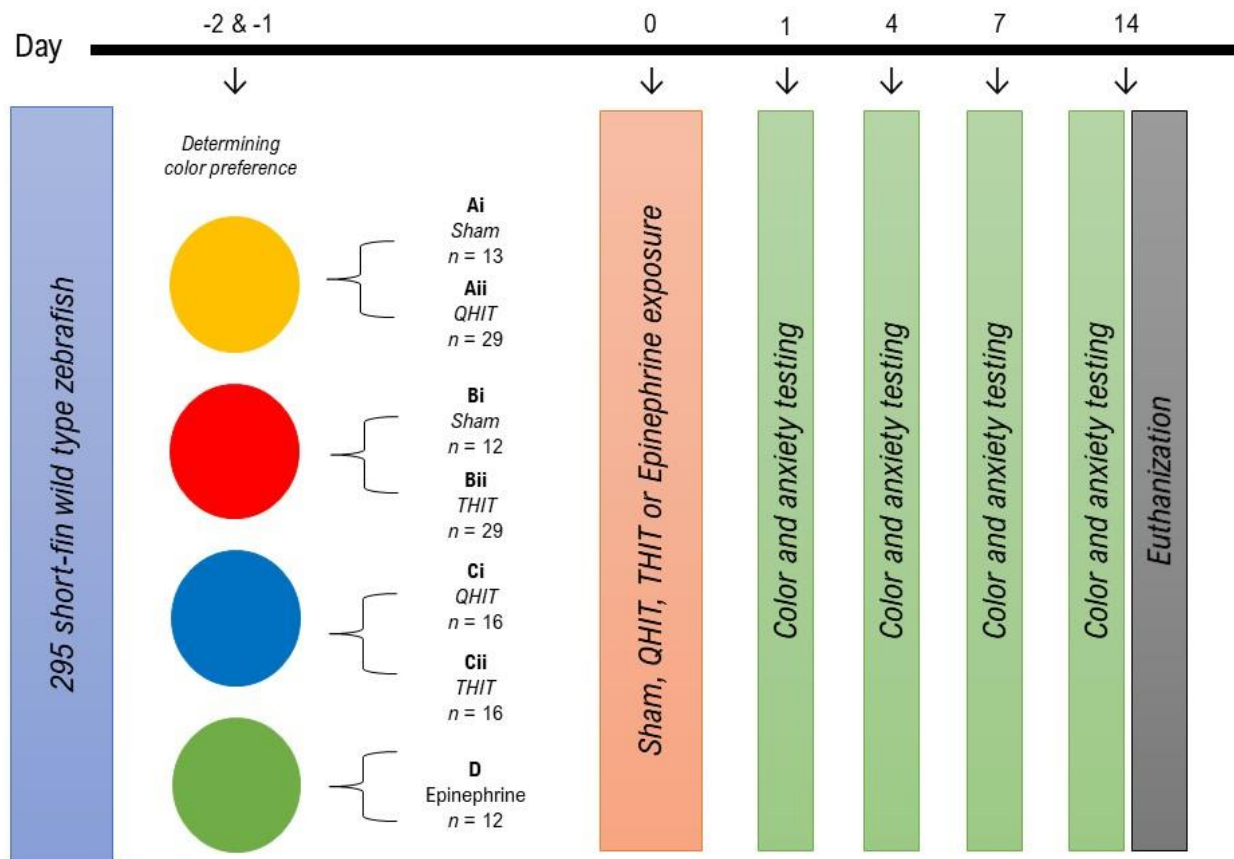


Figure 1-1 - Graphical study layout

Groups A, B and D will be tested for color and anxiety throughout. Group C will only be tested for anxiety on days 1, 4, 7, and 14, while Group D is exposed to a trauma-free protocol, but with the inclusion of EPI. Colors are for illustrative purposes only. Sham: trauma free group; QHIT: quadruple high-intensity trauma; THIT: triple high-intensity trauma; EPI: epinephrine. Fish not indicated in Groups A – D were used as social conspecifics and CAS donors.

1.5.2 Breakdown of experimental fish and fish used as conspecifics and CAS donors

As alluded to above, a total number of 295 zebrafish will be used in the present work. These will be divided as follows:

- 29 zebrafish: QHIT¹ protocol (group Aii);
- 29 zebrafish: THIT² protocol (group Bii);
- 25 zebrafish: sham³ exposure protocol (groups Ai and Bi);
- 32 zebrafish: investigating the role of a contextual reminder on QHIT and THIT-related behavioral sequelae (group C);
- 12 zebrafish: isolating the effect of EPI⁴ on potential later life PTSD⁵-like behaviors protocol (group D);
- 72 zebrafish: used as housing conspecifics in home tanks; and
- 96 zebrafish: euthanized as CAS⁶-donors for use in the QHIT protocol.

1.6 Ethical considerations

The current investigation was approved by the North-West University AnimCare Research Ethics Committee (approval no. **NWU-00526-20-A5**). Throughout the study, we observed and applied the principles of the 3 Rs⁷ as they pertain to experimentation with animals (Arora et al., 2011; Doke & Dhawale, 2015; Ranganatha & Kuppast, 2012). All procedures were in accordance with the code of ethics and complied with national legislation that governs matters of experimental animal welfare (South African National Standard for the Care and Use of Animals for Scientific Purposes; SANS 10386:2008).

1.7 Summary

In summary, we expect this research to deliver a useful and standardized preclinical model platform for the study of PTSD-relevant behavioral changes in zebrafish. We further believe that by providing the field of preclinical neuroscience research with a fully replicable model system, we will bolster future endeavors that are aimed at identifying potentially novel therapeutic targets to improve current PTSD therapies.

¹ quadruple high intensity trauma

² triple high intensity trauma

³ trauma free

⁴ epinephrine

⁵ posttraumatic stress disorder

⁶ conspecific alarm substance

⁷ replacement, refinement, and reduction

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2 Literature Review

2.1 Posttraumatic disorder (PTSD) in the clinic

2.1.1 Description and epidemiology

Posttraumatic stress disorder (PTSD)¹ is a severe and incapacitating mental disorder that manifests in some individuals following a personal encounter with, or experience of a profound threat, and/or witnessing/observing others encountering a traumatic life-threatening event (Miller, Lin, Wolf, & Miller, 2018; Vieweg et al., 2006), e.g. sexual and/or physical assault, war-related incidents, and natural disasters (NIH, 2019). The lifetime prevalence of PTSD ranges between 1.9 % and 8.8 %, while approximately 3 % of the global population are diagnosed with PTSD at any given time (Bisson, Cosgrove, Lewis, & Roberts, 2015). It is noteworthy to mention that the (lifetime) prevalence of PTSD differs across the world as countries in conflict will report higher statistics than others (Atwoli, Stein, Koenen, & McLaughlin, 2015). Importantly, PTSD is distinct from most other neuropsychiatric disorders in that its diagnosis is contingent on the prior involvement of a traumatic experience (Sharma & Ressler, 2019). As such, prior to 1980, PTSD was described as, among others, shell-shock and concentration-camp syndrome (Schnurr, Friedman, & Bernardy, 2002), while the term *posttraumatic stress disorder* was described in the third edition of The Diagnostic and Statistical Manual of Mental Disorders (DSM²), where it was classified as an anxiety disorder (Vieweg et al., 2006).

There are two principal components leading to the development of PTSD, namely 1) encountering a severe, life-threatening and traumatizing event and 2) having a fundamental genetic, clinical or familial predisposition to the disorder (Sharma & Ressler, 2019). The condition is characterized by flashbacks, emotional outbursts (mainly anger), avoidance of contextual reminders of the original traumatic event, and sleep disturbances (Bisson et al., 2015; NIH, 2019). PTSD is clinically heterogeneous and is often accompanied by other psychiatric comorbidities (Albucher & Liberzon, 2002). However, considering its neurobiology which is partly founded in the stress-related noradrenergic and endocrine architecture (refer to **sections 2.2.1.2 & 2.2.1.3**), PTSD also co-presents with conditions of the cardiovascular system, e.g. hypertension (Fait et al., 2018). In general, women seem to be more susceptible to developing PTSD than men (Caramillo, Khan, Collier, & Echevarria, 2015; Garcia, Walker, & Zoellner, 2018; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Ward, 2016), while also recovering from the traumatic experience at a slower rate than men (Caramillo et al., 2015; Meewisse, Reitsma, De Vries, Gersons, & Olf, 2007; Ward, 2016). Patients suffering from PTSD are often functioning within disrupted individual and familial relationships which further puts strain on their social, medical and financial stability (APA, 2013; Miao, Chen, Wei, Tao, & Lu, 2018). It is crucial that more research is done

¹ posttraumatic stress disorder

² Diagnostic and Statistical Manual of Mental Disorders

to find more effective treatment alternatives, since current approaches remain less than optimal (Department of Veterans Affairs, 2020; Galea et al., 2012; Hauger et al., 2012; Lipp, Waters, Luck, Ryan, & Craske, 2020).

Importantly, up to 75 % of all individuals will at some point in time be exposed to a traumatic life event that fits the prodromal picture of PTSD¹ (APA, 2013). However, only a select few, i.e. roughly 12 % (Breslau & Kessler, 2001) of those, will progress to develop PTSD (Skelton, Ressler, Norrholm, Jovanovic, & Bradley-Davino, 2012), pointing to individual variances in the risk for and resilience against the development of PTSD (Nemeroff et al., 2006; NIH, 2019). This suggests that an individual's own subjective experience during and after the traumatic event, i.e. how the person that was exposed to the traumatic event cognitively processes the trauma, plays a noteworthy role in whether or not such exposure will act as an etiopathological trigger of PTSD (Simmons & Granvold, 2005). Risk factors for PTSD have been studied extensively and range from race and religion to child abuse and marital status. Even so, three risk factors were found to be uniform across all populations, namely early-life adversity, as well as own and familial psychiatric history (Breslau, 2009; Kreutzmann et al., 2021). Moreover, a prior diagnosis of anxiety and depression is associated with an increased likelihood of experiencing a traumatic event (Breslau, 2009).

2.1.2 Symptomology and differential diagnosis

The symptoms of PTSD generally manifest from the third month after the exposure to a traumatic event. However, there can be a delay of several years in symptom onset, referred to as “delayed expression PTSD” (APA, 2013; Schnurr et al., 2002). The DSM-5² describes the symptoms of PTSD under four main categories, i.e. i) intrusion; ii) avoidance; iii) negative mood and cognition; and iv) arousal and reactivity (APA, 2013). The symptomatic manifestation of PTSD varies in each individual and many experience only one of the four main symptoms (APA, 2013). The following are the DSM-5 definitions for each category:

Intrusion (or intrusive re-experiencing) refers to reliving the traumatic event in flashbacks, persistent bad dreams or nightmares or any form of prolonged psychological distress cued by an internal or external stimulus (APA, 2013).

Avoidance speaks to the suppression or attempted suppression of distressing internal reminders of the traumatic event such as memories, feelings, or thoughts. It also applies to external reminders such as people, objects, or places. Occasionally specific smells can trigger avoidance, such as in the case of sexual abuse survivors (APA, 2013).

Negative Cognition and Mood symptoms present as dissociative amnesia where the person is incapable of remembering important details of the event. It can also present as distorted perceptions of the

¹ posttraumatic stress disorder

² The Diagnostic and Statistical Manual of Mental Disorders, 5th edition

cause or consequences of the event, leading to self-blame or exaggerated negative beliefs about oneself or others. Other manifestations of this includes decreased interest in once enjoyable activities, isolation from others and the inability to experience positive emotions (APA, 2013).

Arousal and Reactivity symptoms occur as sleep disturbances, hypervigilance, and lack of concentration. Other symptoms include outbursts of aggression and self-destructive or irresponsible behavior, without being aggravated (APA, 2013).

With the addition of specific aspects, e.g. details regarding the nature of the stressor, each of the abovementioned symptom categories are assessed with consideration of specific aspects (APA, 2013):

A. Exposure to stressor in the following manner(s):

- i) Directly experiencing the traumatic event;
- ii) Witnessing the event occurring to others;
- iii) Learning about a traumatic event that occurred to a close family member or friend;
- iv) Undergoing prolonged or/and continuous exposure to aversive details of the traumatic event.

B. Presence of intrusive recollection(s):

- i) Experiencing recurring, involuntary, and invasive distressing memories of the stressor;
- ii) Having recurrent nightmares related to the traumatic event;
- iii) Undergoing dissociative reactions (flashbacks) and reliving/re-experiencing the trauma;
- iv) Extensive psychological distress when exposed to details and reminders of the traumatic event.

C. Persistent avoidance:

- i) Avoidance (or attempted avoidance) of painful memories, thoughts, and feelings related to the traumatic event;
- ii) Avoiding external prompts related with the trauma.

D. Negative cognitions and mood(s):

- i) Incapability to recollect important facets of the traumatic event;
- ii) Experiencing continual exaggerated negative beliefs or expectations;
- iii) Remembering distorted versions about the cause or consequences of the traumatic event;
- iv) Continually in a negative emotional state;
- v) Markedly diminished interest or participation in once enjoyable activities;
- vi) Feeling detached or estranged from others;
- vii) Inability to experience positive emotions.

E. Marked alterations in arousal and reactivity:

- i) Irritable and experiences outbursts of anger and aggression (with little to no provocation);
- ii) Reckless or self-destructive behavior;
- iii) Hypervigilance;
- iv) Exaggerated startle response;
- v) Problems with concentration;
- vi) Sleep disturbances.

Further, a patient will only be diagnosed with PTSD¹ when:

- i) Criteria B, C, D, and E (duration of the disturbance) are present and persistent for at least 1 month;
- ii) The disturbance causes clinically significant distress or impairment in the social, occupational, and other important areas of the individual's functioning; and
- iii) The disturbance is not attributable to the physiological effects of a substance (e.g., medication, alcohol) or another medical condition.

¹ posttraumatic stress disorder

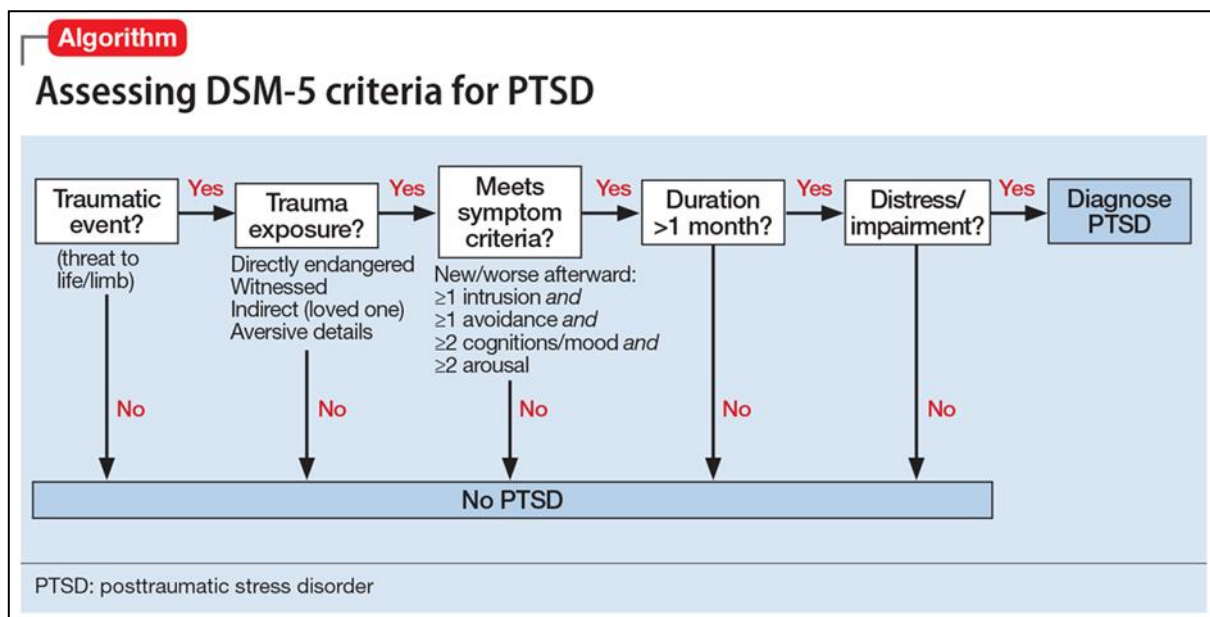


Figure 2-1 - Algorithm for assessing the DSM-5 criteria of PTSD (North et al., 2018)

PTSD¹ is mostly a chronic disorder i.e. lasting longer than one year and requires continuing medical care (Centre for Disease Control, 2021). According to the United States (US)² National Comorbidity Survey, approximately 90 % of the PTSD cases endure for longer than 3 months, 70 % have a duration of over a year, and 33.3 % of cases never reach remission or full recovery (Schnurr et al., 2002). It is thus likely that the manner in which individuals respond to a traumatic event, is shaped by inter-individual differences in early life experiences, which include co-morbid clinical diagnoses, genetic predisposition, and social support structure (Jovanovic & Ressler, 2010; Sharma & Ressler, 2019).

In terms of differential diagnoses, it is notable that most of the specified diagnostic criteria for PTSD are ambiguous, often being applied in the diagnosis of several other mood and anxiety disorders, such as generalized anxiety disorder (GAD³) and major depressive disorder (MDD⁴) (Breslau, 2009; Breslau & Kessler, 2001). The only differentiating criteria are that PTSD requires a fundamental link to an identifiable stressor, and that the corresponding symptoms must be connected to the memory of the stressor (APA, 2013; Breslau, 2009). Therefore, considering the clinical overlap between certain features of PTSD, mood disorders, substance abuse disorders and anxiety disorders, it is often difficult to diagnose patients with PTSD (Vieweg et al., 2006), a skill that requires specialist psychiatric oversight (Vieweg et al., 2006).

¹ posttraumatic stress disorder

² United States

³ generalized anxiety disorder

⁴ major depressive disorder

2.2 The psychobiology of PTSD

Our knowledge and understanding of the pathophysiology of PTSD is far from complete. This may likely be due to the complicated nature of its etiology, as development of the disease is intricately linked to the experience of a totally unpredictable stressor. However, some hypotheses may provide insight into the pathophysiological basis of the condition. These all point to dysfunctional processes underlying the manner in which the human body mitigates the immediate and long-lasting psychobiological responses to acute fear-induced stress following exposure to life-threatening situations (Vieweg et al., 2006).

2.2.1 The human fear response and PTSD

Generally, PTSD¹ is architecturally analogous to other conditions characterized by marked fear responses such as phobias and anxiety disorders (Kreutzmann et al., 2021; Simmons & Granvold, 2005). Humans can experience fear after being exposed to either realistic or unrealistic triggers (Kreutzmann et al., 2021; Stegmann et al., 2019). Such fear-evoking stimuli activate the appropriate fear (and stress) responses during “fight-or-flight” scenarios, which are crucial, as this prepares the individual who is confronting a threat to react in a swift, appropriate, and decisive manner (Agren, Björkstrand, & Fredrikson, 2017; Norrholm & Jovanovic, 2018; Stegmann et al., 2019). Briefly, such responses involve activation of the prefrontal cortex (PFC²) and the amygdala, which launches the organism into “fight-or-flight” mode (Norrholm & Jovanovic, 2018). Simultaneously, the hippocampus applies spatial and temporal information to evaluate the context of the trauma, which is an important foundation of appropriate memory consolidation (Flores, Fullana, Soriano-Mas, & Andero, 2018; O’donnell, Hegadoren, & Coupland, 2004). Further, distressing circumstances are accompanied by the release of stress-responsive hormones and neurotransmitters, e.g. cortisol (CORT³), serotonin (5HT⁴), norepinephrine (NE⁵) and epinephrine (EPI⁶), gamma-aminobutyric acid (GABA⁷), vasopressin, as well as various endogenous opioids (Van der Kolk, 2001). Subsequently, various cognitive pathways which are activated by the cascade of signals initiated by these messaging substances, prime the stress-exposed organism to respond in an appropriate manner. The resulting physiological changes, e.g. inflated sympathetic signaling (Schaefer, Larson, Davidson, & Coan, 2014), hypoalgesia and a hypersensitized startle response (Rosen & Schulkin, 1998), place the organism in a state of optimal responsiveness, previously referred to as the “fight or flight” state. However, while useful during stress exposure, the timely deactivation of such responses is just as critical to mitigate the negative effects of stress on the human body (Agren et al.,

¹ posttraumatic stress disorder

² prefrontal cortex

³ cortisol

⁴ serotonin

⁵ norepinephrine

⁶ epinephrine

⁷ gamma-aminobutyric acid

2017; Hauger et al., 2012). Briefly, PTSD is believed to result from *dysfunctional* fear consolidation and stress mitigation mechanisms which may not only undermine the acute fear response, but also prevent adequate fear desensitization to occur after the threat has passed or subsided (Garcia et al., 2018; Kreutzmann et al., 2021; Van der Kolk, 2001).

2.2.1.1 The neuroanatomy of fear: multiple dysfunctions in PTSD

As briefly highlighted earlier, the main brain areas involved in the pathophysiology of the mammalian fear response are the amygdala, hippocampus, and the PFC¹ (Caramillo et al., 2015; Garcia et al., 2018; Stewart et al., 2012). Each of these brain areas plays a distinct role in the acute fear response, fear memory consolidation, retrieval and the contextual appraisal of future circumstances that may be flagging a potential novel threat. Two routes for processing fear-related stimuli have been proposed, i.e., the so-called “high road” and the “low road” (LeDoux, 2003). The “high road” involves the relay of visual stimuli from the retina via the thalamus to the primary visual and sensory cortices which subsequently activate the amygdala and hippocampus (LeDoux, 2003; Öhman, 2005). The “low road” is activated in the inferior temporal lobe, which recognizes danger and relays the information to the amygdala which is responsible for the ultimate emotional response to be generated (Öhman, 2005). In turn, the hippocampus interacts with both the PFC and the amygdala to achieve an appropriate, potentially lifesaving, fear response and to facilitate the consequent suppression thereof (LaBar, 2007). Indeed, after the aversive stimuli have been avoided, mitigated or neutralized, the PFC will begin to inhibit the amygdala, suspending the emotional and stress response (Bremner et al., 1995) and thereby preventing the further release of NE² (Soeter & Kindt, 2010).

Considering the above, PTSD³ sufferers often present with functional and structural changes to the brain circuits involved in fear processing. In addition to the PFC, amygdala, and hippocampus, several other brain structures are also implicated in PTSD, such as the brain stem, anterior cingulate, corpus callosum and the orbitofrontal cortex (OFC⁴) (Garcia et al., 2018; Norrholm & Jovanovic, 2018). However, the functional significance of these still remains to be elucidated (Van der Kolk, 2001).

The hippocampus is believed to play a role in the emotional processing and recording of short-term memories (Norrholm & Jovanovic, 2018; Van der Kolk, 2001). Therefore, proper functioning of the hippocampus is vital for explicit or declarative memory (that is memory retrieval that involve previous personal experiences, concepts and factual information) (Dew & Cabeza, 2011; Gidron, Barak, Henik, Gurman, & Stiener, 2002; Prera, 2020; Ullman, 2004) and learning from experience, as the hippocampus is vital for the evaluation of stimuli

¹ prefrontal cortex

² norepinephrine

³ posttraumatic stress disorder

⁴ orbitofrontal cortex

relating to previously stored contextual information (Norrholm & Jovanovic, 2018; Van der Kolk, 2001). It has been shown that adults suffering from PTSD present with atrophy on the right side of the hippocampus, which is associated with deficits in the recall of memory and verbal memory (Bremner et al., 1995; Caramillo et al., 2015; Stewart et al., 2012).

The amygdala can be regarded as one of the most important fear-processing central nervous system (CNS¹) structures (Vieweg et al., 2006). While allowing the organism to launch into “fight-or-flight” mode, the amygdala also activates hippocampal signaling, ensuring that under normal circumstances, adequate contextual memories are formed (Caramillo et al., 2015; Stewart et al., 2012; Vieweg et al., 2006). After receiving and interpreting the relevant sensory information, the amygdala directs emotional behavior via the hypothalamus and basal forebrain (LeDoux, 2003; Squire & Zola-Morgan, 1991; Van der Kolk, 2001). In PTSD², the amygdala is typically overreactive when patients are re-exposed to a reminder of the traumatic experience (Caramillo et al., 2015; Stewart et al., 2012). This finding has been corroborated in preclinical literature, where it was found that amygdalar lesions prevented the extinction of fear responses, which resulted in the conclusion that “emotional memory may be forever” (LeDoux, 2003; LeDoux, Romanski, & Xagoraris, 1989; Van der Kolk, 2001).

The PFC³, being involved in learning and problem solving, can exhibit inhibitory control over the amygdala in response to a “fight-or-flight” scenario (Lubit, 2018; Van der Kolk, 2001). Thus, it facilitates the extinction of fear responses by exercising top-down inhibition on the amygdala (Hamann, 2001; Zotev, Phillips, Young, Drevets, & Bodurka, 2013). In PTSD, patients often present with reduced PFC volume and responsivity, which potentially results in working memory deficits, in turn linked to the development of flashbacks (Caramillo et al., 2015; Lubit, 2018; Stewart et al., 2012; Van der Kolk, 2001).

2.2.1.2 The neurobiology of fear – a role for norepinephrine and serotonin

As alluded to earlier, several neurotransmitters are released during a traumatic experience, including NE⁴, EPI⁵, 5HT⁶, endogenous opioids and GABA⁷. These have all been implicated in the neurobiology of PTSD (Asalgoo, Jahromi, Meftahi, & Sahraei, 2015; Harvey & Shahid, 2012; Newport & Nemeroff, 2000; Pervanidou, 2008; Van der Kolk, 2001), though their exact roles still need further investigation. Since the present work involves a

¹ central nervous system

² posttraumatic stress disorder

³ prefrontal cortex

⁴ norepinephrine

⁵ epinephrine

⁶ serotonin

⁷ gamma amino butyric acid

biobehavioral analysis of fear-related behavior and how this potentially relates to bolstered noradrenergic signaling, closer attention will only be afforded to the system here.

NE, being one of the key stress hormones (Cahill, Gorski, & Le, 2003), has been a focus in many PTSD studies, as the neurotransmitter is intricately involved in the processing of emotional memories (Cahill et al., 1996). Specifically, it has been shown that the adrenergic alpha (α)_{1/2} receptors influence processes of working memory, spatial learning and fear appraisal, while beta (β)_{1/2} receptors play a role in the processing of auditory fear, spatial reference and fear memory retrieval (Cahill et al., 1996; Hussain, Reddy, & Maani, 2020). Generally, α_1 and β receptors promote neuronal plasticity and propagates neurotransmission in the fear circuitry, whereas α_2 receptors play an inhibitory role, by for example reducing NE¹ release (Asalgoo et al., 2015; Hussain et al., 2020; Roozendaal, McEwen, & Chattarji, 2009; Van Stegeren, 2008). NE is the primary neurotransmitter of the sympathetic nervous system (SNS²) and is centrally released by the locus coeruleus (LC³) to influence both other nearby brain regions, i.e. the amygdala, as well as peripheral processes once it reaches the systemic circulation in response to a stressful or emotional stimulus (Kodirov, 2012; Niwa, Matsumoto, Mouri, Ozaki, & Nabeshima, 2011; Roozendaal et al., 2009; Van Stegeren, 2008). Additionally, EPI⁴ is also released into the circulation by the adrenal medulla (Asalgoo et al., 2015; Klabunde, 2011).

Evidence points to PTSD⁵ patients often presenting with an increased level of sympathetic functioning, compared to healthy controls (O'donnell et al., 2004). For example, in a study by Geraciotti Jr et al. (2001), NE levels in the cerebrospinal fluid (CSF⁶) of PTSD patients were found to be significantly higher in the PTSD cohort. Further, NE concentrations in the CSF were also positively correlated to PTSD symptom severity. In another investigation, yohimbine (a non-selective α_2 -adrenergic auto-receptor antagonist) caused 40 % of patients to experience flashbacks and 70 % experienced panic attacks (O'donnell et al., 2004). These results indicate a clear role for inflated noradrenergic signaling in PTSD, since α_2 -adrenergic antagonism increases NE release. Importantly, from the available data, it seems that heightened noradrenergic activity is related to the re-experiencing of symptoms (Asalgoo et al., 2015; O'donnell et al., 2004) which are characteristic of PTSD on the one hand, and abnormal prefrontal cortical and amygdalar fear conditioning processes on the other.

Involvement of the altered noradrenergic processes have also been shown in rodent models, which mostly measure locomotor activity and acoustic startle responses as indicators of stressor-associated hyperarousal (Ronzoni, Del Arco, Mora, & Segovia, 2016). For example, when treated with drugs that dampen noradrenergic neurotransmission, i.e. prazosin or clonidine, inescapable foot-shock (IFS⁷)-exposed mice have been shown to

¹ norepinephrine

² sympathetic nervous system

³ locus coeruleus

⁴ epinephrine

⁵ posttraumatic stress disorder

⁶ cerebrospinal fluid

⁷ inescapable foot-shock

present with normal acoustic startle responses (Olson et al., 2011). Additionally, Ronzoni et al. (2016) found that by blocking β -adrenoreceptors through localized amygdalar propranolol injection, IFS-associated reductions in exploration are prevented. Last, and in line with clinical data, a broad base of evidence shows that EPI¹ also modulates memory consolidation in animals (Cahill et al., 2003; McGonigle & Ruggeri, 2014; Van Stegeren, 2008).

Although the noradrenergic system is the only neurobiological construct which is focused on in this study, the serotonergic system also needs mentioning, especially since the first line pharmacotherapeutic therapy for PTSD² is the selective serotonin reuptake inhibitors (SSRIs³) (please refer to **section 2.3**). Originating in the raphe nuclei (RN⁴), the serotonergic system interacts across multiple neuroanatomical targets within both the noradrenergic and dopaminergic systems (Tafet & Nemeroff, 2020). As mentioned earlier, 5-HT⁵ is implicated in the pathophysiology of PTSD, likely due to the known role of the serotonergic system in the processing of emotions such as anxiety, aggression, mood and irritability (Asalgoo et al., 2015). Specifically, 5-HT is involved in the activation of hippocampal pathways, which prevent excessive activation of the fight-or-flight response until it is vitally necessary (Van der Kolk, 2001); thus, 5-HT is believed to blunt the firing of noradrenergic neurons (Newport & Nemeroff, 2000). Indeed, PTSD patients have been shown to present with decreased serum 5-HT concentrations (Newport & Nemeroff, 2000); this has been corroborated in later research (Asalgoo et al., 2015; Kodirov, 2012). It thus stands to reason that SSRIs attenuate PTSD symptomology by blunting hyperadrenergic responses often seen in sufferers.

2.2.1.3 Endocrine involvement

Patients suffering from PTSD often secrete higher concentrations of corticotropin releasing factor (CRF⁶) (Hauger et al., 2012). Still, they generally present with lower levels of cortisol, regardless of exposure to or experiencing continual stressors (Lubit, 2018). Hence, in combination with noradrenergic dysfunction, aberrant hypothalamic-pituitary-adrenal (HPA⁷) axis regulation is a hallmark trait of the condition (Caramillo et al., 2015; O'donnell et al., 2004; Stewart et al., 2011). The HPA axis is not only responsible for the physiological regulation of CORT⁸ secretion but is also vital for the rapid release of CORT under stressful circumstances (Van der Kolk, 2001). In a stressful scenario, the hypothalamus releases CRF, which in turn stimulates the release of adrenocorticotrophic hormone (ACTH⁹) from the anterior pituitary gland. ACTH subsequently circulates to the

¹ epinephrine

² posttraumatic stress disorder

³ selective serotonin reuptake inhibitors

⁴ raphe nuclei

⁵ serotonin

⁶ corticotropin releasing factor

⁷ hypothalamic-pituitary-adrenal

⁸ cortisol

⁹ adrenocorticotrophic hormone

adrenal glands, where it triggers the release of CORT¹ from the adrenal cortex (Newport & Nemeroff, 2000; Van der Kolk, 2001). In healthy individuals, this rapid increase in CORT elicits several stress-related physiological changes in the human body, and importantly, prevents the hypothalamus from persistently secreting CRF².

Considering the lower concentrations of cortisol seen in parallel with higher CRF concentrations, it is likely that—although PTSD³ patients seem to present with heightened stress-related hypothalamic activation—a significant degree of HPA⁴ axis dysregulation, underlies the condition (Caramillo et al., 2015; O'donnell et al., 2004; Stewart et al., 2011). Viewed broadly, increased CRF release in patients does not necessarily translate into increased adrenal CORT release, a clinical finding that still needs elucidation. However, it has been shown that SSRI⁵ treatment may significantly affect HPA axis functioning by inhibiting the effects of CRF release on the amygdala and increasing the density of hippocampal and hypothalamic glucocorticoid receptors (Tafet & Nemeroff, 2020).

On a neurocognitive level, glucocorticoids, like NE⁶, play an important role in fear memory consolidation and retrieval (Caramillo et al., 2015; Stewart et al., 2012). It is believed that prolonged and sustained high levels of glucocorticoids, which may initially prevail under fearful circumstances, result in hippocampal cell death (Van der Kolk, 2001). As such and considering the clear evidence for temporal changes in post-stress CORT regulation, continued investigation is aimed at highlighting and clarifying the role of HPA axis regulation in the psychopathology of PTSD.

2.3 The pharmacotherapy of PTSD

To date, the treatment of PTSD is mainly based on a symptomatic approach, unfortunately offering limited beneficial outcomes (Galea et al., 2012; Hauger et al., 2012). Only 60 % of patients demonstrate mild clinical response (partial symptom attenuation) and of the 60%, symptom remission is achieved in 20 – 30 % of sufferers only (Bailey, Cordell, Sobin, & Neumeister, 2013). Nevertheless, the goal of treatment remains to achieve attenuation of symptom presentation, reduction of other trauma-related comorbidities, improvement of the overall day to day function of patients and prevention of subsequent relapse (Vieweg et al., 2006). Currently, a combination of pharmacological and non-pharmacological interventions (**Figure 2-2**; Miao et al. (2018)) is used to treat PTSD (Department of Veterans Affairs, 2020). Here, we will focus our summary on the pharmacotherapeutic interventions.

¹ cortisol

² corticotropin releasing factor

³ posttraumatic stress disorder

⁴ hypothalamic-pituitary-adrenal

⁵ selective serotonin reuptake inhibitors

⁶ norepinephrine

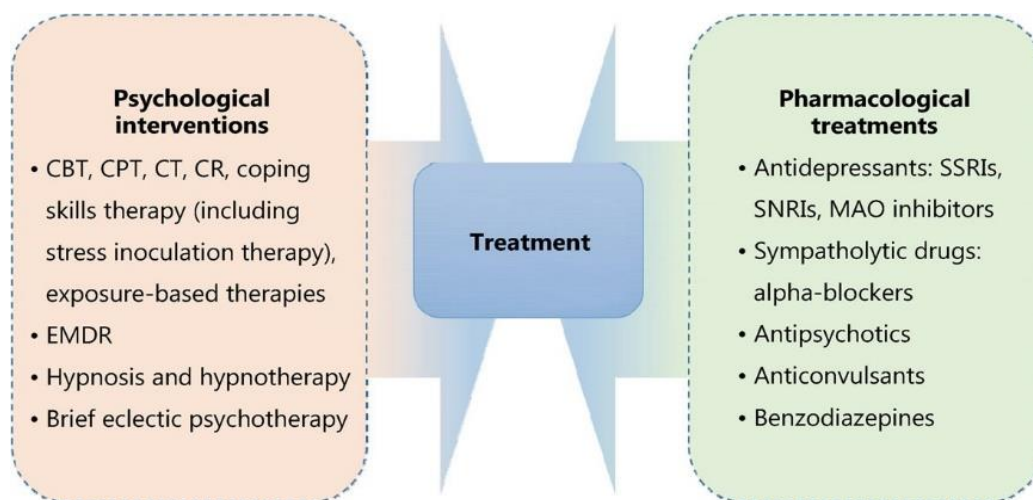


Figure 2-2 - Psychological and pharmacological strategies for treatment of PTSD.

CBT = Cognitive behavioral therapy; CPT = Cognitive processing therapy; CT = Cognitive therapy; CR = Cognitive restructuring; EMDR = Eye movement desensitization and reprocessing; SSRIs = Selective serotonin reuptake inhibitors; SNRIs = Serotonin and norepinephrine reuptake inhibitors; MAO = Monoamine oxidase (Miao et al., 2018)

2.3.1 Drugs targeting serotonergic and adrenergic mechanisms

In terms of the long-term pharmacological treatment of PTSD, SSRIs¹, e.g. fluoxetine, paroxetine, sertraline, citalopram, and escitalopram are used as the first line intervention (Bailey et al., 2013; Charney, Hellberg, Bui, & Simon, 2018). Of note is that the US² is the only country to have particular guidelines for the treatment and management of PTSD, in that SSRIs are the only drugs approved for the management of PTSD (Brunet et al., 2018; Charney et al., 2018; MacNamara et al., 2016; Vieweg et al., 2006). As such, South Africa has also adopted the use of SSRIs as first line treatment. The primary proposed mechanisms of action of SSRIs in the treatment of PTSD can likely be related to their ability to blunt excessive noradrenergic signaling by their involvement in the activation of hippocampal pathways, which prevent excessive activation of the fight-or-flight response until it is vitally necessary (Van der Kolk, 2001). Through this, they attenuate symptoms related to avoidance, negative mood, and reactivity (MacNamara et al., 2016; Vieweg et al., 2006), and regulate HPA³ axis functioning. Nevertheless, most clinical trials involving SSRIs show that their effects are not limited to a particular symptom domain, but rather result in broad improvements across most symptom domains (Albucher & Liberzon, 2002; Charney et al., 2018; MacNamara et al., 2016). Also, the use of SSRIs is favored by their relative low cost and the favorable side-effect profile of therapy (Albucher & Liberzon, 2002; MacNamara et al., 2016; Vaswani, Linda, & Ramesh, 2003). In addition, SSRIs⁴ are much safer in case of accidental overdose, while patient dropout rates in SSRI trials are considerably lower than in trials with tricyclic antidepressants

¹ selective serotonin reuptake inhibitors

² United States

³ hypothalamic-pituitary-adrenal

⁴ selective serotonin reuptake inhibitors

(TCAs¹) and monoamine oxidase inhibitors (MOAIs²), which represent alternate, less commonly employed treatment modalities (Albucher & Liberzon, 2002; MacNamara et al., 2016; Schneier et al., 2015).

Considering that the pathophysiology of PTSD³ is proposed to be founded on SNS⁴ hyperactivity during and following severe trauma, it stands to reason that drugs targeted at adrenergic hyperactivity could likely contribute to improvements in symptom manifestation (Albucher & Liberzon, 2002; Bernardy & Friedman, 2015; Schneier et al., 2015). Against this background, some research into the use of α_1 adrenoceptor blockers, e.g., prazosin, presented promising evidence, especially for the treatment of PTSD-associated nightmares and sleep disturbances (Charney et al., 2018; Galea et al., 2012; North et al., 2018; Vieweg et al., 2006). Moreover, considering the high rate of comorbidity between PTSD and alcohol abuse (Bernardy & Friedman, 2015; Charney et al., 2018; Friedman, 2015), prazosin has also been shown to attenuate the stress response and diminish alcohol cravings in sufferers (Green, 2014). Although these findings point to some involvement of the α_1 receptor in the pathophysiology of PTSD, the effects of α_1 receptor antagonism seem to be short-lived, lasting at most for a few days after treatment cessation (Friedman, 2015; Green, 2014).

Drugs acting on the α_2 adrenoceptor also gained some interest, especially since agonists of this receptor, e.g. clonidine and guanfacine, decrease sympathetic outflow (Belkin & Schwartz, 2015). To date, few studies have focused on these agents as a viable treatment option, although these have delivered mixed results (Belkin & Schwartz, 2015; Giustino, Fitzgerald, & Maren, 2016; Rouillet et al., 2021). That said, α_2 adrenoceptor blockers are also under investigation as potential agents that can be used to interfere with traumatic memory consolidation (Brunet et al., 2018; Giustino et al., 2016; Rouillet et al., 2021). Although much of this approach remains to be elucidated, such interventions may present the field with a valuable alternative, since dysfunctional memory consolidation may contribute to PTSD symptom manifestation as explained earlier (Brunet et al., 2018; Giustino et al., 2016; Rouillet et al., 2021). For the same reason, β adrenoceptor blockers have been trialed in PTSD, since non-selective β -blockers, e.g. propranolol, also impair the inappropriate consolidation of aversive memories (Brunet et al., 2018; Galea et al., 2012). Some studies also suggest that, if administered directly after exposure to the traumatic incident, it may play a role in prevention of delayed PTSD symptoms (Albucher & Liberzon, 2002; Giustino et al., 2016; Pitman et al., 2002). However, Dèbiec, Bush, and LeDoux (2011) reported discrepant results from a preclinical study with only some data indicating successful inhibition of fear memory consolidation and associated fear-related responses. Also, most clinical trials have failed to produce irrefutable evidence of propranolol's effectiveness in PTSD⁵, likely due to

¹ tricyclic antidepressants

² monoamine oxidase inhibitors

³ posttraumatic stress disorder

⁴ sympathetic nervous system

⁵ posttraumatic stress disorder

differences in sample sizes and patient inclusion criteria between the various studies (Bailey et al., 2013; Ravindran & Stein, 2009; Schneier et al., 2015).

Pharmacotherapeutic strategies aimed at both the serotonergic and noradrenergic systems, have also been explored. Serotonin and norepinephrine reuptake inhibitors (SNRIs¹), e.g., venlafaxine and duloxetine, are sometimes used as off-label interventions in PTSD (Department of Veterans Affairs, 2020; Galea et al., 2012; Holder et al., 2021; Vieweg et al., 2006). In fact, SNRIs may be of especial benefit in PTSD patients suffering from comorbid depression; however more research is required to establish the effectiveness of these agents as major therapeutic role players in PTSD (Albucher & Liberzon, 2002; Holder et al., 2021; North et al., 2018). Other drugs that fall in this category are the TCAs², e.g., imipramine, desipramine, amitriptyline and clomipramine, which are also often used. In addition to blocking the reuptake of 5-HT³ and NE⁴ (depending on the specific agent) from the synaptic cleft, these drugs also have other mechanisms of action, including modulation of α_2 adrenoceptor function (Albucher & Liberzon, 2002; Lipinska, Baldwin, & Thomas, 2016; Puetz, Youngstedt, & Herring, 2015). Amitriptyline has been shown to improve both the avoidance- and intrusion-specific symptoms of PTSD, while desipramine seems effective to improve depressive, but not PTSD-specific symptoms (Albucher & Liberzon, 2002; Lipinska et al., 2016; Puetz et al., 2015), per se. Therefore, and considering their well-known side-effect profile, these agents are less preferred in the management of PTSD.

Although MAOIs⁵ have also shown therapeutic benefit with respect to attenuating the hyperarousal, intrusion and avoidance symptoms of PTSD (Albucher & Liberzon, 2002; Department of Veterans Affairs, 2020; Galea et al., 2012; Vieweg et al., 2006), patient compliance remains a clinical dilemma, likely due to the need for diet restrictions and the potential risk of hypertensive crises that may be associated with the use of MAOIs (Albucher & Liberzon, 2002; Hageman, Andersen, & Jørgensen, 2001).

2.3.2 Diverse drugs

Second generation neuroleptic drugs, e.g. risperidone, olanzapine and quetiapine (Bailey et al., 2013), which function primarily by blocking dopamine receptors and are clinically used for the management of psychosis (Charney et al., 2018; Galea et al., 2012), are sometimes added to the first-line treatment of PTSD. Considering recent neurobiological findings, which implicated a role for dopamine in PTSD-related amygdalar processes (Asalgoo et al., 2015; Kodirov, 2012; Niwa et al., 2011; Tafet & Nemeroff, 2020), risperidone was successfully used to treat nightmares in PTSD⁶ patients. However, it is also likely that patients suffering from hallucinatory

¹ serotonin and noradrenaline reuptake inhibitors

² tricyclic antidepressants

³ serotonin

⁴ norepinephrine

⁵ monoamine oxidase inhibitors

⁶ posttraumatic stress disorder

flashbacks, intense paranoia, and self-destructive behavior, could also respond to these agents (Albucher & Liberzon, 2002; Hageman et al., 2001).

Since PTSD is an anxiety disorder, some work into the use of benzodiazepines. e.g., alprazolam and lorazepam have also been done. Used mainly to attenuate acute flares in anxiety and to improve sleep rhythm, the clinical use of these drugs is hampered by their addiction potential, especially since PTSD and substance use disorder show a high degree of comorbidity (Department of Veterans Affairs, 2020). Also, even when patients are slowly downtitrated from these drugs after long periods of use, they still experience severe withdrawal reactions, i.e. hyper-alertness, rage and severe intrusive thoughts (Hageman et al., 2001).

Last, mood stabilizers are often used as an augmentation strategy. The theoretical basis for the use of among others, anti-convulsant drugs, e.g. carbamazepine, lamotrigine and topiramate, is founded on data showing that intensification in seizure activity associated with persistent psychological stresses could cause bioelectric changes in PTSD-relevant brain structures like the amygdala; in turn, these may alter symptom expression (Wang, Woo, & Bahk, 2014). Against this background, valproate and carbamazepine showed some benefit in terms of improving avoidance and intrusive symptoms (Hageman et al., 2001); however, these findings could not be replicated in studies using topiramate (Wang et al., 2014). Another drug that is utilized for the treatment of PTSD is lithium, a mood stabilizer that is used in patients with bipolar disorder. Lithium has shown promise for the treatment of PTSD if used in combination with antidepressants (Albucher & Liberzon, 2002).

2.4 Pre-clinical modelling of PTSD

2.4.1 A brief overview of concepts and validity

Animal models are key instruments used to gain knowledge on the abnormal physiological and neuronal mechanisms that contribute to the development of psychiatric diseases such as PTSD and others. Animal models also constitute valuable tools to identify novel therapeutic targets that interact with the aforementioned factors to bring about symptom attenuation (Flandreau & Toth, 2018; Richter-Levin, Stork, & Schmidt, 2019). Still, PTSD presents preclinical research with a translational challenge, as the majority of animal models to our disposal, fails to deliver clinically useful results (Richter-Levin et al., 2019). This can likely be ascribed to the fact that in the clinical setting, PTSD is diagnosed only by means of patient interviews and observing behavioral symptoms that manifest only *after* the occurrence of a life-threatening, traumatic event (Richter-Levin et al., 2019). These conditions are overly difficult to replicate in an animal model, from both a methodological and an ethical perspective. Fortunately, the specific neurobiological targets that are involved in the processing of fear and anxiety, two vital constructs underlying the psychobiology of PTSD¹, have been conserved between several species throughout evolution. This grants preclinical research with a viable avenue of investigation

¹ posttraumatic stress disorder

(Flandreau & Toth, 2018; Rasmusson & Charney, 1997). In fact, trauma akin to that of human traumatic experiences, can be induced in animals, thereby recreating a real-life scenario that can define the framework for investigation into PTSD-like biobehavioral sequelae (Flandreau & Toth, 2018; Richter-Levin et al., 2019).

Importantly, any preclinical animal framework needs to be as accurate as possible in its resemblance of the human condition being investigated. Thus, when conceptualizing a translational animal model of human conditions, three types of validation criteria must be met (McGonigle & Ruggeri, 2014; Overstreet, 1993; Richter-Levin et al., 2019). With respect to PTSD, these can be summarized as follows:

Construct validity, i.e., the degree to which the model can reproduce the underlying psychobiological and etiological mechanisms of clinical PTSD (McGonigle & Ruggeri, 2014; Overstreet, 1993; Richter-Levin et al., 2019);

Face validity, i.e., the model must represent certain similarities in the symptoms and behavior of PTSD patients that can be perceived in both the human disorder and animal behavior (McGonigle & Ruggeri, 2014; Overstreet, 1993; Richter-Levin et al., 2019); and

Predictive validity, which requires that the model must be able to (eventually) replicate potential treatment responses that are akin to those seen in the clinical condition (McGonigle & Ruggeri, 2014; Overstreet, 1993; Richter-Levin et al., 2019).

For the purposes of this investigation, in which we will aim to develop a novel zebrafish (*Danio rerio*) model of PTSD-like behavior, we will now afford closer attention to this animal species and highlight some key aspects of its biobehavioral profile that may be of value for exploration against the background of PTSD.

2.5 The zebrafish (*Danio rerio*)

2.5.1 Background and ecology

Zebrafish (*Danio rerio*) are small tropical cyprinid fresh-water fish (Khan & Alhewairini, 2018; Pilehvar, Town, & Blust, 2020), which were initially described as “*beautiful fish with several silver and blue stripes on both sides*” by Scottish physician, Francis Hamilton (Parichy, 2015). These fish tend to grow to a maximum length of 4 cm from the tip of the snout to the origin of the caudal fin (Kiyokawa, Kikusui, Takeuchi, & Mori, 2005). They are characterized by the presence of a “danionin notch” in the mandible area and distinctive color patterns (Spence, Fatema, Ellis, Ahmed, & Smith, 2007). The body is laterally compressed and fusiform in shape, i.e. tapering at both ends, with a terminal oblique mouth that is directed upwards (Spence et al., 2007). Zebrafish have recently gained a great deal of interest in the research community since they present with a variety of attributes that allow for experimental manipulation (Khan & Alhewairini, 2018; Spence et al., 2007) and accurate characterization of behavior.

Zebrafish are indigenous to the Himalayan region of South-Asia but are also widely distributed across Bhutan, Pakistan, India, Myanmar, Bangladesh, and Nepal (Khan & Alhewairini, 2018; Lawrence, 2007; McClure, McIntyre, & McCune, 2006) (**Figure 2-3**). The geographic regions where zebrafish occur naturally are characterized by monsoon climates. Such climates are associated by a wide variety of seasonal changes in freshwater habitats (Spence et al., 2007). However, their typical habitat comprises of shallow, slow-moving streams or standing bodies of water which tend to be adjacent to rice-fields (Lawrence, 2007; McClure et al., 2006; Parichy, 2015). These bodies of water are mostly clear and have beds covered by gravel and sand/mud. Cover is provided by both aquatic and terrestrial vegetation overhanging from the banks (Lawrence, 2007; McClure et al., 2006; Parichy, 2015; Spence et al., 2007). Interestingly, since these bodies of water are frequently connected to rice paddies (Lawrence, 2007; McClure et al., 2006; Spence et al., 2007), it has been theorized that agricultural fertilizers, which promote the growth of zooplankton (a component of the zebrafishes diet), are the major reason for the distribution of zebrafish in these waters (Engeszer, Ryan, & Parichy, 2004; McClure et al., 2006; Spence et al., 2007). The most common natural predators are the snakehead fish, freshwater garfish and the Indian pond heron (Spence et al., 2007).

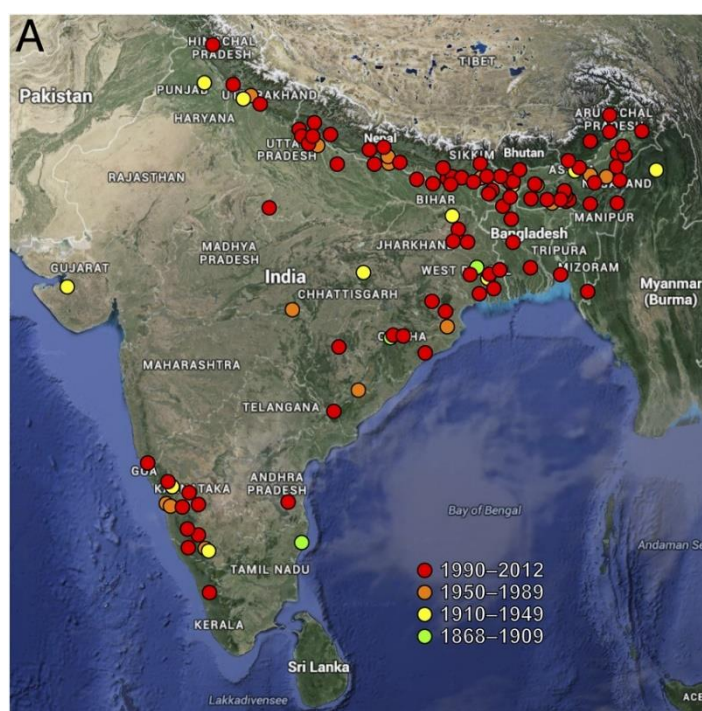


Figure 2-3 – The natural geographical range of zebrafish.

Historic and more recent sites where zebrafish have been reported in India, Nepal, Bangladesh and possibly Myanmar (Parichy, 2015)

Zebrafish can be classified as eurythermal fish, as they demonstrate a high tolerance to a wide temperature range (Lawrence, 2007). This ability assists zebrafish to adapt to the fluctuations in Asian continental temperatures, where in winter, temperatures can fall as low as 6 °C and rise to 38 °C and higher in summer (Spence et al., 2007). Zebrafish can also survive in a variety of water conditions and at geographical elevations of $\pm 8 - 1576$ m (Parichy, 2015). While zebrafish prefer slightly alkaline water, they have been found in pools

with pH values ranging from $\pm 5.9 - 9.8$ (Lawrence, 2007; Parichy, 2015). The species is omnivorous and generally feeds on zooplankton, insects, and small shrimp specimens (Parra, Adrian Jr, & Gerlai, 2009; Piatto et al., 2011; Spence et al., 2007; Rowena Spence, Gerlach, Lawrence, & Smith, 2008). However, gut content analysis has also shown vascular plant materials, invertebrate eggs, fish scales, sand and mud (Spence et al., 2007), while they are known to also prey on their own eggs and larvae (Spence et al., 2007).

In terms of breeding, zebrafish are asynchronous batch spawners, with females able to spawn up to 700 eggs every 2 to 3 days (Lawrence, 2007; Spence et al., 2007) with little to no parental care involved. Zebrafish, both wild and domesticated, normally spawn with the first light of daybreak (Parichy, 2015; Spence et al., 2007). While laboratory-housed zebrafish tend to breed all year round, fish in the wild mainly breed during the summer monsoon season (Lawrence, 2007; Parichy, 2015; Spence et al., 2007). Zebrafish eggs are sensitive to water conditions and hatch between 4- and 7-days post fertilization into free swimming larvae (Lawrence, 2007). From this point onwards their development is rapid (**Figure 2-4**; Holtzman, Iovine, Liang, and Morris (2016)) with food-seeking behaviors and avoidance behaviors appearing 2 to 3 days after hatching (Lawrence, 2007). Growth rate is the most rapid within the first 3 months after hatching and full growth is reached at 18 months (Spence et al., 2007).



Figure 2-4 - Developmental stages of zebrafish; hpf = hours post fertilization (Holtzman et al., 2016; Parichy, 2015)

Naturally occurring genetic mutations yielded four distinct *Danio rerio* phenotypes, i.e. short-fin wild type (WT¹), leopard (Leo²), albino, and long-fin zebrafish (**Figure 2-5**; Egan et al. (2009)). In terms of pre-clinical research, the most used phenotypes are the WT and the Leo (Egan et al., 2009; Quadros et al., 2019; Quadros et al., 2016). The WT is identified by uninterrupted blue stripes across its body and smaller bony segments on the caudal fin (Hoptak-Solga, Klein, DeRosa, White, & Iovine, 2007). It is also widely believed to be characterized by a genetically heterogeneous and well-preserved gene pool (Quadros et al., 2016).

¹ Short-fin wild type

² Leopard

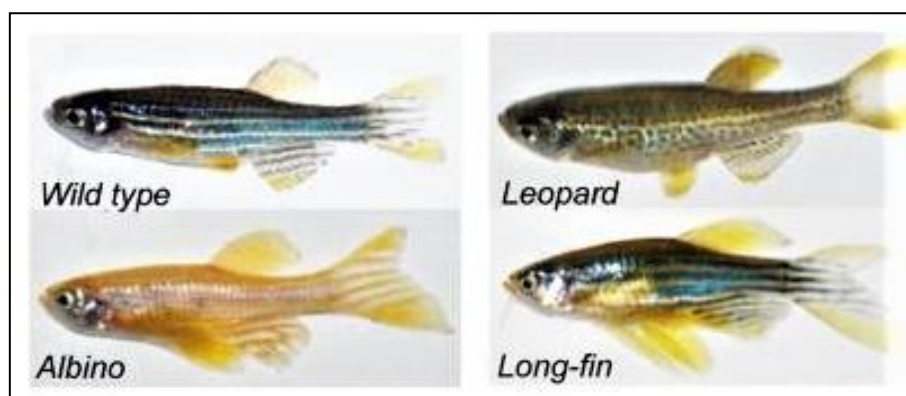


Figure 2-5 - Zebrafish phenotypes (adapted from Egan et al. 2009)

2.5.2 Zebrafish as a novel pre-clinical research platform

The rate of zebrafish-related publications is growing exponentially and currently outpaces that of any other animal model organism (Khan et al., 2017). Despite this, further validation of the species as an animal model system is still required (Khan et al., 2017). Zebrafish present pre-clinical research with several potential benefits, i.e. their relatively quick reproduction rate (Lucon-Xiccato, De Russi, & Bertolucci, 2020; Vaz, Hofmeister, & Lindstrand, 2019), low maintenance costs (Lucon-Xiccato et al., 2020), and a rapid life cycle (Vaz et al., 2019). Thus, zebrafish gained the interest of researchers as an alternative animal model system for the study of the molecular mechanisms and behavioral aspects of brain disorders (Stewart et al., 2011). In fact, as will be alluded to later in this section, such interest is justified since zebrafish also present with comparable neurotransmitter systems and neuroanatomical targets akin to those found in mammals (Maximino et al., 2011). Both larval and adult zebrafish are extensively used for psychopharmacological drug screening (Stewart et al., 2011) and as pre-clinical *in-vivo* models (Khan et al., 2017). In comparison to rodent models, zebrafish fertilization and embryonic development take place ex-utero (Vaz et al., 2019), which favors the use of larval zebrafish for neurodevelopmental studies with or without genetic manipulation due to their small size and transparency (Khan et al., 2017; Stewart et al., 2011; Vaz et al., 2019). Nevertheless, it is important to note that larval zebrafish are morphologically and behaviorally less complex than their adult counterparts (Stewart et al., 2011).

Aside from the advantages of using larval zebrafish as mentioned above, adult fish are employed due to their widely recognized, favorable biobehavioral traits. Although only reaching their fully grown form at 18 months, zebrafish are considered mature at 90 days post-fertilization (dpf¹). Moreover, they can then be used throughout their lifespan of approximately 42 months (Gerhard, 2003). A substantial body of research (Khan et al., 2017; Kiyokawa et al., 2005) has confirmed broad genetic overlap—approximately 70 %—between

¹ days post fertilization

zebrafish and humans (Howe et al., 2013). Further, because the genome¹, transcriptome², and proteome³ (Maximino, da Silva, Gouveia Jr, & Herculano, 2011) of zebrafish have been defined and mapped (Khan et al., 2017), useful insights into the genetic components underlying certain anxiety-like manifestations which may be reminiscent of human illness, may be gained from zebrafish (Quadros et al., 2019). WT⁴ zebrafish appear to be genetically heterogenous and well-preserved (Quadros et al., 2016).

2.5.3 Zebrafish behavior within the context of PTSD

Zebrafish display a few noteworthy behavioral characteristics that are akin to that of humans and other mammals (Stewart et al., 2011). These characteristics form a valuable background for the study of PTSD⁵-like phenomena. For example, zebrafish can contextualize fear in response to certain stressors such as predation (Ogawa et al., 2014), which is valuable for studies of conditioned and unconditioned fear responses (Piato et al., 2011). Further, zebrafish can adapt their own preferences based on external feedback (Blank et al. 2009), for example by refraining from swimming into a dark compartment—normally an instinctive choice—to avoid being shocked in the prior shock-cued dark compartment. In addition, zebrafish can recruit processes of working memory to learn associations between certain contextual cues and related tasks (Gaikwad et al., 2011).

When in distress, zebrafish present with a wide range of unmistakable, quantifiable behaviors (Kiyokawa et al., 2005; Maximino et al., 2010; Stewart et al., 2011), such as freezing, shoaling cohesion, diving, bottom dwelling, and thigmotaxis⁶ (Maximino et al., 2018; Stewart et al., 2011). These stress-induced behavioral changes can easily be compared to the instinctive behaviors of zebrafish which normally dwell at the bottom of a novel environment until they are habituated (Stewart et al., 2011). Importantly, with respect to this investigation, Quadros et al. (2019) showed that both WT and Leo⁷ zebrafish present with observable anxiety-like responses such as freezing, escaping behavior and erratic movements in response to a repeated exposure protocol. However, in a study by Egan et al. (2009) that characterized all four phenotypes of zebrafish, it was found that compared to WT⁸, the *other* mutant strains demonstrated reduced exploratory behavior, suggesting these phenotypes present a higher baseline anxiety. This is important, since the development of a robust fish model of PTSD⁹ would benefit from studying a generalized, i.e. the WT, and not an overly anxious population. This

¹ the entire genetic material of an organism

² the total of all the messenger RNA molecules of an organism, i.e. RNA that has been transcribed from a DNA sequence

³ the entire complement of all the proteins expressed by an organism

⁴ Short-fin wild type

⁵ posttraumatic stress disorder

⁶ near or close dwelling to a tangible surface

⁷ Leopard

⁸ Short-fin wild type

⁹ posttraumatic stress disorder

would be more in line with the clinical picture of traumatic life experiences, which affects all human beings, irrespective of psychiatric history.

To date and to the best of our knowledge, no truly successful zebrafish model of PTSD has yet been developed. Nevertheless, a number of tests of general stress and anxiety have been documented (Caramillo et al., 2015), e.g. the novel tank test—being akin to the rodent open field test (Egan et al., 2009; Stewart et al., 2011)—which is based on the natural tendency of zebrafish to protect themselves from unfamiliar environments by diving and remaining at the bottom of an environment until they are habituated (Blaser, Chadwick, & McGinnis, 2010; DePasquale & Leri, 2018; Stewart et al., 2010). In a more analogous version of the rodent open field test, a similar design can be applied for zebrafish in which behaviors such as thigmotaxis and exploration can be measured (Stewart et al., 2011). As zebrafish habituate to unfamiliar testing environments, they tend to explore more. In contrast, increased time spent wall-hugging (thigmotaxis) is purportedly indicative of anxiety and stress (Caramillo et al., 2015; Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Stewart et al., 2011). Additionally, social preference and shoaling tests performed in such environments assess social behavior and the effects of anxiety on sociability. Since zebrafish are highly sociable by nature, they tend to shoal together; however, while moderate stressors can cause the shoal cohesion to increase, fish also tend to isolate themselves from the group (Engeszer et al., 2004; Stewart et al., 2011) after severe stress.

While the abovementioned tests are by no means indicative of PTSD, they can provide a manner of assessing PTSD-like behavioral manifestations in zebrafish after a traumatic experience, which in turn can further the conceptualization of a valid and reliable PTSD model using zebrafish (Caramillo et al., 2015).

2.5.4 The zebrafish brain

Owing to pharmacological screening, genetic characterization, and behavioral analysis, significant and valuable insights into the neurobiological processes underlying zebrafish functioning (Speedie & Gerlai, 2008) have been made. Initially it was thought that zebrafish only possessed primitive brains (DePasquale & Leri, 2018; Stewart et al., 2011) and that their behavior was primarily instinct-driven (Stewart et al., 2011). However, recently this idea has been refuted (Egan et al., 2009). It is important to appreciate that even though the zebrafish brain is complex, it is not as developed and intricate as mammalian equivalents (Vaz et al., 2019). However, despite the differences in certain structures, most mammalian brain structures and neural cell types are represented in the zebrafish brain in some manner (Vaz et al., 2019). These include the cerebellum, tectum (which is responsible for the startle and reflex responses), tegmentum (which receives external sensory stimuli and triggers motivated behavior), hippocampus and the diencephalon, which ultimately regulate alertness, attention, and circadian behaviors. Each of these are implicated to a certain extent in PTSD¹ (Vaz et al., 2019).

¹ posttraumatic stress disorder

Additionally, the medial teleost pallium of zebrafish is structurally analogous to the mammalian amygdala (Khan et al., 2017).

In terms of neurotransmitter function, zebrafish possess intact noradrenergic, serotonergic, dopaminergic and purinergic systems which are organized in a similar manner to that found in mammalian animal species (Nabinger, Altenhofen, & Bonan, 2020). This characteristic is of benefit for stress-related studies, especially since zebrafish also present with a stress cascade system similar to the mammalian HPA¹ axis, namely the hypothalamus-pituitary-interrenal (HPI²) axis (Alderman & Bernier, 2009; Alsop & Vijayan, 2009; Alsop & Vijayan, 2008). As in mammals, this system, also activated by the hypothalamus, regulates circulating CORT³ levels. Moreover, the neuroendocrine system of zebrafish can easily be modified through genetic and pharmacological manipulation (Khan et al., 2017), while cortisol can be sampled by means of both non-invasive and invasive techniques (Canavello et al., 2011; Félix, Faustino, Cabral, & Oliveira, 2013; Pavlidis, Sundvik, Chen, & Panula, 2011). That zebrafish secrete human-like CORT, instead of rodent-like corticosterone, further contributes to its desirability as a potential preclinical model of PTSD (Piato et al., 2011).

2.6 In summary: key concepts of relevance for the current investigation

2.6.1 Towards the development of a PTSD model of accurate translational relevance

As alluded to earlier, PTSD is a debilitating anxiety disorder that manifests in some individuals after experiencing a life-threatening, traumatic event (Miller et al., 2018; Vieweg et al., 2006). However, the exact pathophysiology of PTSD is still unknown, resulting in patients responding sub-optimally to currently available treatments. Since the available treatments for PTSD are largely based on controlling symptomatic outcomes and not addressing the core dysfunction *per se*, very little is known about the progression of the illness from cause to illness (Galea et al., 2012; Hauger et al., 2012). Hence, attempts to improve our understanding of PTSD are urgently needed, which in turn may result in the development of more adequate treatment options. To date, PTSD models have mainly been based on rodent studies (Stewart, Yang, Nguyen, & Kalueff, 2014). Regrettably, these models are expensive while also presenting research with a narrow window of opportunity for investigation since laboratory-housed rodents age and mature rapidly. As such, it is not viable to study the long-term sequelae of trauma in rats and mice. To this end, zebrafish may be a viable alternative for rodent studies. *Danio rerio* is a relatively low-cost, rapidly reproducing and long-living species, which makes it a potentially useful candidate for modelling psychiatric illnesses, including PTSD⁴. Hence, the current investigation will seek to explore the

¹ hypothalamus-pituitary-adrenal

² hypothalamus-pituitary-interrenal

³ cortisol

⁴ posttraumatic stress disorder

existing gap in PTSD literature by validating a novel zebrafish model of PTSD with robust face validity for further exploration in terms of neurobiology and treatment response in later studies.

2.6.2 General anxiety vs. the fear of death

Although PTSD and generalized anxiety disorder (as well as other anxiety disorders) appear to be very similar due to the great degree of shared symptomology, e.g. elevated levels of anxiety, they differ with respect to several other aspects (Chen & Etkin, 2013). According to the DSM-5¹ to be diagnosed with GAD², patients must experience (APA, 2013):

- A. Excessive worry and anxiety, which occurs more commonly than not for a minimum of 6 months, about several events or activities (such as daily routines and work);
- B. Difficulty in regulating the worry;
- C. The anxiety and worry must be associated with three (or more) of the following six symptoms:
 - 1. Feelings of restlessness
 - 2. Becoming fatigued easily
 - 3. Struggling to concentrate
 - 4. Irritability
 - 5. Muscle tension
 - 6. Recurrent sleep disturbances;
- D. The anxiety and worry cause noteworthy distress and possible impairment in social, occupational, or other important areas of functioning;
- E. The disturbance must not be caused by effects of a substance or another medical condition;
- F. The disturbance must be explained better by another mental disorder (e.g., panic disorder, social anxiety disorder, obsessive-compulsive disorder, etc.)

These symptoms may seem akin to that of PTSD³. However, as stated earlier, the diagnosis of PTSD relates directly to a specific experience during which the patient experienced a significant traumatic or life-threatening event (APA, 2013; Miller et al., 2018; Vieweg et al., 2006). Thus, the first and foremost difference between PTSD and GAD⁴ is that GAD occurs due to unspecific worries about routine and normal experiences, whereas PTSD pertains to a specific event (APA, 2013; Miller et al., 2018; Vieweg et al., 2006). On a psychobiological

¹ The Diagnostic and Statistical Manual of Mental Disorders, 5th edition

² generalized anxiety disorder

³ posttraumatic stress disorder

⁴ generalized anxiety disorder

level also, only PTSD sufferers have chronic and intrusive fear-based memories relating to a specific trauma (Chen & Etkin, 2013). This distinction is important for the present work.

2.6.3 A new direction forward: quadruple high intensity trauma (QHIT) – the conceptualization of a bolstered fear response

Many plants and animals communicate information pertaining to potential danger via multiple methods, including alarm calls, visual signals and chemical cues (Maximino et al., 2019). Zebrafish are also known to alert one another of danger by means of secreting a chemical cue, that is conspecific alarm substance (CAS¹) (Maximino et al., 2019; Quadros et al., 2016; Waldman, 1982). CAS is a pheromone produced in the epithelial club cells of the fish when they are injured or damaged (Lima et al., 2016; Parra et al., 2009; Waldman, 1982). To date, several laboratories have attempted to create PTSD in zebrafish by using CAS. However, the results have at most been unreliable and inconsistent (Parra et al., 2009; Quadros et al., 2019; Speedie & Gerlai, 2008), likely due to the lack of standardized and replicable methods of CAS extraction and that the message it conveys is non-specific in terms the nature of the threat it is meant to forewarn (Lima et al., 2016). Further, although there have been several attempts to synthesize a standardized, synthetic form of CAS, this has yielded mixed and suboptimal results (Parra et al., 2009).

As early as the late 1980's, Gray (1987) proposed that different categories of stressors could be used to induce fear in animals, e.g. exposure to novelty, intense sensory stimuli, mild electric shocks, predatory stimuli, and alarm cues from conspecifics (Gray, 1987; Quadros et al., 2016). Yet, a robust body of research has shown that a single, e.g., electric shock, or even a double stressor, does not elicit a sustained fear response in zebrafish (Oliveira et al., 2014; Parra et al., 2009; Quadros et al., 2016), likely due to the robust and overly non-anxious nature of WT² zebrafish. In the present work, we will attempt to expand upon this work by investigating whether a life-threatening, severe stress paradigm, i.e., quadruple high intensity trauma (QHIT³), aiming to combine the various constructs of life-threatening trauma, will be sufficient to induce lasting PTSD-like fear in a WT zebrafish model.

2.7 Conclusion

PTSD⁴ places a substantial burden on sufferers and society due to the several mental, physical, and socio-economic implications (Bailey et al., 2013). However, due to the many hypothetical dysfunctions that may contribute to causing PTSD, the pathophysiology remains unknown, and the treatment of PTSD should be considered ineffective because it lacks a reliable and convincing scientific evidence base (Galea et al., 2012;

¹ conspecific alarm substance

² Short-fin wild type

³ quadruple high intensity trauma

⁴ posttraumatic stress disorder

Hauger et al., 2012). Another stumbling block in the diagnosis and treatment of PTSD is the individual variability in the risk for and resilience against the development of PTSD (Nemeroff et al., 2006; NIH, 2019). Hence, attempts to improve our understanding of PTSD are urgently needed, which in turn will result in the development of more adequate treatment options. As such, this work is aimed at addressing this need by attempting to develop a robust and standardized model of PTSD-like behavior in zebrafish.

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3 Research article

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

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Abstract

Posttraumatic stress disorder (PTSD¹) transpires in some individuals after exposure to or experiencing a severe life-threatening event. While the condition is primarily founded upon aberrant adrenergic processes, our understanding of how said processes influence the etiopathology of the condition, remains inconclusive. Here, we aimed to develop and describe a novel zebrafish (*Danio rerio*) model of life-threatening trauma-induced anxiety that may be representative of clinical PTSD. Briefly, four groups of zebrafish were each exposed to different and unique stress-related paradigms, i.e. i) a sham² (trauma free), protocol, ii) exposure to high-intensity trauma in the presence of epinephrine (EPI³) exposure (quadruple high intensity trauma; QHIT⁴), iii) exposure to high-intensity trauma in the absence of EPI exposure (triple high intensity trauma; THIT⁵), and iv) EPI exposure on its own. Novel tank anxiety was subsequently assessed in the absence and presence of a contextual cue, i.e. a trauma-paired color, at 1, 4, 7 and 14 days after the traumatic event. The present results demonstrate that 1) trauma-paired EPI exposure consistently blunted the delayed anxiety-like manifestations of severe trauma, but bolstered persistent anxiety-like behavior when paired with exposure to a novel circumstance in the absence of additional stressors, 2) exposure to a trauma-paired context prior to anxiety testing strengthened the subsequent anxiety-like behavior of THIT, but not QHIT-exposed fish, and 3) despite this, THIT- and EPI-exposed fish showed a lesser degree of contextual avoidance behavior, compared to sham- or QHIT-exposed fish. The results reported here show that the stressors were robust enough to create a novel model of PTSD in zebrafish, while a dichotomous effect of EPI is highlighted in the consolidation of mild, vs. severe stress-associated feedback.

Keywords

Posttraumatic stress disorder; zebrafish; anxiety; trauma; stress; epinephrine

¹ posttraumatic stress disorder

² trauma free

³ epinephrine

⁴ quadruple high intensity trauma

⁵ triple high intensity trauma

3.1 Introduction

Posttraumatic stress disorder (PTSD¹) is a chronic and debilitating mental disorder that affects up to 3 % of the global population at any given time (Bisson, Cosgrove, Lewis, & Roberts, 2015; NIH, 2019). Our knowledge and understanding of the pathophysiology of PTSD, which manifests after experiencing or witnessing a traumatic, life-threatening event, is incomplete. To date, the treatment of PTSD is mainly symptomatic and directed at attenuating post-stress anxiety, preventing other trauma-related comorbidities, improving the overall functioning of the patient, and preventing subsequent relapse (Vieweg et al., 2006). Currently, different combinations of pharmacological and non-pharmacological interventions are used to treat PTSD (Department of Veterans Affairs, 2020). Unfortunately, the response to drug treatment is less than adequate with only 60 % of patients demonstrating mild clinical response, while remission is rarely achieved, i.e. in 20 – 30 % of sufferers only (Bailey, Cordell, Sobin, & Neumeister, 2013).

The extent to which individuals experience stress and anxiety is dependent on specific personal vulnerabilities and the inherent degree of resilience that individuals show (Fink, 2016). Stress can loosely be defined as “an observation of a potential threat which results in discomfort, difficulty adjusting, and emotional tension” (Adell, Garcia-Marquez, Armario, & Gelpi, 1988; Fink, 2016). Stress and anxiety are intricately linked in terms of their association with the noradrenergic system. In fact, norepinephrine (NE²) plays a fundamental role in conditions characterized by marked fear responses such as phobias and anxiety disorders, including PTSD (Adell et al., 1988; Fink, 2016). Being a key stress hormone that guides the mammalian response to both implicit and external stressors (Cahill, Gorski, & Le, 2003), NE has been the focus of many PTSD and other anxiety disorder studies (Cahill et al., 1996; Goddard et al., 2010). NE, the primary neurotransmitter of the sympathetic nervous system (SNS³), is centrally released by the locus coeruleus (LC⁴) in response to a stressful or emotional stimulus (Kodirov, 2012; Niwa, Matsumoto, Mouri, Ozaki, & Nabeshima, 2011; Roozendaal, McEwen, & Chattarji, 2009; Van Stegeren, 2008), while epinephrine (EPI⁵), also an adrenergic agonist, is simultaneously released into the systemic circulation by the adrenal medulla (Asalgoo, Jahromi, Meftahi, & Sahraei, 2015; Klabunde, 2011). The main purpose of stress- or trauma-associated NE and EPI release is to prepare the individual who is confronting a threat to react in a swift, appropriate, and decisive manner, i.e. the so-called “fight-or-flight” response (Agren, Björkstrand, & Fredrikson, 2017; Norrholm & Jovanovic, 2018; Stegmann et al., 2019). While such effects of centrally released NE are vital at the time of experiencing a traumatic life event, perpetuating dysregulated noradrenergic processes may contribute to the manifestation of among others, anxiety and panic attacks (Derrick, Green, & Wand, 2019; Goddard et al., 2010). When individuals experience

¹ posttraumatic stress disorder

² norepinephrine

³ sympathetic nervous system

⁴ locus coeruleus

⁵ epinephrine

trauma, spatial and temporal information of the event is contextualized by the hippocampus in a process often referred to as memory consolidation (Flores, Fullana, Soriano-Mas, & Andero, 2018; O'donnell, Hegadoren, & Coupland, 2004). This is a pivotal mechanism underlying the way in which humans and other animals learn from fearful experiences and avoid and/or manage similar scenarios in future. However, when the actions of NE¹ are not adequately regulated and terminated, psychobiological complications, e.g. spontaneous re-experiencing of the stressful event and anxiety-related responses, manifest (Seki, Yoshida, & Jaiswal, 2018). In this regard, PTSD² is believed to result from dysfunctional fear consolidation and stress mitigation mechanisms which may prevent adequate post-stressor fear desensitization to occur (Garcia, Walker, & Zoellner, 2018; Kreutzmann et al., 2021; Van der Kolk, 2001). Nevertheless, PTSD shows inconsistent treatment response to drugs aimed at blunting noradrenergic signaling, e.g. clonidine (Belkin & Schwartz, 2015), prazosin (Simon & Rousseau, 2017), and propranolol (Argolo, Cavalcanti-Ribeiro, Netto, & Quarantini, 2015). Thus, and considering that the majority of trauma-exposed individuals remain resilient against developing later-life trauma-related psychopathologies, our understanding of the role that NE plays in the etiopathology of PTSD and other trauma-related anxiety disorders, remain inconclusive. To this end, the use of animal model systems, in which species-specific and ethologically relevant traumatic events can be replicated, may be useful.

Animal models of anxiety-like behavior have provided several insights into the complex processes underlying the manifestation of stress-induced psychobiology (Cohen, Matar, & Zohar, 2014; Daskalakis, Yehuda, & Diamond, 2013; Richter-Levin, Stork, & Schmidt, 2019; Yehuda & Antelman, 1993). Still, modeling PTSD is complex for two reasons. First, as opposed to biologically or pharmacologically induced models, animals presenting with PTSD-like behaviors should preferably do so only because of trauma exposure. However, inter-individual variance with respect to how organisms will overcome or succumb to the effects of trauma, highlights the second complexity of modeling PTSD, i.e. distinguishing 'normal' trauma-related anxiety, from 'aberrant' biobehavioral profiles. To date, different putative rodent models of PTSD overcome these difficulties with varying levels of success (**Table 3-1**). Yet, no truly robust model of PTSD has been described in which the naturalistic contributions of the noradrenergic system to the development of trauma-related anxiety, can be investigated (Daskalakis & Yehuda, 2014; Daskalakis et al., 2013; Richter-Levin et al., 2019; Zhang et al., 2019).

¹ norepinephrine

² posttraumatic stress disorder

Table 3-1 - Commonly used rodent models for the study of PTSD

Adapted from (Cohen et al., 2014; Flandreau & Toth, 2018; B. H. Harvey, L. Brand, Z. Jeeva, & D. J. Stein, 2006; Harvey, Naciti, Brand, & Stein, 2003; Richter-Levin et al., 2019; Zhang et al., 2019).

Model	Procedure	PTSD-like behaviors produced
Inescapable electric foot shock	Acute or chronic exposure to electric shocks of varying intensity.	• Open-arm avoidance in a plus-maze • Aggression towards conspecifics • Sleep disturbances • Freezing episodes • Anhedonia and related depressive-like behaviors
Restraint/immobilization	Single period of prolonged immobilization.	• Anxiety – freezing in a plus maze • Sleep fragmentation
Single prolonged stress (SPS¹)	Multimodal stressor consisting of i) 2-h restraint, ii) 20-min forced swimming, and iii) exposure to noxious gas until loss of consciousness.	• Altered hypothalamus-pituitary-adrenal (HPA²)-axis functioning • Time-dependent behavioural, HPA- and brain monoamine changes • Exaggerated startle responses • Stress-induced nociceptive sensitivity • Anxiety – freezing in plus a maze • Cognitive impairment
Underwater trauma	Subjects are allowed to swim for 30 sec before forcibly being submerged for 30 – 45 sec.	• Impaired learning in Morris water maze • Increased anxiety in plus maze • Reduced corticosterone levels 7 days after stress exposure
Predator-based stress	Exposure to a natural predator or predator scent	• Compromised spatial and working memory • Enhanced startle responses • Freezing episodes

¹ single prolonged stress

² hypothalamus-pituitary-adrenal

Although most PTSD¹ models have been developed in mammalian species, such models are typified by some noteworthy practical and ethical caveats. For example, since it can be expected that only a handful of subjects of a larger population will succumb to trauma and progress to PTSD-like behavior, a significant number of animals have to be trauma-exposed so as to clearly, albeit subjectively, distinguish between animals showing ‘normal’ and ‘aberrant’ trauma-related behaviors (Cohen & Zohar, 2004). Also, PTSD-like behavior should arguably be the result of exposure to a severe, life-threatening traumatic event, e.g. physically exposing rodents to a live predator. Such interventions are difficult to execute under standard laboratory settings. However, given its direct association with a life-threatening event, the question begs whether other non-mammalian species, in which these difficulties can be addressed, can be suitable replacements for or complement mammalian models. In fact, in cases where the main objective of PTSD-research would be to ascertain how life-threatening events and its association with noradrenergic processes can contribute to later-life trauma-related biobehavioral abnormalities, any species with a similar neurobiological footprint to mammals and in which a life-threatening event can be mimicked, might be suitable to inform such questions.

Over the past three decades, zebrafish (*Danio rerio*) emerged as a potentially useful preclinical model species. With respect to PTSD, anxiety-like behaviors are motivated by the same environmental influences as is observed 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, that underlie the manner in which fish learn from aversive and rewarding circumstances (Agetsuma et al., 2010; Jesuthasan, 2012). Zebrafish also display robust and easily quantifiable anxiety-related behaviors and endocrine responses akin to that of humans and other non-human mammals (Stewart et al., 2011), some of which are especially suited for the study of PTSD. Since these have been extensively reviewed before (Blaser, Chadwick, & McGinnis, 2010; Stewart et al., 2012), they will not be discussed here also.

Several attempts have been made at developing a zebrafish model of PTSD. However, these mostly employed a single stressor, e.g. electric shock, water drainage, or exposure to the danger-signaling, conspecific-derived chemical, conspecific alarm substance (CAS²), which as of yet, has not been suitable to produce a consistently reproducible and sustained fear response (Oliveira et al., 2014; Parra, Adrian Jr, & Gerlai, 2009; Quadros et al., 2016). It further stands to reason that none of these stressors, as they are normally applied, truly resemble a life-threatening event, hence also failing to impart a PTSD-like behavioral signature, i.e. trauma-dependent, sustained, and context-driven anxiety. Thus, in this work, we aimed to develop an ethologically relevant zebrafish model of persistent and context-driven anxiety that is contingent on experiencing a truly life-

¹ posttraumatic stress disorder

² conspecific alarm substance

threatening event, and that demonstrates temporal stability. We further aimed to investigate how the noradrenergic system contributes to the potential behavioral sequelae of such exposure.

3.2 Materials and methods

3.2.1 Experimental layout

Initially 295 shortfin wildtype (WT¹) zebrafish of both sexes were selected for experimentation from the zebrafish colony housed in the National Aquatic Bioassay Facility (NABF²) of the North-West University (NWU³), Potchefstroom, South Africa (Ethics Approval No. **NWU-00526-20-A5**). Since zebrafish show high variability in terms of color preference, we first established the individual and naturalistic preference for each fish on experimental days -2 and -1. These preferred colors have later been cued with the control (sham⁴), quadruple high-intensity trauma (QHIT⁵), triple high-intensity trauma (THIT⁶), or EPI⁷ protocols, so as to ensure that potential context-related avoidance observed in post-trauma assessments, were not confounded by the inherent and naturalistic aversion that some fish may show to specific colors. Zebrafish were subsequently divided into different color groups and housed as per their color preference. For illustrative value, these colors were green, yellow, red, and blue (although not an equal number of fish were allocated to each group due to differences in color preference shown; data not shown). Where applicable, fish in each of the chosen color groups (A, B, C and D) were divided into different groups (**Figure 3-1**), viz. Ai ($n = 13$) + Aii ($n = 29$), Bi ($n = 12$) + Bii ($n = 29$), Ci ($n = 16$) + Cii ($n = 16$) and D ($n = 12$). On day 0, experimental testing commenced according to the different exposure paradigms (**see paragraphs 3.2.4 – 3.2.7**). A handheld digital video-camera (Panasonic® HC-V180) used, and all trials were digitally recorded. Recordings were subsequently analyzed by means of EthoVision® XT 14 (Noldus® Information Technologies, Wageningen, The Netherlands) digital tracking software.

¹ Short-fin wildtype

² National Aquatic Bioassay Facility

³ North-West University

⁴ trauma free

⁵ quadruple high-intensity trauma

⁶ triple high-intensity trauma

⁷ epinephrine

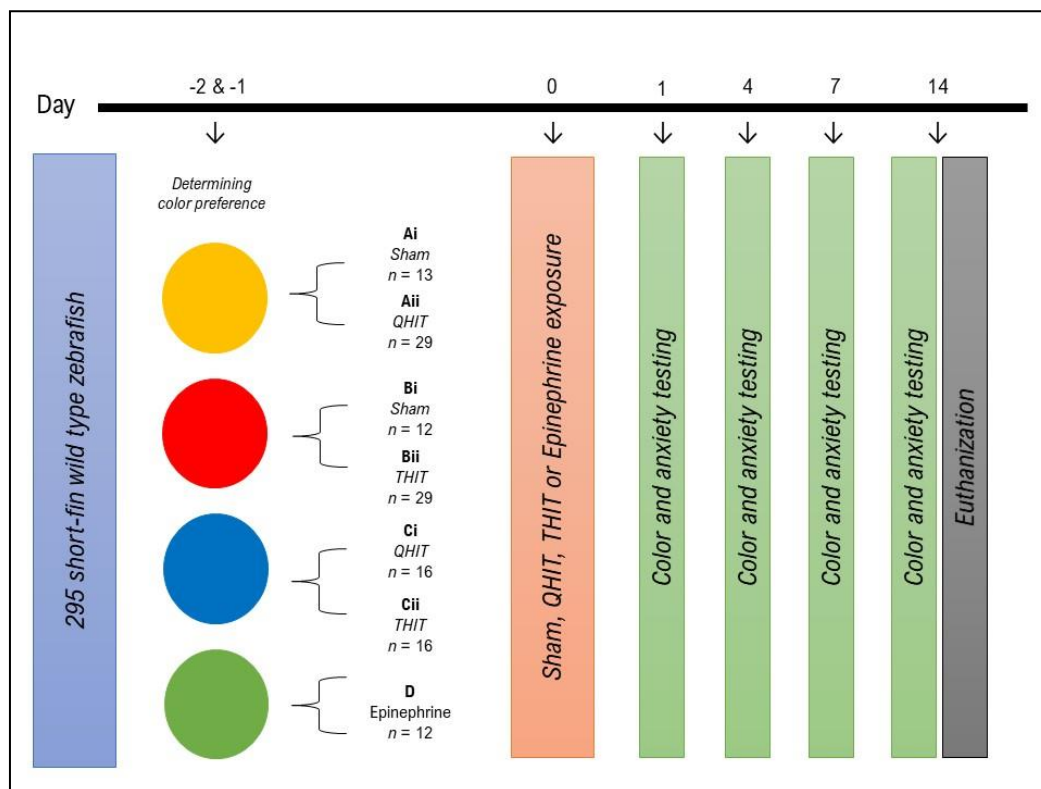


Figure 3-1 - Experimental layout.

Sham: Control group; QHIT: quadruple high-intensity trauma; THIT: triple high-intensity trauma.

Groups Ai and Bi: fish that underwent the same sham protocol and that were tested for anxiety in the presence of a contextual trigger; Aii: QHIT-exposed fish tested for contextually-triggered anxiety; Bii: THIT exposed fish tested for contextually-triggered anxiety; Ci: QHIT-exposed exposed fish tested for anxiety in the absence of a contextual trigger; Cii: THIT-exposed fish tested for anxiety in the absence of a contextual trigger; D: fish exposed to EPI only and tested for anxiety in the presence of a contextual reminder. Fish not indicated in Groups A – D were used as social conspecifics and CAS donors.

3.2.2 Animals and housing

As alluded to in **paragraph 3.2.1**, two-hundred-and-ninety-five (295) adult short-fin wildtype (WT¹) zebrafish (*Danio rerio*) of both sexes ($\pm 30 - 40$ mm in length; $\pm 5 - 8$ fish used per exposure group on each specific day) were assessed in this investigation. Of these, seventy-two (72) zebrafish were used as social conspecifics and ninety-six (96) fish were used as CAS² donors (see below). The progenitor stock was originally obtained from Aquaworld® Tropical Fish (Singapore) via a national South African importing supplier (WCB Imports Ltd, Pretoria, South Africa). All fish were subsequently bred and housed in the NABF³, NWU⁴, Potchefstroom, South Africa. Fish were group housed ($n = 8$ per tank) in a modified version of the home tank that allowed for the

¹ Short-fin wildtype

² conspecific alarm substance

³ National Aquatic Bioassay Facility

⁴ North-West University

separation and continuous identification, but not social isolation, of the each of the eight fish into individual cuboid compartments (**Figure 3-2**; spatial volume of at least 500 mL each, as modified based on home cage dimensions). At the terminal ends of the eight housing compartments for the eight experimental fish, two additional compartments with more or less the same volume have been constructed (green enclosures; **Figure 3-2**) to house four conspecifics not used in further experimentation. These fish, i.e. social conspecifics, have only been housed in the home tank so that each of the eight experimental fish could each continuously interact with three other fish (one to each side and one directly in front). Compartments between the 12 (eight experimental and four conspecific) fish in each home tank were separated by clear Plexiglass® grid walls, so that each fish had sight of the three other surrounding fish and that water and semiochemicals could flow and diffuse freely (**Figure 3-2**). These 8 L 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 O₂/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.

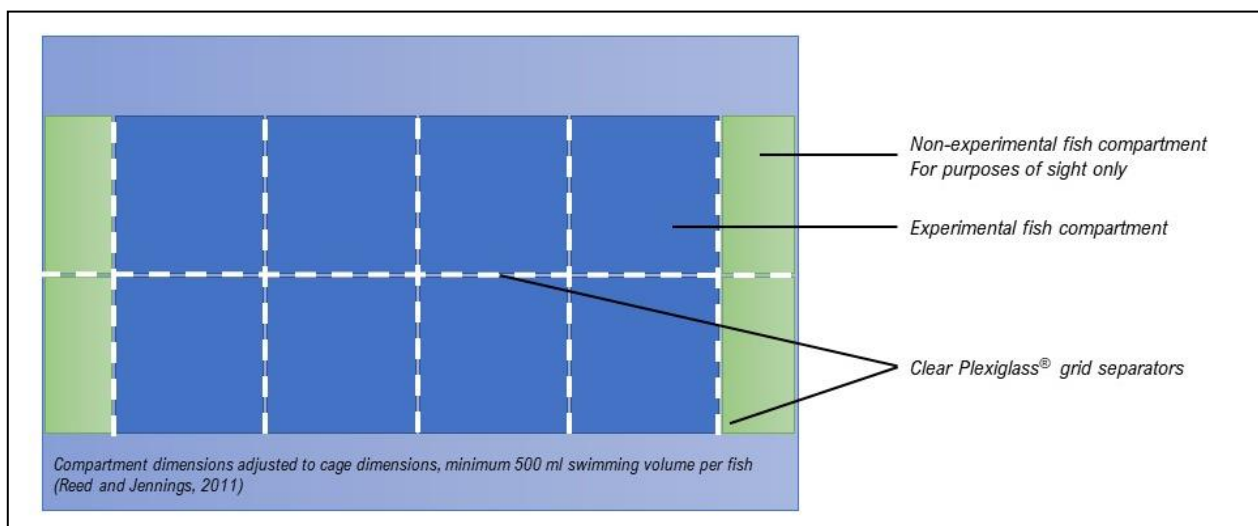


Figure 3-2 - Schematic (above) and photographic (below) view of home cage setup and separators, respectively, allowing for simultaneous fish identification and social interaction.

3.2.3 Preparation of CAS-releasing zebrafish

The chemical composition of CAS¹ 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. Fresh CAS was extracted from eight donor fish before each QHIT²/THIT³-protocol (during which eight fish were simultaneously exposed, to trauma) to ensure consistency throughout the protocols. CAS-donating fish were euthanized by rapid decapitation with a scalpel and excess water was removed from specimens using paper towel. Three shallow lateral incisions were made into epidermis on each side with a razor blade, ensuring that as little blood as possible was introduced to the sample. The eight donor fish were then placed back into a single petri dish to which 10 mL of distilled water was added. The concentration of CAS was thus 'standardized' as 8 fish per 10 mL distilled water. After adding the euthanized donors to each of the trauma-paired compartments (see **Figure 3-4**, later), the remaining liquid in the petri dish was also dispersed in the apparatus.

3.2.4 Chemicals and apparatus

EPI⁴ was obtained from Fresenius Kabi® South Africa (Pty.) Ltd. (Midrand, South Africa). All experimental and behavioral testing apparatuses were custom manufactured according to specification by the Instrument Making and Electronic Services departments of the NWU⁵, South Africa. Behavioral analyses were performed with Ethovision XT 14 (Noldus® Information Technology, Wageningen, The Netherlands).

3.2.5 Days -2 and -1: Color preference testing

To determine the inherent, naturalistic color preference of each of the initially selected 295 zebrafish, each fish was assessed in a rectangle Plexiglass® plus maze comprising of four compartments [**Figure 3-3**; 10 cm (*l*) x 5 cm (*w*) x 15 cm (*h*) each], arranged around an opaque and neutral-colored removable start box (5 x 5 cm). The four arms of the maze were constructed from clear Plexiglass® so that the different colored 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 days -2 and -1, each fish was allowed to habituate in the start box for 1 min, after which the start box was removed. Fish 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. This procedure was repeated once daily for two consecutive days. 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

¹ conspecific alarm substance

² quadruple high-intensity trauma

³ triple high-intensity trauma

⁴ epinephrine

⁵ North-West University

time, i.e. 10 min. The colored arm in which each fish spent most of its time during the total 10 min window was recorded as the preferred color (data not shown). This color was paired with trauma or EPI¹ exposure on experimental day 0 (see **paragraph 3.2.5**). The colored arm in which each fish spent the least amount of time was recorded as the non-preferred color (data not shown). This color was used in combination with the preferred color in subsequent contextual trigger- and avoidance analyses (**see paragraph 3.2.7.1**).

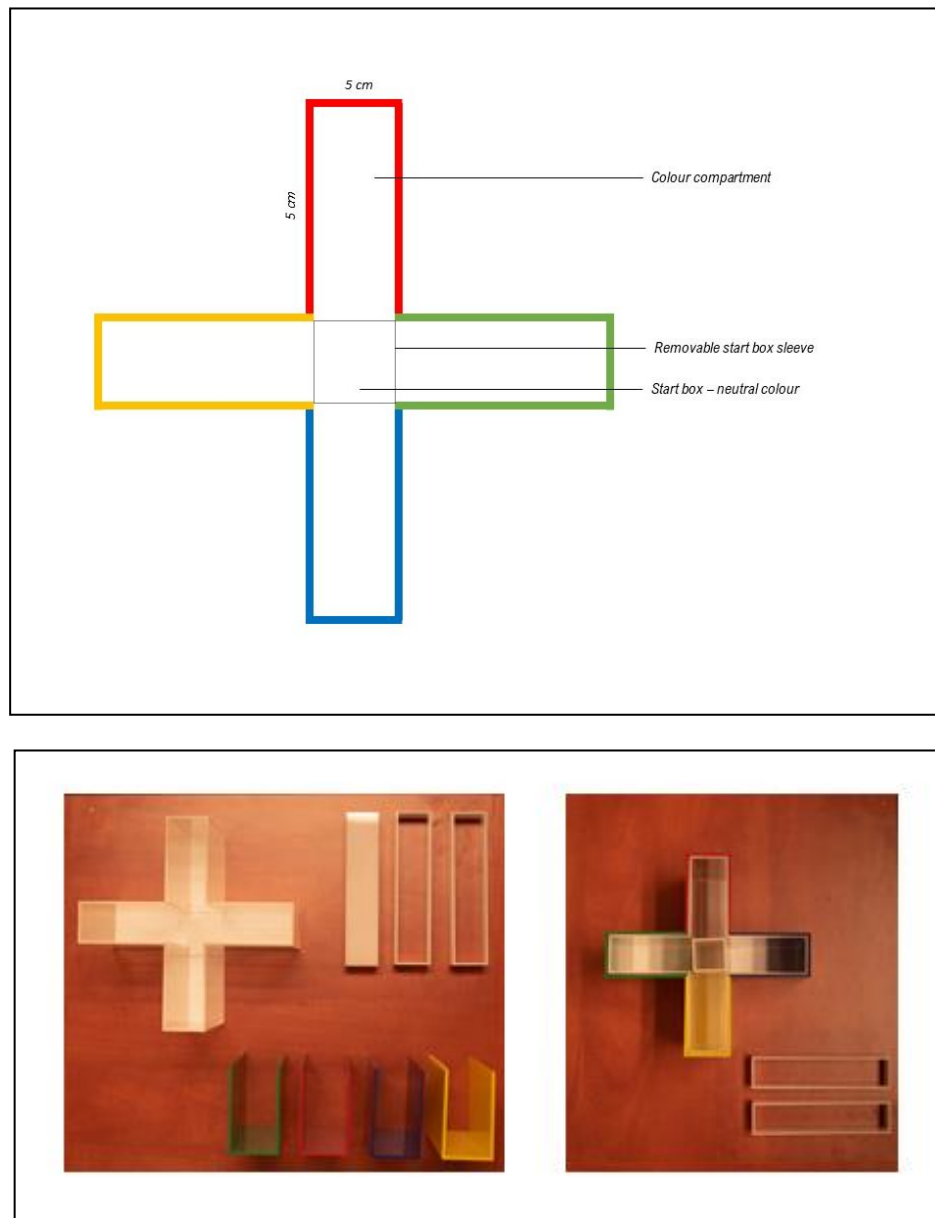


Figure 3-3 – Schematic (above) and photographic representation of color preference testing maze.

Important: Only two arms were used in repeated contextual trigger and avoidance analyses on experimental days 1, 4, 7, and 14.

¹ epinephrine

3.2.6 Day 0: QHIT, THIT, epinephrine and sham exposure

3.2.6.1 Apparatus

The exposure apparatus consisted of 8 x 500 mL cuboid compartments [**Figure 3-4**; 7 cm (*l*) x 7 cm (*w*) x 10 cm (*h*); arranged in a 4 x 2 configuration] constructed from clear Plexiglass®. Individual compartments were separated by clear Plexiglass® grids so that fish had sight of other fish. Further, water and semiochemical substances could flow and diffuse freely, while electricity could be 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 x 2 arrangement, the apparatus was flanked by two additional compartments, each 14 cm (*l*) x 3.5 cm (*w*) x 12 cm (*h*). These were used to administer EPI¹ and drain water. A grid of electrical wiring that conducted at ≈ 166 A at 3 ± 0.2 V AC uniformly throughout the entire apparatus, was affixed throughout the apparatus (**Figure 3-4**).

The internal floor of the apparatus underneath the fish-containing compartments (false floor) consisted of a Plexiglass® grid to facilitate water drainage. 2 cm below this floor, another solid floor attached and sealed the apparatus to its walls. The space between the false inner floor and the solid floor was used as a conduit for water drainage. On top of the apparatus, a gantry constructed with eight hatch-like Plexiglass® trapdoors allowed for the simultaneous introduction of a single euthanized zebrafish to each of the compartments. Last, during any of the given procedures (QHIT², THIT³, EPI, or sham⁴ exposure), the outer walls of the compartments, as well as the floor of the whole arena, were covered in the preferred color of the respective exposed groups of zebrafish. In other words, fish were exposed in groups of eight with the same color preference. If this color was red for a specific group, the apparatus was covered in red.

¹ epinephrine

² quadruple high-intensity trauma

³ triple high-intensity trauma

⁴ trauma free

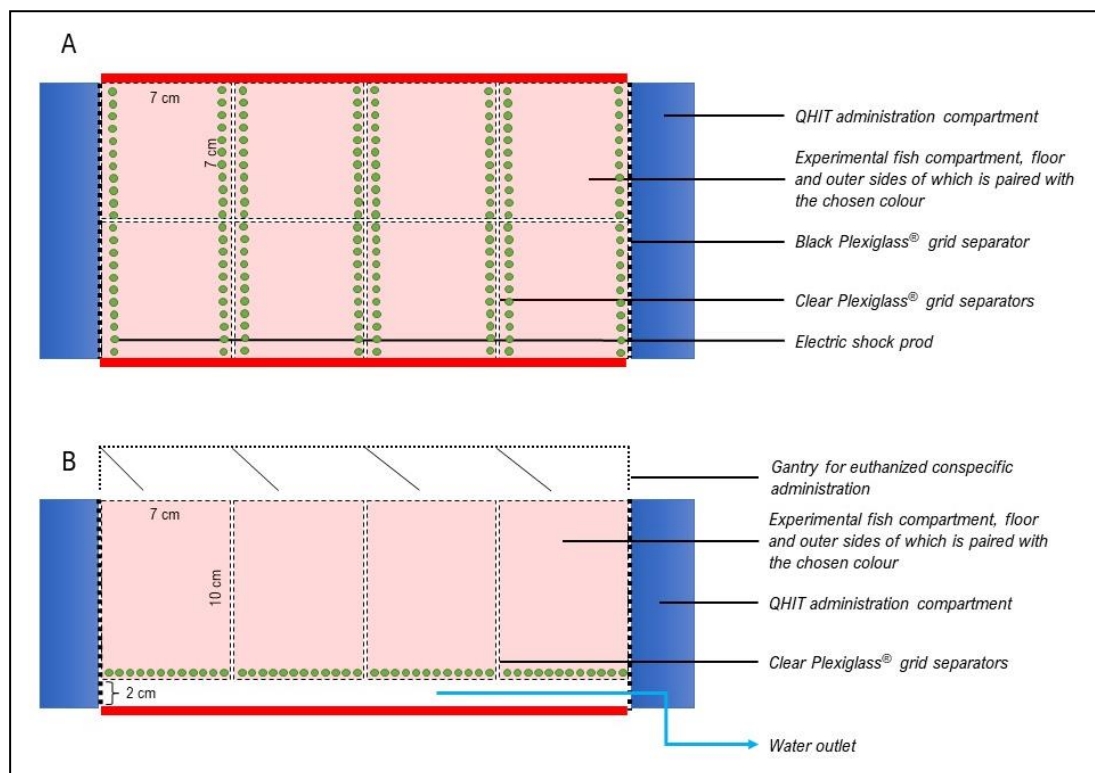


Figure 3-4 – Schematic (above) and photographic (below) representation of the exposure apparatus. Above (A) - top view; Above (B) - side view

QHIT = quadruple high-intensity trauma.

3.2.6.2 QHIT exposure

The QHIT¹ procedure broadly consisted of i) 1-h EPI² exposure in a replica of the home tank, ii) water drainage up to a level of dorsal fin air-exposure in the exposure apparatus, iii) electric shock in the exposure apparatus, and iv) direct exposure to a euthanized and CAS³-releasing donor, also in the exposure apparatus. On any of the specific assessment days, fish were transferred for 60 min to a replica of the home tank to which 2 L of water and 2 mg of EPI (1 mg/L) were added. After 1 h, fish were introduced to the already preferred color-coated exposure apparatus, filled with system water up to a level of 5 cm (2.5 L), and left for 1 min. Subsequently, the following sequential steps were followed:

- a. the water level was slowly drained (over the span of 3 min) up to the level of dorsal fin air-exposure (1.1 L remained). All the following steps then followed while fish had their dorsal fins exposed to air for 2 min (Piato et al., 2011);
- b. the apparatus was electrocuted for 5 sec at 166 A and 3 ± 0.2 V AC (Piato et al., 2011), the first shock having been administered while—
- c. euthanized, CAS releasing zebrafish were dropped into each compartment to contextualize the triad of electric shock, lowering water level and the prior EPI exposure to collectively evoke a response akin to a presumed life-threatening event;
- d. the apparatus was electrocuted again for 5 sec at 166 A and 3 ± 0.2 V AC; and
- e. fish were left for 1 min before being transferred back to the home tank.

The complete procedure took approximately 6 min to complete for eight fish.

3.2.6.3 THIT exposure

For the THIT⁴ protocol, the same procedures as described above were followed. However, zebrafish were not exposed to EPI prior to the exposure session.

3.2.6.4 Epinephrine exposure

EPI-only-exposed zebrafish were exposed to EPI for 1 h as explained for the QHIT protocol. However, afterward, fish were transferred to the exposure apparatus which was prepared as explained for the QHIT protocol and left for 6 min. Fish were then, without having been exposed to any of the abovementioned stressors, relocated to their home tanks.

¹ quadruple high intensity trauma

² epinephrine

³ conspecific alarm substance

⁴ triple high-intensity trauma

3.2.6.5 *Sham protocol*

For the sham¹ protocol, zebrafish were exposed to the same procedures as QHIT²-exposed fish, but in the absence of EPI³ or traumatic intervention.

3.2.7 Days 1, 4, 7 and 14: Investigating the immediate and delayed effects of trauma

3.2.7.1 *Repeated contextual (color) exposure*

Where contextual re-exposure was applicable, zebrafish were gently netted from their home tanks and placed into the start-box of the color preference testing maze (**Figure 3-3**). However, during these assessments only two color-compartments opposite to one another were used. The other two arms were consequently 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 **paragraph 3.2.5**). In order to force exposure to the trauma-, EPI-, or sham-paired color, fish were first introduced to the trauma-paired color and left for 1 min. The arm separator was then removed so that fish could freely move between the two colors for 6 min. Color preference (or aversion) was measured by calculating the time spent in each of the color compartments as a fraction of the total time in both color compartments. Following completion of the assessment, fish were gently netted into novel tanks (see below). This procedure was repeated on days 1, 4, 7 and 14 post-trauma, EPI-, or sham-exposure.

Importantly, to explore the effect of contextual reminding on subsequent anxiety-like behavior, two groups of fish, one of the QHIT- and THIT⁴-exposed groups respectively, were not contextually reminded prior to anxiety testing. These fish were only assessed for novel tank behavior.

3.2.7.2 *Repeated anxiety testing*

Immediately following contextual re-exposure where applicable, zebrafish were assessed for anxiety-like novel tank diving behavior. Since we conducted four novel tank diving tests for each fish over days 1, 4, 7 and 14, four different tank designs were used to prevent habituation to the testing tank (**Figure 3-5**). On each testing day, one specific tank design was employed to ensure test reproducibility. All tanks were constructed of Plexiglass® 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.

¹ trauma free

² quadruple high intensity trauma

³ epinephrine

⁴ triple high-intensity trauma

On any specific assessment day, fish were moved either from their home tanks (in the case of no-contextual reminding), or from the color preference testing maze. The test was conducted over 6 min. Novel tank anxiety was characterized by calculating the average time spent in the bottom third of the tank.

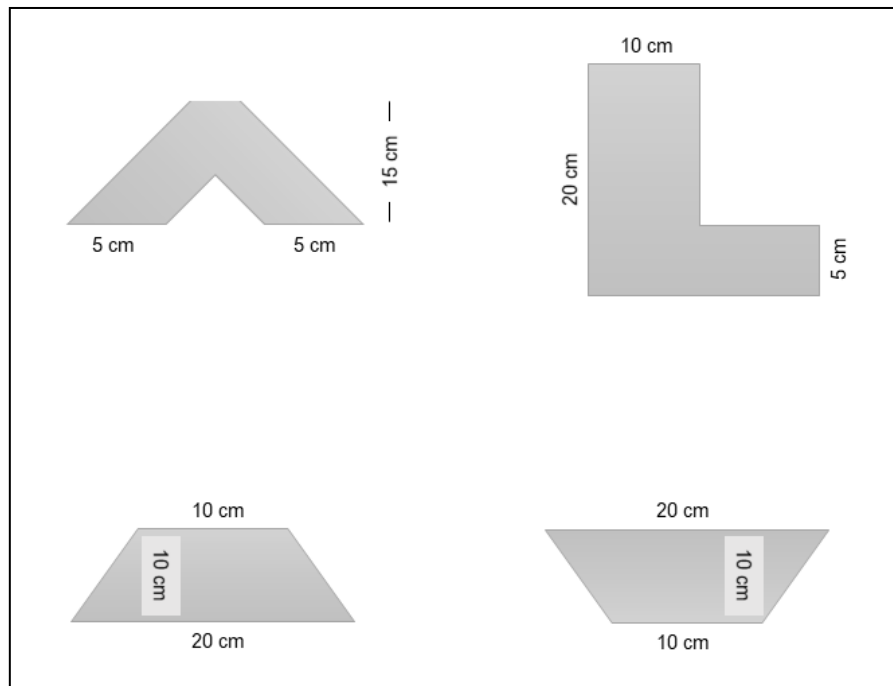


Figure 3-5 – Schematic (above) and photographic (below) representation of the tank shapes and dimensions of the 4 novel tank anxiety assessments.

The front-to-back depth of all tanks was 5 cm. Tanks have a clear front side, but opaque sides, back and floor. Tanks used on the respective testing days are above, clockwise from left to right: top, d1, d4; bottom, d7, and d14.

3.2.8 Study endpoint

After the behavioral tests were completed on day 14, the fish were netted back into the home tanks and fed. After 1 h, the fish were taken to another room in the facility to be euthanized. Each fish was individually netted and euthanized via rapid decapitation with a scalpel. The remaining fish in each home tank were not able to see other fish being euthanized.

3.2.9 Statistical analysis

Statistical analyses were performed with GraphPad Prism®, version 9.3.0. Two-way repeated measures analysis of variance (2-way RM ANOVA)¹ 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 contextual avoidance were set as the respective dependent variables. All analyses were followed up by Bonferroni post-hoc testing for pairwise differences and calculations of Cohen's *d* to establish the magnitude of the effects observed. Descriptive statistics (median, interquartile range) were used to explore the distribution of data points and to inform the various observations made.

3.3 Results

Important – The results of color-preference testing are not shown, since this was a categorical experiment performed to expose fish to trauma in a simulated naturally-preferred environment. Irrespective of which colors were chosen, the experimental procedures were the same.

3.3.1 Figure 3-6, Table 3-1: Novel tank anxiety of context-exposed fish over time

Here, the reported results apply to zebrafish that were exposed to either a sham² protocol, QHIT³, THIT⁴ or EPI⁵ on day 0 of the investigation. All fish in this data cluster were exposed in an identical setup, differing only with respect to the stressogenic protocol that was followed (**refer paragraph 3.2.6**). Said setup was cladded in the color of preference for each of the exposed fish, while fish were reminded of this color-context on the subsequent testing days (days 1, 4, 7, and 14; **paragraph 3.2.7**).

¹ two-way repeated analysis of variance

² trauma free

³ quadruple high-intensity trauma

⁴ triple high-intensity trauma

⁵ epinephrine

Two-way RM-ANOVA¹ failed to reveal a significant time-exposure interaction with respect to the time spent in the bottom third zone of a novel tank by context-exposed zebrafish [$F(9,279) = 1.32, p = 0.227$]. However, while time narrowly missed being a significant influence on novel tank bottom-dwelling [$F(2.7,253.9) = 2.41, p = 0.073$], exposure had a significant main effect on the results reported here [$F(3,93) = 6.28, p = 0.0006$]. This was confirmed by a follow-up comparison of bottom-dwelling behavior of fish exposed to the different paradigms, irrespective of time [$H(3) = 10.48, p = 0.003$; medians and interquartile ranges reported in **Table 3-1**]. Subsequent within-day, between-exposure group pairwise comparisons revealed significant differences mainly between fish exposed to THIT² or EPI³ and QHIT⁴ or sham⁵ (**Table 3-2**).

Importantly, whereas the medians and interquartile distributions of sham- and QHIT-exposed fish largely demonstrated similar distributions (**Table 3-2**), the bottom-dwelling behavior of at least 75 % of THIT-exposed zebrafish was consistently more than the median sham and QHIT values (**Figure 3-6**). The same trend was observed for the EPI-exposed groups over all testing days, except day 14.

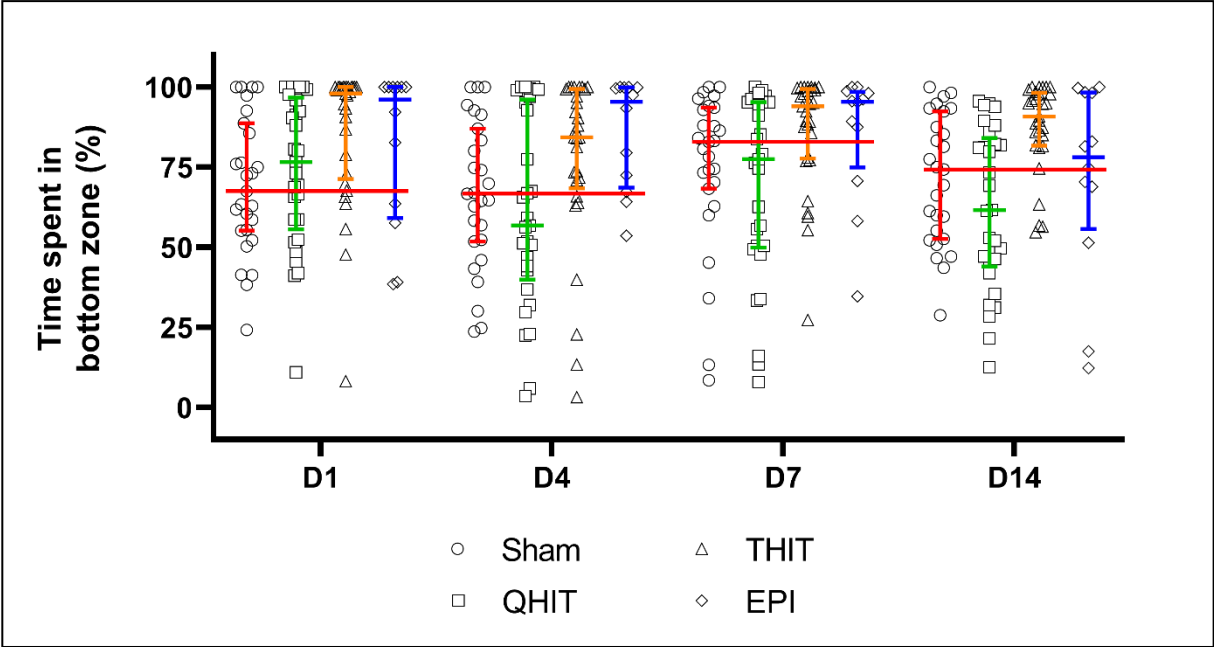


Figure 3-6 – Anxiety-like bottom-dwelling behavior of context-exposed sham-, trauma-, and EPI-exposed fish over time.

Horizontal lines: median values of sham-exposed fish. Sham: control; QHIT: quadruple high-intensity trauma; THIT: triple high-intensity trauma; EPI: epinephrine.

¹ two-way repeated analysis of variance

² triple high intensity trauma

³ epinephrine

⁴ quadruple high intensity trauma

⁵ trauma free

Table 3-2 – Descriptive statistics and pairwise comparisons with respect to the novel tank bottom-dwelling behavior of fish in the different exposure groups.

Descriptive Statistics					
Day	Median % time spent in bottom zone (interquartile range)	Sham	QHIT	THIT	EPI
1		67.52 (55.3, 88.8)	76.62 (55.6, 96.8)	98.10 (71.4, 100.0)	96.10 (59.2, 100.0)
4		66.80 (51.8, 87.0)	56.82 (39.9, 96.2)	84.39 (68.5, 99.4)	95.49 (68.7, 100.0)
7		82.99 (68.4, 93.7)	77.56 (50.0, 95.4)	94.05 (77.7, 99.5)	95.49 (74.9, 98.6)
14		74.31 (52.7, 92.4)	61.65 (44.1, 84.2)	90.85 (81.77, 98.2)	78.18 (55.8, 98.4)
Pairwise Comparisons					
Day	Comparison	Difference	<i>p</i>	<i>d</i>	<i>dCI</i>
1	<i>Sham vs THIT</i>	69.32±21.31 vs 85.84±21.75	0.0351	0.77	0.22, 1.31
4	<i>Sham vs EPI</i>	66.71±22.93 vs 85.65±17.17	0.0481	0.89	0.17, 1.60
	<i>QHIT vs EPI</i>	61.20±30.22 vs 85.65±17.17	0.0147	0.87	0.16, 1.57
14	<i>Sham vs THIT</i>	71.42±20.11 vs 87.42±13.91	0.0075	0.93	0.375, 1.48
	<i>QHIT vs THIT</i>	62.50±25.03 vs 87.42±13.91	0.0002	1.231	0.66, 1.79

3.3.2 Figure 3-7, Table 3-3: Effect of context on novel-tank behavior over time

To determine whether contextual (trauma-paired color) exposure influences anxiety-like behavior in the novel tank diving test, we compared the percentage time spent bottom-dwelling by separate groups of QHIT¹- (**Figure 3-7A; page 73**) and THIT²-exposed (**Figure 3-7B; page 73**) fish that were tested in the absence or presence of such exposure. Thus, the results in this section pertain to the context-exposed fish that underwent QHIT and THIT, of which the repeated anxiety data are presented in **paragraph 3.3.1** also. However, the no-context-exposed fish were separate groups of QHIT- and THIT-exposed fish that were also subjected to the stress protocol in the presence of the preferred color, but that were tested for anxiety in the absence of prior exposure to such color again.

In terms of QHIT, a significant time-context interaction was revealed [$F(3,129) = 3.67, p = 0.014$], while time [$F(2.6,113.1) = 3.45, p = 0.024$], but not context [$F(1,43) = 0.59, p = 0.445$], had a significant main effect on the result reported, with significant pairwise comparisons shown only in the ‘no-context’ group between different days of exposure (**Table 3-3**). In THIT-exposed fish, no significant two-way interaction was shown [$F(3,129) = 0.55, p = 0.648$]. However, in this instance, context [$F(1,43) = 5.47, p = 0.024$], but not time [$F(2.34,100.8) = 2.45, p = 0.082$], significantly impacted bottom-dwelling behavior. Statistically, no pairwise differences between the bottom-dwelling of no-context- and context-exposed fish were shown on any of the testing days. Rather, isolating context as a main effect, a Mann-Whitney *U*-test revealed a difference between

¹ quadruple high intensity trauma

² triple high intensity trauma

the overall bottom-dwelling of no-context- (median: 73.24 %) and context-exposed (median: 86.17 %), that narrowly missed statistical significance ($p = 0.057$).

Table 3-3 - Descriptive statistics and pairwise comparisons with respect to the novel tank bottom-dwelling behavior of fish in the QHIT and THIT groups in the absence and presence of contextual (trauma-paired color) exposure.

Descriptive Statistics					
Day	Median % time spent in bottom zone (interquartile range)	QHIT No Context	QHIT Context	THIT No Context	THIT Context
1		87.23 (59.45, 97.88)	76,62 (55.62, 96.80)	60,72 (53.35, 98.64)	98,01 (71.38, 100.0)
4		79.18 (63.09, 92.99)	56,82 (39.94, 96.16)	70,36 (61.00, 83.79)	84,39 (68.50, 99.45)
7		47.95 (35.03, 93.05)	77,55 (49.96, 95.36)	77,07 (63.29, 87.85)	94,05 (77.74, 99.46)
14		65.73 (85.89, 90.53)	61,65 (44.10, 84.18)	84,26 (74.50, 91.22)	90,85 (81.77, 98.21)
Pairwise Comparisons					
Day	Comparison (QHIT Group)	Difference	<i>p</i>	<i>d</i>	<i>dCI</i>
1 vs 7	<i>No context only</i>	79.65±21.20 vs 58.06±30.63	0.0243	0.820	-1.54, -0.09
4 vs 7	<i>No context only</i>	77.46±17.14 vs 58.06±30.63	0.0266	0.782	-1.50, -0.06

With respect to the data distribution, two trends are shown. In the QHIT¹-exposed-group, time spent in the bottom third zone of the respective novel tanks were near similar between no-context- and context exposed fish, except on day 7, when 75 % of context-exposed fish generated higher bottom-dwelling scores than the median score generated by no-context exposed fish (**Figure 3-7A, Table 3-3**). This trend changed in THIT²-exposed fish, in that most context-exposed zebrafish spent more time in the bottom third zone of a novel tank than the median values generated by no-context-exposed fish on all testing days (**Figure 3-7B, Table 3-3**).

3.3.3 Figure 3-8, Table 3-4: Contextual avoidance over time

To establish whether sham³-, trauma-, or EPI⁴-exposed fish would avoid a previously preferred, but exposure-paired color upon subsequent exposures to the same color, two-way RM ANOVA⁵ was run. While no time-exposure interaction was shown [$F(9,279) = 0.83, p = 0.590$], both time [$F(2.9,266.3) = 3.99, p = 0.009$] and exposure [$F(3,93) = 3.32, p = 0.023$] significantly impacted color avoidance behavior of the different groups. Subsequent pairwise comparisons revealed only minor statistical differences with respect to some of the comparisons drawn (**Table 3-4**).

¹ quadruple high intensity trauma

² triple high intensity trauma

³ trauma free

⁴ epinephrine

⁵ two-way repeated analysis of variance

In this experiment, the medians and interquartile distributions of sham¹- and QHIT²-exposed fish again demonstrated similar distributions (**Table 3-4**), whereas the majority of THIT³- and EPI⁴-exposed fish showed a lesser degree of trauma-paired color avoidance on most testing days, except day 4 (**Figure 3-8, Table 3-4**).

Table 3-4 - Descriptive statistics and pairwise comparisons with respect to context-avoiding behavior of fish in the different exposure groups.

Descriptive Statistics					
Day	Median % time spent in the non-preferred color zone (interquartile range)	Sham	QHIT	THIT	EPI
1		27,36 (9.58, 39.00)	26,19 (9.68, 47.77)	12,47 (3.58, 30.13)	8,94 (0.07, 23.11)
4		34,94 (13.16, 50.66)	31,22 (16.16, 53.46)	29,50 (14.40, 50.41)	23,73 (4.55, 65.08)
7		37,35 (22.12, 57.27)	38,39 (24.01, 65.27)	22,26 (8.68, 37.06)	10,83 (3.72, 22.13)
14		26,46 (15.99, 43.83)	38,80 (22.36, 51.12)	26,93 (9.53, 34.99)	14,18 (3.94, 39.48)
Pairwise Comparisons					
Day	Comparison	Difference	<i>p</i>	<i>d</i>	<i>dCI</i>
14	<i>QHIT vs THIT</i>	37.13±23.32 vs 23.04±13.86	0.0450	0.735	-1.26, -0.20
1 vs 7	<i>Sham only</i>	27.27±20.74 vs 40.44±24.46	0.0344	0.581	0.03, 1.12

¹ trauma free

² quadruple high intensity trauma

³ triple high intensity trauma

⁴ epinephrine

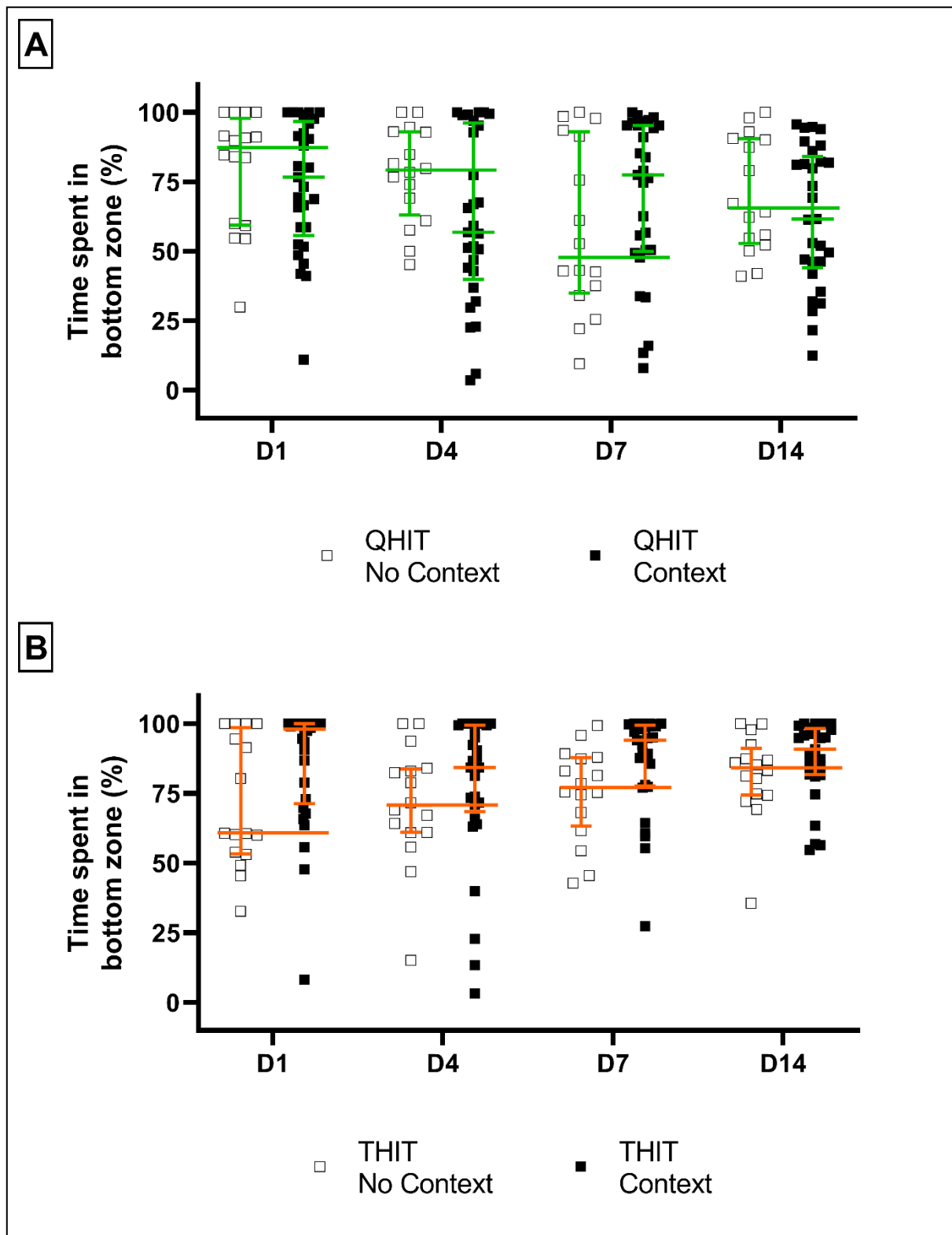


Figure 3-7 – Anxiety-like bottom-dwelling behavior (A) QHIT- and (B) THIT-exposed fish in the presence or absence of pre-test exposure to the trauma-paired color.
Horizontal lines: median values of fish that were not contextually reminded. QHIT: quadruple high-intensity trauma; THIT: triple high-intensity trauma.

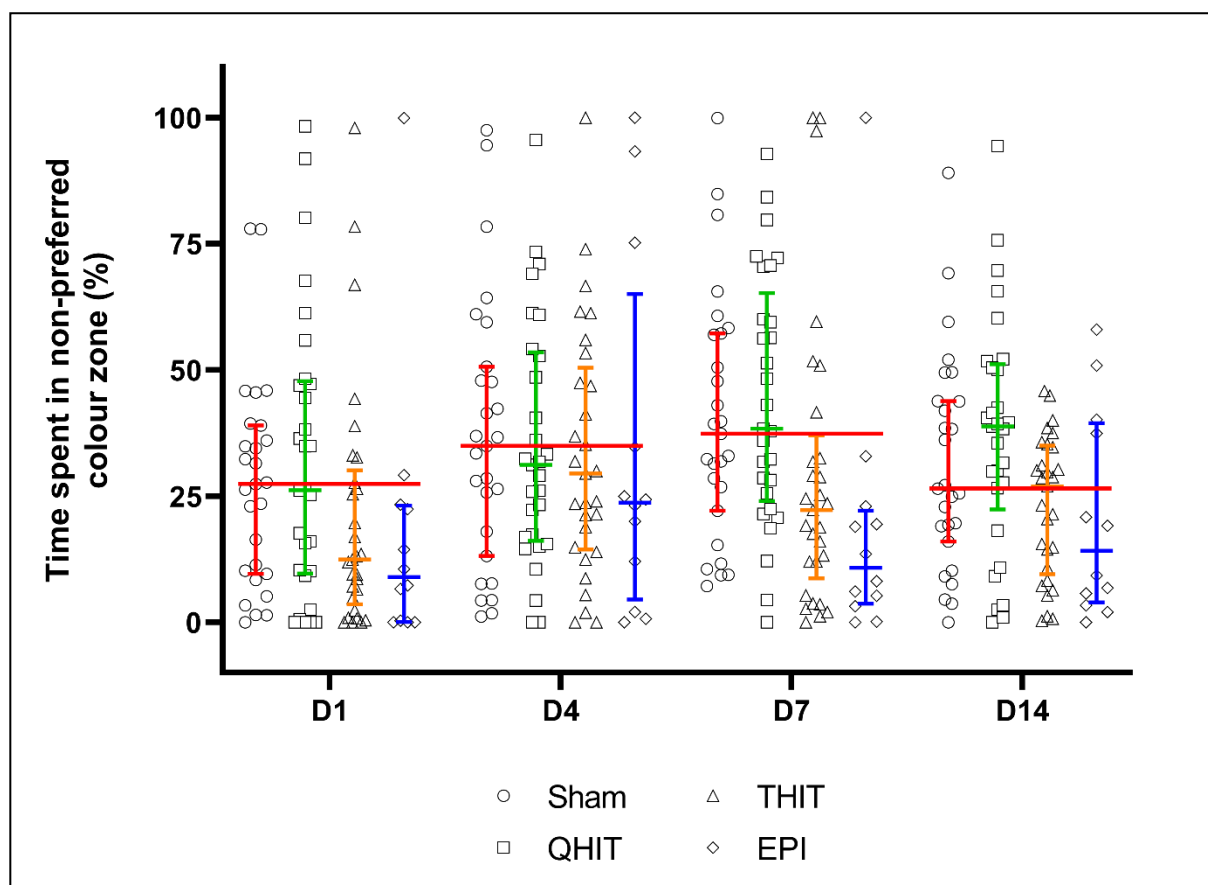


Figure 3-8 – Time spent by sham-, trauma-, and EPI-exposed fish in the naturalistically least-preferred color, i.e. as opposed to the procedure-paired (preferred) color.

Horizontal lines: median values of sham-exposed fish. Sham: control; QHIT: quadruple high-intensity trauma; THIT: triple high-intensity trauma; EPI: epinephrine.

3.4 Discussion

In this work, we attempted to develop a naturalistic zebrafish model of PTSD¹ that is etiologically and clinically relevant by applying a combination of stressors that together, constitute a truly 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) trauma-paired EPI² exposure consistently blunted the delayed anxiety-like sequelae associated with severe trauma (QHIT³ paradigm), but bolstered persistent neophobia-related anxiety when paired with novelty (exposure to a novel circumstance) in the absence of additional stressors (EPI only paradigm), 2) exposure to a trauma-paired context prior to testing strengthened the subsequent anxiety-like

¹ posttraumatic stress disorder

² epinephrine

³ quadruple high intensity trauma

behavior of THIT¹, but not QHIT²-exposed fish, and 3) despite this, THIT- and EPI³-exposed fish showed a lesser degree of contextual avoidance behavior, compared to sham⁴- or QHIT⁵-exposed fish.

Although the actions of NE⁶ has been the focus of many PTSD⁷ studies, its exact role in fear processing and the manifestation of fear-related delayed anxiety still needs further investigation. According to our most basic understanding, alpha-1 (α_1) and beta (β) receptor stimulation generally promotes neuronal plasticity and activates the fear circuitry, whereas α_2 receptor agonism inhibits such responses, supposedly by blunting NE release (Asalgoo et al., 2015; Hussain, Reddy, & Maani, 2020; Roozendaal et al., 2009; Van Stegeren, 2008). The first finding of this study shows that pre-trauma EPI exposure (as applied in the QHIT protocol), was associated with a delayed anxiety-like profile similar to that observed in sham-exposed fish (**Figure 3-6**), that is, eliciting a post-traumatic behavioral signature akin to that which was observed in non-stressed fish. In contrast, both trauma which was inflicted in the absence of EPI (as in the THIT protocol), as well as EPI administered on its own under novel circumstances, persistently bolstered anxiety-like bottom-dwelling over all testing days. These results are interesting for two reasons. First, it shows that life-threatening trauma in zebrafish can be applied as a useful model for the temporal study of delayed trauma-associated behavioral sequelae. Secondly, it highlights a clear dichotomous role for adrenergic signaling on the processing and later effects of acute fear.

Peripheral EPI secretion is known to induce central NE release via its actions on vagal afferents which indirectly influences forebrain regions that are implicated in fear learning and memory consolidation, e.g. hippocampus and amygdala (McGaugh & Roozendaal, 2002). This peripheral-central pathway is believed to mediate the effects of EPI and NE on fear memory consolidation. Since EPI that is administered to zebrafish by aqueous immersion is unlikely to cross the blood-brain barrier (Jeong et al., 2008), it is likely that EPI modulated the observed behavioral manifestations via similar mechanisms. However, a dichotomous role for the actions of EPI in the consolidation of fear memories is highlighted in this work. Specifically, pre-trauma EPI exposure prevented the manifestation of delayed and sustained context-triggered anxiety. However, EPI exposure prior to a sham (i.e. novel) procedure, elicited a similar degree of delayed anxiety than did trauma exposure in the absence of pre-traumatic EPI exposure (**Figure 3-6**). A potential explanation for this finding might related to the manner in which EPI and NE is synthesized and released, i.e. in that EPI is primarily synthesized in the chromaffin cells of the adrenal gland with only a small percentage being produced in the brain.

¹ triple high intensity trauma

² quadruple high intensity trauma

³ epinephrine

⁴ trauma free

⁵ quadruple high intensity trauma

⁶ norepinephrine

⁷ posttraumatic stress disorder

To explain, the final conversion of NE¹ to EPI² involves the methylation of NE by phenylethanolamine N-methyltransferase (PNMT³), which is primarily expressed in the adrenal medulla of most species, including in zebrafish, in which, though differing to some extent from mammals with respect to their physiology, have a similar adrenal gland architecture (DePasquale & Leri, 2018; Piato et al., 2011). Elevated PNMT expression is involved in the feed-forward effect of the stress response, where increased stress hormones like corticotrophin (CRF⁴) and cortisone (CORT⁵), enhance the conversion of NE to EPI, thereby increasing sympathetic output and worsening a pre-existing stress response (Wurtman, 2002). In support of this, acute stress has been shown to increase PNMT activity in rodents (Cahill, Eertmoed, Mangoura, & Perlman, 1996). Escalating the allostatic load prior to and during exposure to an adverse stimulus will therefore increase the subsequent responses to a reminder and to a co-presenting stressor, resulting in a self-sustaining feedforward loop, involving a high level of NE and CRF outflow (Elman & Borsook, 2019). With CRF having a potent central effect to bolster anxiety and stress responses, maladaptation and allostatic load present in the animal during sustained uncontrolled stress, may play a prominent role in the behavioral findings presented here.

The above mechanism implies then that peripheral administration of acute response stress hormones, like CRF and CORT, can, by suppressing endogenous processes, abrogate the later development of PTSD⁶ over time. This has been demonstrated clinically (Hadad, Schwendt, & Knackstedt, 2020) as well as in pre-clinical models (Gresack & Risbrough, 2011). Here, we propose that early or prior trauma exposure to EPI may result in the same. Where α_1 and β_1 blockers abrogate the stress response when administered early after experiencing trauma (Krauseneck et al., 2010; Pitman et al., 2002), EPI could have the same dampening effect on the noradrenergic system by promoting α_2 -mediated feedback inhibition. Since sympathetic outflow is blunted by the activation of post-synaptic α_2 adrenoreceptors in the vasomotor center (VMC⁷) of the medulla (nucleus tractus solitarius) (Katzung and Taylor, 2015), increased EPI in the periphery, as used in this study, may be toning down the noradrenergic response centrally, as noted following QHIT⁸. However, its apparent *worsening* effect in zebrafish exposed to EPI alone, concurs with a recent paper that suggests that EPI to contribute to persistent traumatic memories in a mouse model of PTSD (Martinho et al., 2020), although in our work, this group was only exposed to a novel context. There is also a case report where pheochromocytoma, which presents with hypersecretion of EPI and NE, exacerbates PTSD symptomology.

¹ norepinephrine

² epinephrine

³ phenylethanolamine N-methyltransferase

⁴ corticotrophin

⁵ cortisone

⁶ posttraumatic stress disorder

⁷ vasomotor center

⁸ quadruple high intensity trauma

The α_{2A} and α_{2C} adrenoceptors are the main auto-receptors involved in presynaptic feedback inhibition of NE¹, with especially the latter being selectively distributed in the stress-regulatory regions of the brain (Uys, Shahid, & Harvey, 2017). However, the potency of NE at the α_{2C} receptor is higher than that for the α_{2A} receptor (Uys et al., 2017), which inhibits NE release at low [10 – 100 nM] endogenous concentrations of NE with the opposite effect seen with stimulation of the α_{2A} receptor (Uys et al., 2017). The α_{2C} receptor also displays much slower deactivation upon removal of NE than the α_{2A} receptor (Uys et al., 2017), thus potentially taking the proverbial center stage in mediating the behavioral stress response.

In line with our theory that adrenergic signaling via the α_2 receptor class might provide the key to our findings, previous work demonstrated that administration of the α_2 agonist, dexmedetomidine, prior to trauma exposure in mice, prevented cued fear encoding, without affecting context appraisal. However, when it was administered after such exposure, memory of the contextual circumstance of the traumatic exposure was suppressed. Therefore, it seems that α_2 receptor stimulation at the time of experiencing a traumatic event, as was arguably the case in the QHIT²-exposed group, can potentially prevent the immediate encoding of fear memory; this theory is supported by the present data. Sadly, explanations pertaining to the exact role of the adrenergic system via its actions on various receptors on fear processing, are conflicting, which clouds a more complete understanding of the present results. Furthermore, that EPI³ administration in the absence of trauma, albeit before exposure to a ‘novel’ sham procedure, elicited a similar behavioral response than THIT⁴, highlights an important aspect of adrenergic system functioning in the processing of stress: the actions of EPI and NE are likely concentration, timing, and context specific. Suffice to conclude that with respect to life-threatening trauma in zebrafish, naturalistic processes contribute to the consolidation of contextual fear memory, while pre-traumatic EPI exposure prevents this phenomenon.

Our second main finding pertains to the effect of contextual re-exposure prior to testing of post-traumatic anxiety-like behavior. In this work said re-exposure strengthened the anxiety-like behavior of THIT, but not QHIT-exposed fish (**Figure 3-7**), likely since EPI exposure prevented the proper encoding of fear memory, as explained above. This is an important result, because it demonstrates that zebrafish not only contextualize traumatic events, but also remember traumatic cues for at least two weeks after trauma exposure. This is not unlike findings from rodent models of PTSD⁵ that emphasize the contingency between an initial severe stressor and later contextual reminders (Harvey, Brand, Jeeva, & Stein, 2006; Harvey et al., 2003). Considering the purpose of this investigation, this finding is vital. One of the main symptoms of clinical PTSD pertains to flashbacks and avoidance behaviors, which entails re-experiencing and attempts to suppress or avoid

¹ norepinephrine

² quadruple high intensity trauma

³ epinephrine

⁴ triple high intensity trauma

⁵ posttraumatic stress disorder

contextual reminders of the traumatic event, respectively (APA, 2013). Still, while zebrafish display heightened anxiety after exposure to the trauma-paired context, they did not avoid voluntary exposure to said contexts. This is interesting and might speak to the species-specific appraisal of color. In fact, zebrafish display highly individual preferences for color, a caveat that was accounted for in the present investigation by first establishing the naturalistic color preference of each fish employed in this work. While the purpose of this procedure was to prevent 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 in itself. Thus, while zebrafish recognize the contextual value of said trauma-paired colors in showing heightened post-color-reminded anxiety-like behavior, such colors do not necessarily possess aversive properties in itself.

Some noteworthy shortcomings of this work need mentioning. First, since we did not employ different adrenergic receptor targeting drugs, detailed conclusions regarding the exact mechanisms underlying the effects of EPI¹ in this model cannot be drawn. That said, since we show that a THIT² model of PTSD³-like anxiety 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 NE⁴ and cortisol over all days of traumatic exposure and anxiety testing would have been beneficial to yield insights into how temporal crosstalk between central and peripheral stress-related biology influence behavioral manifestation. Last, while the data presented are promising, additional PTSD-relevant behavioral tests, i.e. tests of sociability, aggression and anhedonia, would have been informative.

3.5 Conclusion

Here, we show that zebrafish can contextualize a severe and realistic life-threatening stressor. We further show that the resultant post-traumatic anxiety, which is context-sensitive, manifests for at least two weeks after trauma exposure. Since the concepts of persistent and delayed post-trauma anxiety, as well as contextual relevance are pivotal diagnostic symptoms of clinical PTSD, the present work extends current efforts in preclinical PTSD research by describing a readily reproducible zebrafish model system in which the psychobiological mechanisms that may underlie the clinical condition, can be explored. Further, the present investigation also highlighted a dichotomous role for adrenergic processes in the acquisition and consolidation of fear memory, which needs continued investigation.

¹ epinephrine

² triple high intensity trauma

³ posttraumatic stress disorder

⁴ norepinephrine

3.6 References

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4 Conclusion

The aim of this study was to develop a naturalistic zebrafish model of posttraumatic stress disorder (PTSD¹) that is etiologically and clinically relevant by applying a combination of stressors that together, constitute a truly life-threatening scenario. We also aimed to explore the effect of trauma-associated adrenergic processes in the manifestation of post-trauma anxiety.

Mainly, we showed that 1) trauma-paired epinephrine exposure consistently blunted the delayed anxiety-like sequelae associated with severe trauma (quadruple high intensity trauma (QHIT²) paradigm), but bolstered persistent anxiety-like behavior when paired with exposure to a novel circumstance in the absence of additional stressors (epinephrine (EPI³) only paradigm), 2) exposure to a trauma-paired context prior to testing strengthened the subsequent anxiety-like behavior of triple high intensity trauma (THIT⁴), but not QHIT-exposed fish, and 3) despite this, THIT- and EPI-exposed fish showed a lesser degree of contextual avoidance behavior, compared to trauma free (sham⁵)- or QHIT-exposed fish.

* * *

Although the actions of norepinephrine (NE⁶) at times of trauma has been the focus of many PTSD studies, its exact role in fear processing and the delayed manifestation of fear-related anxiety still needs further investigation. In general, alpha-1 (α_1) and beta (β) receptor stimulation promotes neuronal plasticity and activates the fear circuitry, whereas α_2 inhibit such responses, supposedly by blunting NE release (Asalgoo, Jahromi, Meftahi, & Sahraei, 2015; Hussain, Reddy, & Maani, 2020; Roozendaal, McEwen, & Chattarji, 2009; Van Stegeren, 2008).

The first finding of this study shows that pre-trauma EPI exposure (as in the QHIT protocol), was associated with a delayed anxiety-like profile similar to that observed in sham-exposed fish that is, eliciting a post-traumatic behavioral profile akin to that observed in non-stressed fish. In contrast, both trauma which was inflicted in the absence of EPI (as in the THIT protocol), as well as EPI administered on its own under novel circumstances, persistently bolstered anxiety-like bottom-dwelling over all testing days. These results are interesting for two reasons. First, it shows that life-threatening trauma in zebrafish can be applied as a useful model for the temporal study of delayed trauma-associated behavioral sequelae. Secondly, it highlights a clear dichotomous role for adrenergic signaling on the processing and later effects of acute fear, which are likely founded upon α_2 receptor mediated processes.

¹ posttraumatic stress disorder

² quadruple high intensity trauma

³ epinephrine

⁴ triple high intensity trauma

⁵ trauma free

⁶ norepinephrine

Our second main finding pertains to the effect of contextual re-exposure prior to the testing of post-traumatic anxiety-like behavior. In this work said re-exposure strengthened the anxiety-like behavior of THIT¹, but not QHIT²-exposed fish, likely since EPI³ exposure prevented the proper encoding of fear memory, as explained above. This is an important result, because it demonstrates that zebrafish not only contextualize traumatic exposure, but also remember traumatic cues for at least two weeks after trauma exposure. Considering the purpose of this investigation, this finding is vital. One of the main symptoms of clinical PTSD⁴ pertains to flashbacks and avoidance behaviors, which entails re-experiencing and attempts to suppress or avoid contextual reminders of the traumatic event, respectively. Still, while zebrafish display heightened anxiety after exposure to the trauma-paired context, they did not avoid voluntary exposure to said contexts. This is interesting and might speak to the species-specific appraisal of color. While the purpose of this procedure was to prevent 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 in itself. Thus, while zebrafish recognize the contextual value of said trauma-paired colors in showing heightened post-color-reminded anxiety-like behavior, such colors do not necessarily display aversive properties in itself.

* * *

Here, we show that zebrafish can contextualize a severe and realistic life-threatening stressor. We further show that the resultant post-traumatic anxiety, which is context-sensitive, manifests for at least two weeks after trauma exposure. Since the concepts of persistent and delayed post-trauma anxiety, as well as contextual relevance are pivotal diagnostic symptoms of clinical PTSD, the present work extends current efforts in preclinical PTSD research by describing a readily reproducible zebrafish model framework in which the psychobiological mechanisms that may underlie the clinical condition, can be explored. Further, the present investigation also highlighted a dichotomous role for adrenergic processes in the acquisition and consolidation of fear memory, which needs continued investigation.

¹ triple high intensity trauma

² quadruple high-intensity trauma

³ epinephrine

⁴ posttraumatic stress disorder

4.1 Summary of expected vs actual outcomes

Table 4-1 – Final outcomes matched with a summary of the hypothesis study objectives as provided in Chapter 1.

Objective	Result
To explore and describe a novel model of PTSD in zebrafish by exposing zebrafish to a severe ethologically and etiologically relevant, life-threatening stressor.	✓ Zebrafish are able to show delayed post-trauma anxiety after being exposed to a severe and life-threatening stressor.
To establish if zebrafish will show said anxiety-like behavior after trauma in a widely used zebrafish test of anxiety, i.e. the novel tank diving test.	✓ Our findings indicate that zebrafish display anxiety-like behavior in the novel tank anxiety test, showing that currently available anxiety tests are adequate for the assessment of post-traumatic anxiety in this species as well.
To establish whether zebrafish will display delayed avoidance of the context in which they have been exposed to a life-threatening stressor.	✗ This was not the case. However, while this finding may seem counterintuitive for the purposes of modeling PTSD, this could likely be ascribed to species specific differences in how zebrafish appraise and value color. Also, see below.
To establish if zebrafish, exposed to a life-threatening traumatic experience, value a contextual reminder of such event in that it would act as a trigger of post-traumatic anxiety.	✓ Indeed, zebrafish reminded to the context in which they were exposed to trauma, displayed a higher degree of anxiety-like behavior compared to non-reminded fish. This finding further supports the use of the model for preclinical PTSD research.
To explore the effects of adrenergic processes in the manifestation of post-trauma anxiety.	≈ Here we show that adrenergic processing plays a dichotomous role in different levels of stress/trauma. Specifically, we show that norepinephrine (NE) bolsters delayed novelty-associated anxiety-like manifestations, while preventing memory consolidation after severe traumatic exposure. This finding needs further elucidation.

4.2 Study shortcomings and future directions

Some noteworthy shortcomings of this work need mentioning. First, we did not employ different adrenergic receptor targeting drugs. Therefore, detailed conclusions regarding the exact mechanisms underlying the effects of EPI¹ in this model cannot be drawn. That said, since we show that a THIT² model of PTSD³-like anxiety 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 NE⁴ and cortisol over all days of traumatic exposure and anxiety testing would have been beneficial to yield insights into how temporal crosstalk between central and peripheral stress-related biology influence behavioral manifestation. Last, while the data presented are promising, additional PTSD-relevant behavioral tests would have been informative.

For future directions, additional behavioral tests i.e. tests of sociability and anhedonia could be useful, since these changes in these psychological constructs are also hallmark traits of PTSD.

¹ epinephrine

² triple high intensity trauma

³ posttraumatic stress disorder

⁴ norepinephrine

4.3 References

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Addendum A - Description of supplementary methods

The methods discussed in this addendum are supplementary to the detailed methods reported in Chapter 3 but were regarded to be excessive in terms of publication purposes. As such, Addendum A only contains additional information that was not required by the “*Journal of Neuroscience Research*”.

* * *

Layout of groups for the behavioral investigation

Throughout each of the 18 days of the investigation (with respect to each fish), each group were exposed to a sham- or trauma paradigm and behavioral testing on any specific day (specific for each cluster of fish tested). Two to three groups of fish (e.g. groups Ai(1), Ai(2) and Ai(3)) were tested on a single day (see **Table A-1**).

Table A-1 - Dates of behavioral tests and group layout

Group	Start date	Sham/Stressor date	End date	Group description
Ai(1)	29/03/2021	01/04/2021	15/04/2021	QHIT – $n = 8$
Ai(2)	29/03/2021	01/04/2021	15/04/2021	QHIT – $n = 8$
Ai(3)	29/03/2021	01/04/2021	15/04/2021	QHIT – $n = 8$
Ai(4)	09/04/2021	12/04/2021	26/04/2021	QHIT – $n = 8$
Aii(1)	09/04/2021	12/04/2021	26/04/2021	Sham – $n = 8$
Aii(2)	09/04/2021	12/04/2021	26/04/2021	Sham – $n = 8$
Group	Start date	Sham/Stressor date	End date	Group description
Bi(1)	17/04/2021	21/04/2021	05/05/2021	THIT – $n = 5$
Bi(2)	17/04/2021	21/04/2021	05/05/2021	THIT – $n = 8$
Bi(3)	17/04/2021	21/04/2021	05/05/2021	THIT – $n = 7$
Bi(4)	29/04/2021	16/05/2021	02/05/2021	THIT – $n = 8$
Bii(1)	29/04/2021	16/05/2021	02/05/2021	Sham – $n = 8$
Bii(2)	08/05/2021	10/05/2021	24/05/2021	Sham – $n = 8$
*Bi_{replacement}	29/04/2021	16/05/2021	02/05/2021	THIT – $n = 8$
Group	Start date	Stressor date	End date	Group description
Ci(1)	13/07/2021	16/07/2021	30/07/2021	QHIT – $n = 8$
Ci(2)	13/07/2021	16/07/2021	30/07/2021	QHIT – $n = 8$
Cii(3)	13/07/2021	16/07/2021	30/07/2021	THIT – $n = 8$
Cii(4)	13/07/2021	16/07/2021	30/07/2021	THIT – $n = 8$
Group	Start date	Stressor date	End date	Group description
Di(1)	08/05/2021	10/05/2021	24/05/2021	Epinephrine – $n = 7$
Di(2)	08/05/2021	10/05/2021	24/05/2021	Epinephrine – $n = 7$

**Bi_{replacement}* were randomly selected from the zebrafish colony to replace fish (B1.2/B1.3/B1.7/B3.2) that died before completing behavioral tests.

Routines during the behavioral investigation

- Each day began with monitoring the quarantine (where the zebrafish colony was housed) and breeding rooms (of which the water was used for behavioral experimentation tanks) for water quality. These measurements included checking the following: the control unit see (**Figure A-i**) of the automated ZebTec® Zebrafish Housing System, Tecniplast®, Varese, Italy® housing system to ensure that the readings for water quality (pH: 7 ± 0.2 ; conductivity: 595-605 μS), temperature (27.9 ± 0.3 °C), and aeration (7.2 mg O₂/L) were correct. Zebrafish were also examined in the home tanks to confirm animal health.
- Thereafter (and where and when applicable), fish were transported into the behavioral room and allowed to habituate in their home tanks for a minimum of 30 minutes.
- Depending on the experimental phase and day, behavioral testing commenced with color aversion testing (2 fish were tested simultaneously), which was followed by novel tank anxiety testing (4 fish were tested simultaneously; each in its own tank) (see **Figure A-ii**).
- Upon completion of behavioral testing, fish were gently placed back in their home tanks, transported back to the quarantine room and fed. This process was repeated on all experimental days.
- On “stressor” days, fish which were exposed to the quadruple high intensity trauma (QHIT¹)/epinephrine (EPI²) protocols, were allowed to habituate in the experimental apparatus for 10 minutes before receiving adrenaline administration.
- On non-experimental days, routine checks were conducted, and fish fed at approximately 10am.

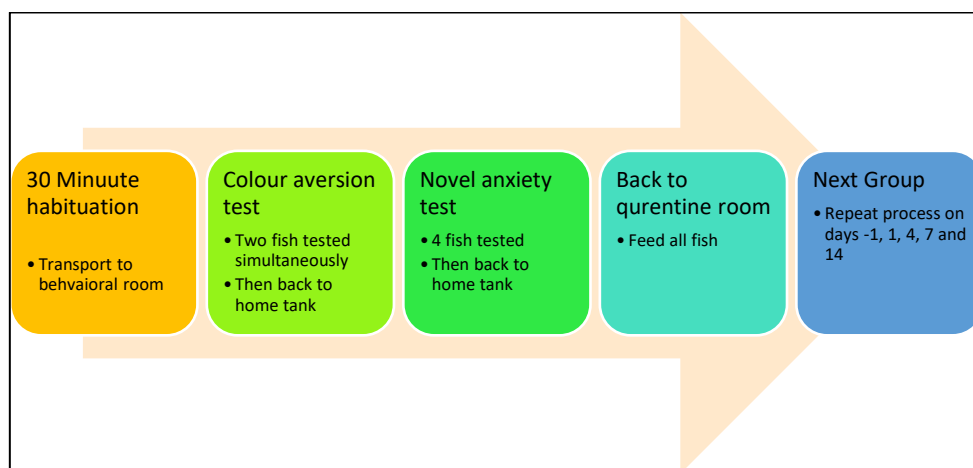


Figure A-i – Order of experimental events on each specific assessment day

¹ quadruple high intensity trauma

² epinephrine



Figure A-ii – The automated ZebTec® Zebrafish Housing System, Tecniplast®, Varese, Italy® housing system to ensure that the readings for water quality and the corresponding control panel,

Detailed methodology of behavioral tests

Color- baseline determination / aversion test

1. Collect water from breeding room.
2. Transport fish from quarantine room to behavioral room.
3. Habituation for a minimum of 30 min.
4. Place correctly colored arm sleeves onto arms of tank (see supplementary **Figure A-iii**).
5. Fill two tanks to line marked as “6 cm”
6. Fill two beakers to line marked as “8 cm”.
7. Net respective fish and place into beaker marked “1”. Repeat this and net into next beaker marked “2”.
8. Place fish into center sleeve and allow for 1 min habituation.
9. Remove center sleeve and allow another 1 min habituation time for water movement to settle.
10. Record behavior for 5 min.
11. Net fish and place back into respective home tank compartments.
12. For color determination, wait 150 min before commencing next color determination test.
13. Change tank water after each set of tests.

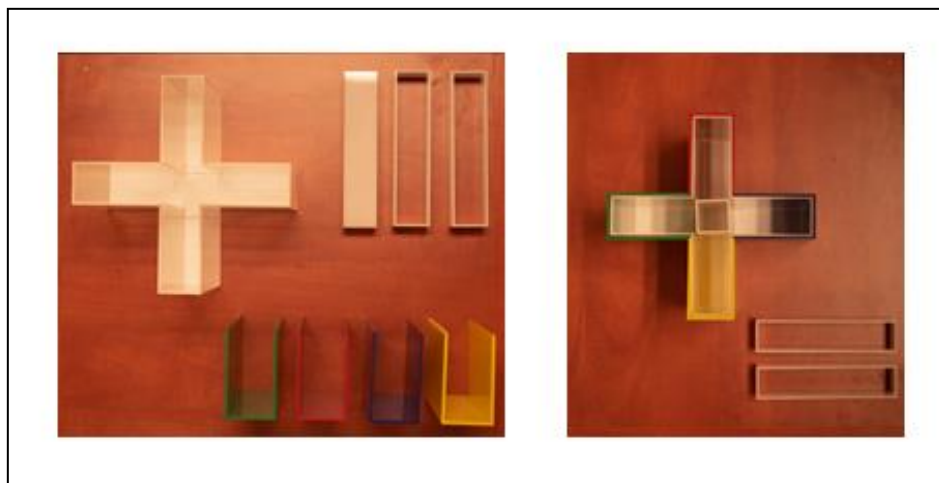


Figure A-iii – Schematic (above) and photographic representation of color preference testing maze.
Important: Only two arms were used in repeated contextual trigger and avoidance analyses on experimental days 1, 4, 7, and 14.

Novel anxiety test

1. Occurs after finishing color aversion test.
2. Collect water from breeding room.
3. Place identification cards of fish next to the respective tanks.
4. Fill four novel tanks to marked line (cylinder: 18 cm; inverse "V": 11 cm; "L-shape": 18 cm; trapezium: 8 cm; and inverse trapezium: 8 cm).
5. Fill four beakers to line marked as "1 cm".
6. Net first tested fish and place into beaker marked "1". Repeat and net fish into next beakers marked "2", "3" or "4" respectively.
7. Begin recording.
8. Show identifying cards of fish to the camera.
9. Pour fish into respective tanks.
10. Allow the test to run for 7 min.
11. Net fish and place back into respective home tank compartments.
12. Change tank water after each set of tests.

QHIT protocol

1. Collect water from breeding room.
2. Prepare “EPI¹home tank” by placing in a divider and filling tank with 2 L water.
3. Transport fish from quarantine room to behavioral room.
4. Habituate for 10 min.
5. Net fish into the respective compartments of the “EPI home tank”.
6. Administer 2 vials (1 mg/mL) of EPI into the “EPI home tank”.
7. Allow fish to be exposed to EPI for 60 min.
8. While exposure occurs, prepare QHIT² tank by adding 2L water into tank.
9. Euthanize 8 fish for conspecific alarm substance (CAS³) donation.
10. After 60 min have passed, net fish from “EPI home tank” into respective QHIT tank compartments.
11. Place the trap door equipment onto QHIT tank; then place one CAS donor onto each of the individual trap doors.
12. While completing step 11, allow 900 mL water to drain out of apparatus (water drainage up to a level of dorsal fin air-exposure).
13. Electrocute tank with a 1 sec pulse of ≈ 166 A at 3 ± 0.2 V AC.
14. Drop CAS donors into tank by releasing trap doors.
15. Electrocute tank again as in (13) above.
16. Allow 1 min to pass.
17. Net fish back into respective home tank divisions.
18. Clean QHIT tank.

¹ epinephrine

² quadruple high intensity trauma

³ conspecific alarm substance

THIT protocol

1. Collect water from breeding room.
2. Transport fish from quarantine room to behavioral room.
3. Habituate for 10 min.
4. While habituation occurs, prepare QHIT¹ (or THIT² in this instance) tank by adding 2 L water into tank.
5. Euthanize 8 fish for CAS³ donation.
6. After 10 min have passed, net fish from home tank into respective THIT tank compartments.
7. Place the trap door equipment onto QHIT tank; then place one CAS donor onto each of the individual trap doors.
8. While completing step 11, allow 900 mL water to drain out of apparatus.
9. Electrocute tank with a 1 sec pulse of ≈ 166 A at 3 ± 0.2 V AC.
10. Drop CAS donors into tank by releasing trap doors.
11. Electrocute tank again.
12. Allow 1 min to pass.
13. Net fish back into respective home tank divisions.
14. Clean THIT tank.

¹ quadruple high intensity trauma

² triple high intensity trauma

³ conspecific alarm substance

Epinephrine protocol

1. Collect water from breeding room.
2. Prepare “EPI¹ home tank” by inserting a divider and filling the tank with 2 L water.
3. Transport fish from quarantine room to behavioral room.
4. Habituate for 10 min.
5. Net fish into the respective compartments of the “EPI home tank”.
6. Administer 2 vials (1 mg/mL) EPI into “EPI home tank”.
7. Allow fish to be exposed to EPI for 60 min.
8. While exposure occurs, prepare QHIT² (not to be used for additional stressors at this time) tank by adding 2 L water into the tank.
9. After 60 min have passed, net fish from “EPI home tank” into respective QHIT tank compartments.
10. Allow 6 min and 30 sec to pass (same time as that transpiring during the QHIT/THIT³ protocols).
11. Allow a further 1 min to pass.
12. Net fish back into respective home tank divisions.
13. Clean QHIT tank.

¹ epinephrine

² quadruple high intensity trauma

³ triple high intensity trauma

Sham protocol

1. Collect water from breeding room.
2. Transport fish from quarantine room to behavioral room.
3. Habituate for 10 min.
4. While habituation occurs, prepare QHIT tank (not to be used for additional stressors at this time) by adding 2 L of water into tank.
5. After 10 min have passed, net fish from home tank into respective QHIT tank compartments.
6. Allow 6 min and 30 sec to pass (same time as that transpiring during the QHIT/THIT protocols).
7. Then allow a further 1 min to pass.
8. Net fish back into respective home tank divisions.
9. Clean QHIT¹ tank.

¹ quadruple high intensity trauma

Additional Information

- Prior to the onset of the study, zebrafish were bred and housed in the National Aquatic Bioassay Facility (NABF¹) and fed once daily from the larval to adult (3-4 months old) stages according to the same schedule used during this study (**Chapter 3**).
- All tanks used for the duration of the behavioral study were properly cleaned before a new group of fish were introduced. Home tanks were also cleaned on day 7, after behavioral testing.
- All behavioral test tanks and beakers used during the experimental process were cleaned immediately after tests were completed. The tanks were cleaned with salt water and left to dry upside-down overnight.
- Double LED fluorescent tube lights (400 lux) were used in the behavioral room as the lighting source.
- Zebrafish were euthanized in a different room than the exposure room.

¹ National Aquatic Bioassay Facility