Development and validation of a post-traumatic stress disorder model in zebrafish

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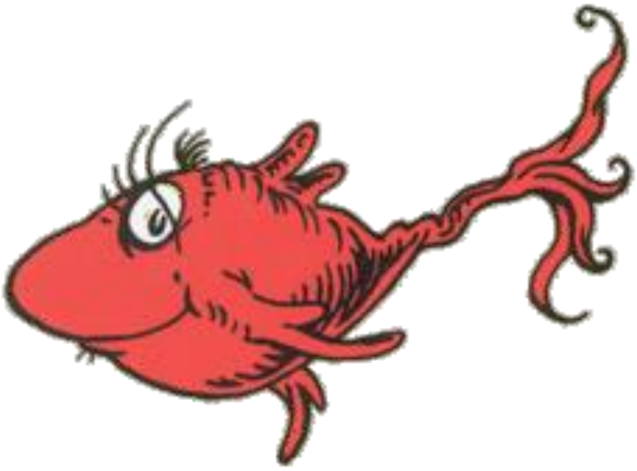
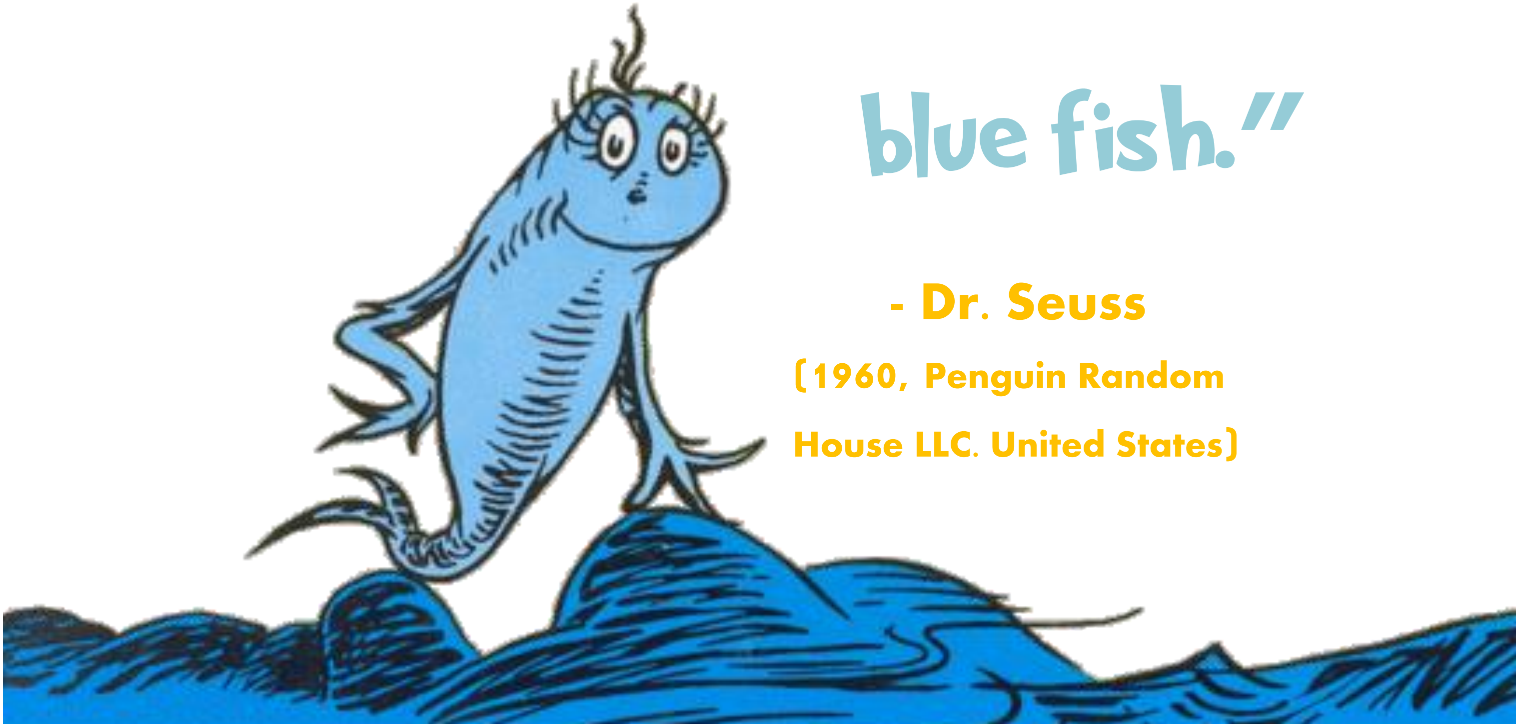
“One fish

two fish



red fish



blue fish.”

- Dr. Seuss

(1960, Penguin Random
House LLC. United States)

For Sonia. Thanks Mom ❤

ABSTRACT

Post-traumatic stress disorder (PTSD) is a severely debilitating chronic mental disorder brought about by encountering an actual or perceived, life-threatening or traumatic experience or series of events, like violence or combat. Specifically, PTSD is intrinsically associated with dysregulated fear conditioning, a form of Pavlovian conditioning where a neutral conditioned stimulus is paired with an aversive unconditioned stimulus.

Lifetime prevalence of PTSD differs across regions of the world and is higher in countries emerging from conflict, with a reported lifetime PTSD prevalence of 2.3% in South Africa. Moreover, the pathophysiology of PTSD development is largely unknown and requires further examination. These hindrances necessitate innovative experimental apperception so that novel treatment strategies may be developed. One approach is to extend the range of translational model species used in PTSD research as to enable analysis of overlapping behavioural phenotypes.

Because zebrafish exhibit evolutionary conserved homologies in neuronal circuitry and mediator systems with humans and rodents, this species has emerged as a useful model in translational research of complex neuropsychiatric disorders such as PTSD. Congruently, the advantages of using zebrafish in pre-clinical neuropsychiatric research also include homologous gene sequences and vertebrate-specific physiological processes, like organogenesis, shared with mammals.

A commonly used animal model of PTSD is the predator exposure model (PEM), in which predator-related cues (visual or chemical) serve as warnings to animals about potential threats in the surrounding area. To this end, exposure to a pheromone-like exudate, conspecific alarm substance (CAS), mediates significant anti-predatory responses in zebrafish. CAS is produced by specialized epidermal club cells in the zebrafish skin whereupon it is secreted into the water consequent to skin damage. Released CAS is detected through olfaction by neighbouring zebrafish and alarm reactions are elicited. Accordingly, CAS-exposed zebrafish display exacerbated anxiety and fear behaviours and, thus, CAS may be useful in the analysis of fear and anxiety in zebrafish.

In this study we aimed to develop a novel translational model of PTSD in zebrafish, in order to expand the PTSD research capabilities at the North West University (NWU). The primary objectives of the study were based on assessing the anxiety-like behavioural responses in zebrafish following CAS exposure. Furthermore, building on evidence in stress-restress models of PTSD in rodents, we aimed to explore the effect of re-experience in zebrafish. This would inform on whether CAS is capable of evoking a sustained anxiogenic response up to 2 days post stress exposure, with or without a visual reminder, thus emulating fear conditioned learning seen in clinical PTSD. In this regard, CAS was paired with contextual reminders to induce fear conditioning and perpetuated fear-like behaviour in the absence of the original stressor. Finally, we aimed to confer face (behavioural) validity to the model for future construct (biological) and predictive (treatment response) validity testing and subsequent application in pre-clinical drug screening initiatives at the NWU.

A total of 72 zebrafish were employed in this study, of which 32 zebrafish were used as CAS donors. The remaining 40 fish were randomly divided into 4 groups ($n = 10$ per group), *viz.* 'vehicle/no cue', 'CAS/no cue', 'vehicle/cue' and 'CAS/cue'. Zebrafish behaviour in the test tanks was recorded during both conditioning and re-experience. For conditioning on day 1, ensuing a 1-h habituation period, two groups were exposed to the vehicle (distilled water), and two groups were exposed to CAS. The exposure lasted for 6 min, with groups 'vehicle/no cue' and 'CAS/no cue' exposed in the absence of a specific visual cue, while groups 'vehicle/cue' and 'CAS/cue' were exposed in the presence of the visual cues (black and white stripes). For re-experience on day 2, zebrafish were returned to the test tanks for 6 min, groups 'vehicle/no cue' and 'CAS/no cue' in the absence of the visual cue, and groups 'vehicle/cue' and 'CAS/cue' in the presence of the visual cue, thereby allowing the conditioned stimulus to be assessed. Zebrafish were then captured and euthanised.

The behavioural video recordings were analysed using EthoVision XT 14 tracking software to virtually divide the test tanks into 2 equal horizontal sections (bottom and top zones) and measuring frequency (number of entries) and total duration (s) in the top of the test tank. By that the position within the test tank was considered as a general index of anxiety with geotaxis indicative of greater anxiety. Further, total duration of immobility (s) and mean meandering (degrees/cm – degree of turning over distance travelled) were also scored as measurements of anxiety.

CAS exposure significantly reduced frequency and time spent in the top zone, while immobility and meandering significantly increased following CAS exposure. Overall, this illustrates that CAS-exposed zebrafish displayed definite anxiety-like behaviour immediately after CAS exposure compared to non-exposed zebrafish. Thereon, the observed anxiety-like behaviour was sustained on day 2 under both cued and non-cued conditions.

The current project establishes that CAS exposure evokes fear- and anxiety-like behaviour in zebrafish, not only during initial exposure, but also thereafter when zebrafish are presented with a contextual reminder in the absence of CAS. This 're-experiencing' phenomenon is characteristic of PTSD-like fear conditioning and confirms the face validity of CAS ± contextual reminder as an effective stressor ± re-experience model of PTSD in zebrafish. That said, anxiety responses were independent of time and cue, implying that further development is required to firmly validate the perpetuation of behavioural fear responses over time using this protocol. Additionally, for future exploratory research to be meaningful, it is imperative that this model be assessed with respect to construct and predictive validity.

Keywords:

Conspecific alarm substance (CAS); Fear conditioning; Zebrafish; Animal model; Post-traumatic stress disorder (PTSD); Anxiety; Predator exposure model (PEM)

CONGRESS PROCEEDINGS

Poster presentation

- Development of a post-traumatic stress disorder model in zebrafish: Conspecific alarm substance induced fear conditioning. *Heslie Loots, De Wet Wolmarans, Marli Vlok, Stephan Steyn, Allan Kalueff, Brian Harvey*. Presented at the South African Neuroscience Society (SANS) Symposium as part of the Biological Psychiatry Congress 2019, 20-23 September, Century City Conference Centre, Cape Town, South Africa (Addendum A).

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"So long, and thanks for all the fish."

– Douglas Adams, The Hitchhiker's Guide to the Galaxy (1979)

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LIST OF ABBREVIATIONS

5HT	Serotonin
ACTH	Corticotropin
AGM	Agomelatine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
CAS	Conspecific alarm substance
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DA	Dopamine
DNA	Deoxyribonucleic acid
dpf	days post-fertilisation
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
ELISA	Enzyme-linked immunosorbent assay
FLX	Fluoxetine
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptors
HPA	Hypothalamic-pituitary-adrenergic
HPI	Hypothalamic-pituitary-interrenal
HPLC	High performance liquid chromatography
HTS	High throughput screening
Hypothal	Hypothalamus
iNOS	Inducible nitric oxide synthase
LC	Locus coeruleus
LTP	Long-term potentiation
MAO	Monoamine oxidase
MR	Mineralocorticoid receptors
MRC	Medical Research Council
NA	Noradrenaline

NAT	Noradrenaline transporter
NABF	National Aquatic Bioassay Facility
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NWU	North-West University
PEM	Predator exposure model
PFC	Prefrontal cortex
PSNS	Parasympathetic nervous system
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
RSF	Russian Science Foundation
SAMRC	South African Medical Research Council
SNRI	Serotonin and noradrenaline reuptake inhibitors
SNS	Sympathetic nervous system
SRI	Serotonin reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
TDS	Time-dependent sensitisation
VEH	Vehicle
WGR	Water Research Group
ZNRC	Zebrafish International Neuroscience Research Consortium

GLOSSARY

Agonist	A drug which binds to and activates a receptor to initiate a biological response.
Allostasis	The process of maintaining homeostasis through internal physiological changes to adapt after acute stress
Anhedonia	The inability to feel pleasure in normally pleasurable activities
Antagonist	A drug which binds to a receptor to prevent binding by other molecules and thus inhibits the biological response of the receptor.
Anxiogenic	Used to cause anxiety.
Anxiolytic	Used to reduce anxiety.
Apoptosis	Programmed cell death.
Autoreceptors	Presynaptic receptors that respond to the primary transmitter substance released by the nerve ending.
Baroreceptor reflex	The mechanism responsible for rapid, moment-to-moment adjustments in blood pressure.
Circadian rhythms	The internal 24-hour clock that regulates the sleep-wake cycle.
Conditional prevalence (PTSD)	The prevalence of post-traumatic stress disorder among those specifically exposed to trauma, as opposed to the overall prevalence.
Construct validity	The model must replicate the underlying neurobiological mechanisms of clinical PTSD.
Disengagement	Efforts to avoid the situation
Dysphoria	A psychological state of unease or dissatisfaction.
Ephemeral	Transitory; existing only briefly.
Face validity	The model must replicate behavioural symptoms associated with clinical PTSD.
Geotaxis (zebrafish)	A preference for, and subsequent movement toward the bottom of the tank.
Heteroreceptors	Receptors that respond to substances released by nerve endings other than its own.
Hyperalgesia	Increased sensitivity to pain.
Hypervigilance	A state of heightened alertness; being exceptionally aware of your environment.
Locomotor activity	Movement from one place to another.
Long-term potentiation	A persistent strengthening of synapses based to produce a long-lasting increase in signal transmission between two neurons.
Minnows	The common name for a number of species of small freshwater fish
Neuroendocrine	Cells that release hormones into the blood in response to stimulation of the nervous system.
Neurogenesis	The process by which new neurons are formed by neural stem cells in the brain.

Olfaction	The sense of smell.
Peritraumatic	Occurring during and/or immediately after a traumatic event.
Phenotype	Observable characteristics or traits of an organism.
Polymorphisms	Variations in DNA sequences between individuals
Predictive validity	The model must replicate the treatment response seen in clinical treatment of PTSD
Primary control engagement	Efforts to influence the situation or modulating one's emotional reaction to it.
Retrograde signalling	The process by which retrograde transmitters are released from the postsynaptic neuronal compartment and travel in a retrograde manner to across the synapse to bind to presynaptically expressed receptors.
Scototaxis (zebrafish)	A preference for, and subsequent movement toward a dark area.
Secondary control engagement	Efforts to adapt to the situation.
Thigmotaxis (zebrafish)	A preference for, and subsequent movement toward the peripheral walls of the tank.

1. INTRODUCTION

1.1. Dissertation layout

The current dissertation is prepared in article format in compliance with the requirements for the MSc degree in pharmacology from the North-West University (NWU). Therefore, the main body of this dissertation is presented as a single concept manuscript for submission to an accredited, peer reviewed scientific journal. The in-text citations and reference lists in this dissertation have been prepared according to Harvard referencing style using the reference management tool, Endnote, as prescribed by the NWU. The text of this dissertation was written in British English and has been spellchecked in Microsoft Word.

The current dissertation comprises of Chapter 1-4. Chapter 1 (Introduction) summarises the research problem, study questions, project aims and objectives, study design, and expected outcomes. Chapter 2 (Literature review) constitutes a concise literature review of post-traumatic stress disorder (PTSD) and zebrafish as translational model, as relevant to this project. Chapter 3 (Manuscript A) provides the key findings of the research conducted in this project as a concept article. This manuscript has been prepared in accordance to the 'Author Information Pack' (Addendum B) of the scholarly journal identified by the supervisor, in consultation with the student (myself) and co-authors (*viz. Journal of Neuroscience Methods*). Chapter 4 (Conclusion) concludes the dissertation and outlines shortcomings of this study along with future recommendations. Finally, the following supplementary evidence is presented in the addenda:

- Addendum A – Biological Psychiatry Congress 2019 participation certificate.
- Addendum B – *Journal of Neuroscience Methods* 'Author Information Pack'.
- Addendum C – Letters of consent.
- Addendum D – Zebrafish handling and ethics certification.
- Addendum E – Power analysis report.
- Addendum F – AnimCare committee approval letter.
- Addendum G – Sample of monitoring sheet.

1.2. Research problem

PTSD is a severely debilitating chronic mental illness caused by an actual or perceived life-threatening or traumatic event or series of events (First *et al.*, 2015, Hoffman *et al.*, 2011, Kang *et al.*, 2003). PTSD develops in consequence of dysregulated processing of emotional stimuli related to the trauma, with the risk exacerbated as the intensity and duration of trauma increases (Kang *et al.*, 2003, Stevens *et al.*, 2013).

The Diagnostic and Statistical Manual of Mental Disorders (5th Edition; DSM-5) categorises PTSD as a trauma- and stressor-related disorder, with symptoms generally emerging within 3 months after experiencing trauma (American Psychiatric Association, 2013). PTSD predominantly manifests in adverse alterations in anxiogenesis and working- and fear memory (Caramillo *et al.*, 2015, Honzel *et al.*, 2014). Accordingly, PTSD is widely described as a disorder of affective memory, characterised by over-consolidated traumatic memory and reduced explicit memory (Bentz *et al.*, 2013, Bowers and Ressler, 2015, Parsons and Ressler, 2013, Pittenger, 2013). Furthermore, diagnosis of PTSD typically includes negative thoughts, moods and beliefs (e.g., detachment, guilt, fear, anger and anhedonia), persistent perceptions of heightened current threat (hypervigilance or enhanced startle reaction), re-experiencing or reliving the trauma (in the form of intrusive memories, flashbacks or nightmares), avoidance of thoughts and memories of the trauma, as well as avoidance of places, people and situations reminiscent of it (First *et al.*, 2015, Hoffman *et al.*, 2011). These symptoms typically last for at least a month and lead to significant impairments in personal, family, social, educational and occupational life, or other important areas of functioning (First *et al.*, 2015, Hoffman *et al.*, 2011). Moreover, symptoms such as abnormalities in mood, arousal, cognition and memory link PTSD to a variety of comorbid neuropsychiatric disorders, including major depressive disorder, anxiety disorders, substance use disorder, chronic fatigue syndrome, suicidal ideation and psychosis (Anderson *et al.*, 2014, Elhai *et al.*, 2011, Gallagher and Brown, 2015, Kang *et al.*, 2003, Rojas *et al.*, 2014, Zoellner *et al.*, 2014).

Lifetime prevalence of PTSD varies across different regions of the world (Atwoli *et al.*, 2015). These variations may be explained either by different trauma exposure rates or the use of different definitions and methodologies in the determination of prevalence, although the USA has arguably the most accurate PTSD statistics (Carriere, 2014). Therefore, the lifetime prevalence of PTSD in the adult population of the USA (7.8%) (Kessler *et al.*, 1995) is pertinent. However, the relative burden of PTSD in the adult population of South Africa (2.3%) (Atwoli *et al.*, 2013), our homeland, is our principal concern. The conditional prevalence (prevalence among those specifically exposed to trauma, as opposed to the overall prevalence) of PTSD in the adult population of South Africa is 3.5%, with a cross-national conditional prevalence of 9.2% (Atwoli *et al.*, 2015, Atwoli *et al.*, 2013).

The pathophysiology of PTSD development is largely unknown and requires further examination (Sherin and Nemeroff, 2011, Stewart *et al.*, 2014b). Additionally, the current pharmacological treatments for PTSD are sub-optimal (Kelmendi *et al.*, 2016) due to the limited research portfolio of novel pharmacotherapy for PTSD (Krystal *et al.*, 2017, Lisieski *et al.*, 2018). In order to address these limitations, it is important to establish models that can mimic the human condition. These models must possess face (behaviour), construct (biology) and predictive (treatment response) validity. Currently, PTSD research relies on mammalian (especially rodent) models of PTSD (Stewart *et al.*, 2014b). Validated rodent models of PTSD include the predator exposure model (PEM) (Cohen *et al.*, 2003, Cohen *et al.*, 2004) and time-dependent sensitization (TDS) (Harvey *et al.*, 2005, Harvey *et al.*, 2006, Harvey *et al.*, 2003, Harvey *et al.*, 2004), amongst others (see Daskalakis *et al.* (2013) and Deslauriers *et al.* (2018) for comprehensive reviews). However, these models are hampered by high cost, low throughput and long breeding periods, as well as being time- and labour intensive (Caramillo *et al.*, 2015, Freudenberg *et al.*, 2018, Kafkafi *et al.*, 2018, Planchart *et al.*, 2016).

Zebrafish (*Danio rerio*) exhibit high homology in neuronal mechanisms and mediator systems with mammals, thus emerging as a useful model of complex neuropsychiatric disorders, including PTSD (Kaslin and Panula, 2001, Stewart *et al.*, 2014b). The zebrafish habenula, implicated in the stress response, is analogous to that of mammals (Okamoto *et al.*, 2012), while the fish hypothalamic-pituitary-interrenal (HPI) axis parallels the human hypothalamic-pituitary-adrenergic (HPA) axis, both sharing similar cortisol (not corticosterone as in rodents) driven stress responses (Cachat *et al.*, 2013, Stewart *et al.*, 2014b). These attributes make zebrafish an excellent model of altered cortisol regulation associated with PTSD (Alsop and Vijayan, 2009, Stewart *et al.*, 2014b). Additionally, zebrafish models are also ideal for high throughput screening (HTS) as they can easily be genetically and pharmacologically manipulated, great numbers can be housed in a small area, and they are easy to breed and maintain (Gerlai, 2010).

As mentioned, the PEM is a commonly used animal model of PTSD (Uys *et al.*, 2003). In this regard, zebrafish display robust anti-predatory responses to predator-related cues such as conspecific alarm substance (CAS) (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart *et al.*, 2014b). CAS is a pheromone-like substance produced by specialized epidermal club cells and is released in the water upon skin damage of zebrafish (Maximino *et al.*, 2018, Speedie and Gerlai, 2008). Upon release CAS is detected through olfaction by neighbouring zebrafish, hence alarming them (Speedie and Gerlai, 2008, Waldman, 1982). Therefore, CAS treatment is effective in inducing fear responses and anti-predatory behaviour in zebrafish and may be useful in the analysis of fear and anxiety in these animals (Speedie and Gerlai, 2008). Furthermore, a linear relationship exists between CAS concentration and the frequency and duration of alarm reactions (Speedie and Gerlai, 2008), likely indicating that a stronger alarm response is elicited when an injured conspecific (and presumably the predator) is nearby. Thus, a higher concentration of CAS is detected by the neighbouring zebrafish, compared to a diminished alarm response to a smaller concentration of CAS when an injured conspecific (and presumably the predator) is further away (Speedie and Gerlai, 2008).

Zebrafish display a wide range of clearly discernible and complex behaviours induced by a variety of stimuli, which are similar to stress-related behaviours exhibited by humans (Blaser *et al.*, 2010, Caramillo *et al.*, 2015, Stewart *et al.*, 2014b), while they also possess the ability to contextualise fear (Ogawa *et al.*, 2014). In this regard, fear conditioning has been demonstrated in zebrafish by pairing visual cues with CAS (Blaser and Vira, 2014, Hall and Suboski, 1995). Moreover, fear- and anxiety-like behaviours displayed by zebrafish in reaction to stressors such as CAS exposure, include increased scototaxis (dark preference), geotaxis (bottom dwelling), thigmotaxis (preference for peripheral regions), freezing (immobility), and erratic movements (Caramillo *et al.*, 2015, Egan *et al.*, 2009). These behavioural phenotypes are remarkably cognate with the fear response of rodents and humans exposed to stress and trauma (Caramillo *et al.*, 2015, Stewart *et al.*, 2014a, Stewart *et al.*, 2014b, Yang *et al.*, 2020). Consequently, zebrafish models present a useful means of comparing normal social behaviour in unstressed animals to pathological social behaviour in stressed animals (Stewart *et al.*, 2014a, Stewart *et al.*, 2014b), and how these responses may be modulated by pharmacological treatment or other intervention.

For the purpose of expanding the platform of PTSD research, we launched this project to establish a novel translational zebrafish model of PTSD at the NWU. We initiated this project by first assessing the anxiety-like behavioural responses following CAS exposure. Thereon, similar in construct to rodent models of PTSD that utilise contextual reminders to perpetuate fear-induced behaviour in the absence of the original stressor (Brand *et al.*, 2008, Harvey *et al.*, 2005, Harvey *et al.*, 2006, Harvey *et al.*, 2003, Harvey *et al.*, 2004), we evaluated the impact of re-experience in zebrafish using a contextual reminder that has previously been paired with CAS.

1.3. Study questions, project aims and expected outcomes

The current project is designed to develop a PTSD model in zebrafish primarily with respect to face validity. In keeping with this, the study is conceptualised to re-examine fear- and anxiety-like behaviour displayed by zebrafish in reaction to predator-related cues. Furthermore, a key objective is to perpetuate zebrafish fear responses in the absence of the original stressor through fear contextualisation, not unlike that noted in clinical PTSD. Thereon, table 1-1 outlines the fundamental study questions of this project with the corresponding aims and expected outcomes.

Table 1-1: *Study questions, project aims and expected outcomes.*

Study questions	***Project aims***	***Expected outcomes***	***Applicable literature***
Akin to clinical PTSD patients, will zebrafish display fear- and anxiety like behaviours (including increased geotaxis, freezing, and erratic movements) in response to trauma (CAS exposure)?	To address this study question, we aimed to establish a translational zebrafish model of PTSD at the NWU, particularly with respect to anxiety behaviour.	It is expected that this study will prompt the setting up of a translational zebrafish model of PTSD for further validation and study.	(Egan *et al.*, 2009, First *et al.*, 2015, Hoffman *et al.*, 2011, Speedie and Gerlai, 2008)
Based on evidence in rodent models of PTSD, will zebrafish exhibit the ability to contextualise fear by pairing a visual cue (black and white stripes) with CAS to perpetuate PTSD-like symptoms?	To address this study question, we aimed to establish the face validity of stressor ± re-experience in a simulated PTSD model in zebrafish.	This model of PTSD will be based on fear conditioning, with CAS ± a contextual reminder as an effective stressor ± re-experience to evoke PTSD-like symptoms in zebrafish.	(American Psychiatric Association, 2013, Blaser and Vira, 2014, Brand *et al.*, 2008, Hall and Suboski, 1995, Harvey, 2005, Harvey *et al.*, 2006, Harvey *et al.*, 2003, Harvey *et al.*, 2004, Ogawa *et al.*, 2014, Ziani *et al.*, 2018)
In view of the limited efficacy of current treatments for PTSD, will the development of a PTSD model in zebrafish introduce a reliable, cost-effective HTS platform for novel drug discovery?	To address this study question, we aimed to establish this zebrafish model of PTSD as a pre-clinical HTS platform for future construct and predictive validity testing, with subsequent application in novel anti-PTSD drug discovery initiatives at the NWU.	Given that a great number of zebrafish can be housed in a small area and that they are easy to breed and maintain, it is expected that a zebrafish model of PTSD will allow cost-efficient HTS of novel PTSD treatments.	(Gerlai, 2010, Kelmendi *et al.*, 2016, Krystal *et al.*, 2017, Lisieski *et al.*, 2018, Stewart *et al.*, 2014b)

1.4. Study design

A total of 72 zebrafish were required to complete this study, of which 32 zebrafish were used as donors for the preparation of CAS. For the behavioural tests the remaining 40 fish were randomly divided into 4 groups (n = 10 per group). The sample size for the behavioural experiments of this project was established in a power analysis done in collaboration with the Statistical Consultation Service (Prof Suria Ellis) of the NWU, Potchefstroom, South Africa, based on previously published studies on zebrafish stress (Demin *et al.*, 2017, Meshalkina *et al.*, 2018, Volgin *et al.*, 2018, Wang *et al.*, 2020). To address the study questions outlined in table 1-1 the study comprised of the following groups, as depicted in the study design in figure 1-1:

- **Group 1** (vehicle/no cue) – Control group exposed to the vehicle (distilled water) without a specific visual cue on day 1, then returned to testing tanks in the absence of a visual cue on day 2 to measure basal behaviour.
- **Group 2** (vehicle/cue) – Control group exposed to the vehicle in the presence of the visual cue on day 1, then returned to testing tanks with the visual cue on day 2 to measure stress-related behaviour in response to the visual cue.
- **Group 3** (CAS/no cue) – Predator cue group exposed to CAS without a specific visual cue on day 1, then returned to testing tanks in the absence of a visual cue (and in the absence of CAS) on day 2 to measure the maintenance of fear- and anxiety behaviour without a conditioned stimulus.
- **Group 4** (CAS/cue) – Predator cue group exposed to CAS in the presence of the visual cue on day 1, then returned to testing tanks with the visual cue (and in the absence of CAS) on day 2 to measure fear- and anxiety behaviour in response to the visual cue as conditioned stimulus.

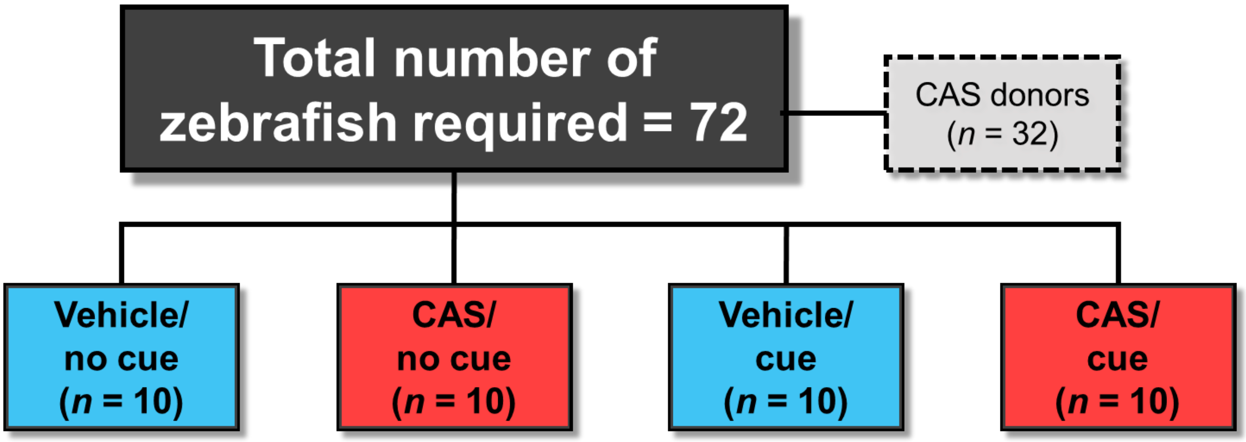

Figure 1-1: *Study design and animal groups.*

1.5. Project layout

For conditioning on day 1, two groups were exposed to the vehicle (distilled water), and two groups were exposed to CAS. Groups ‘vehicle/no cue’ and ‘CAS/no cue’ were exposed without a specific visual cue, with only external cues (i.e. the curtains around this area, the cameras, the structure to which the cameras were fixed, etc.) acting as spatial reminders. Groups ‘vehicle/cue’ and ‘CAS/cue’ were exposed in the presence of the visual cues (black and white stripes (Ziani *et al.*, 2018). For re-experience on day 2 (24-h after conditioning), zebrafish were returned to their testing tanks for 6 min, groups ‘vehicle/no cue’ and ‘CAS/no cue’ in the absence of the visual cue, and groups ‘vehicle/cue’ and ‘CAS/cue’ in the presence of the visual cue, thus allowing the conditioned stimulus to be assessed. The sequence of events on day 1 and 2 for all zebrafish were as follows (see figure 1-2):

- On day 1, zebrafish were individually transferred from the housing tank to the test tank and allowed to explore freely for 1-h.
- Immediately following the habituation period, the lateral walls and floor of each test tank were covered with either plain white laminated paper (groups ‘vehicle/no cue’ and ‘CAS/no cue’) or laminated paper with the visual cue (groups ‘vehicle/cue’ and ‘CAS/cue’).
- In this context zebrafish were exposed to either 3.5 ml/L of the CAS solution (stressor; groups ‘CAS/no cue’ and ‘CAS/cue’) or distilled water (control; groups ‘vehicle/no cue’ and ‘vehicle/cue’) for 6 min.
- On day 2, behavioural responses of zebrafish were analysed again by returning fish to the test tanks with (groups ‘vehicle/ cue’ and ‘CAS/cue’) or without (groups ‘vehicle/no cue’ and ‘CAS/no cue’) the visual cue 24-h after conditioning, but in the absence of vehicle/CAS, for 6 min.
- Thereafter zebrafish were captured and immediately frozen in liquid nitrogen for 30 s and stored at −80°C for future bioanalysis.

Figure 1-2: *A timeline of experimental procedures.*

1.6. Ethical consideration

The estimated animal experience category for this study was 3, or “severe”, due to the fact that zebrafish were exposed to CAS, which may result in long-term alterations in behaviour and stress biomarkers. However, this animal model makes it possible to investigate the behavioural and biological aspects of PTSD. Furthermore, this study may contribute to future studies using zebrafish models as a rapid screening tool for novel drug discovery in PTSD. Therefore, the benefits of this study exceed the risks to the animals.

All experiments and procedures reported fully complied with national legislation and international guidelines. This research project was approved by the NWU AnimCare committee (NHREC registration AREC-130913-015; approval number NWU-00169-18-A5), while the strategy of the 4R’s (replacement, reduction, refinement and responsibility) was used to ensure that experiments and analyses were conducted in an ethical manner (Arora *et al.*, 2011, Doke and Dhawale, 2015, Ranganatha and Kuppast, 2012).

1.6.1. Replacement

Replacement refers to substituting higher order animals with lower order organisms or alternative methods (Doke and Dhawale, 2015, Ranganatha and Kuppast, 2012). In this study rodent models are replaced with a lower order animal, namely zebrafish. Zebrafish models are more time- and cost-efficient and will allow for HTS of drugs before testing the most effective drugs in rodent models.

1.6.2. Reduction

Reduction refers to minimising the number of animals used in an experiment (Doke and Dhawale, 2015). In this study the sample size for the behavioural experiments was established in a power analysis done in collaboration with the Statistical Consultation Service (Prof Suria Ellis) of the NWU, Potchefstroom, South Africa, based on previously published studies on zebrafish (Demin *et al.*, 2017, Meshalkina *et al.*, 2018, Volgin *et al.*, 2018, Wang *et al.*, 2020).

1.6.3. Refinement

Refinement refers to planning an experiment carefully in order to minimise pain and distress of animals (Doke and Dhawale, 2015). This study refines future anti-PTSD drug discovery initiatives by first establishing a translational zebrafish model for PTSD in terms of behavioural determinations. While this work will provide proof of concept, only after sufficient validation through further studies will this model be used for novel drug screening.

1.6.4. Responsibility

Responsibility refers to the appropriate use of laboratory animals to ensure that animals are only used when necessary for biomedical advancement (Arora *et al.*, 2011). We believe that the use of animals in this study was justified, since it added to the knowledge base of PTSD research. Indeed, the PTSD zebrafish model may become a platform for novel drug discovery in the future. Furthermore, responsibility also refers to integrity and honesty in research (Arora *et al.*, 2011). In this regard, we upheld the highest standard of scientific integrity and honesty throughout this project. I received training in zebrafish handling while animals were monitored daily by making use of monitoring sheets, to ensure that animals did not experience distress.

1.7. Research team

This project was a collaborative effort to expand the platform of PTSD research at the NWU. Table 1-2 outlines the contributions made by each member of the research team.

Table 1-2: *Research team involved in the project.*

Name	Role	Affiliations	Contributions
Miss Heslie Loots	Postgraduate student	1. Department of Pharmacology, School of Pharmacy, NWU, Potchefstroom, South Africa 2. Centre of Excellence for Pharmaceutical Sciences, NWU, Potchefstroom, South Africa	• Evolution of overarching research goals and aims set by the supervisor. • Obtaining ethical approval. • Planning of the project. • Development and design of methodology. • Creation of the translational model. • Performing experiments. • Behavioural analyses. • Data collection. • Application of statistical and computational techniques to analyse study data. • Substantive translation of results. • Visualisation and presentation of the work as a poster presentation at a local congress (see ‘Congress Proceedings'). • Assistance with the creation of zebrafish slideshows used by the supervisor in non-project related presentations. • Writing the initial draft of the concept article and overall dissertation.
Prof Brian Harvey	Supervisor	1. Department of Pharmacology, School of Pharmacy, NWU, Potchefstroom, South Africa	• Conceptualisation of the project. • Formulation of overarching research goals and aims. • Acquisition of the financial support for the project. • Oversight and leadership responsibility for the planning and execution of research activities.

Table 1-2: *Research team involved in the project (Continued).*

Name	Role	Affiliations	Contributions
		2. Centre of Excellence for Pharmaceutical Sciences, NWU, Potchefstroom, South Africa 3. MRC Unit on Risk and Resilience in Mental Disorders, University of Cape Town, Cape Town, South Africa	• Provision of study materials, equipment, laboratory animals, instrumentation, computing resources, and other analysis tools. • Visualisation and presentation of parts of this work in slideshows in non-project related presentations. • Creation of the slideshows. • Critical review, commentary and revision of concept article and overall dissertation. • Maintaining research data for initial use and later reuse. • Mentorship.
Dr Marli Vlok	Co-supervisor	1. Department of Pharmacology, School of Pharmacy, NWU, Potchefstroom, South Africa 2. Centre of Excellence for Pharmaceutical Sciences, NWU, Potchefstroom, South Africa	• Laboratory set-up. • Evolution of overarching research goals and aims set by supervisor. • Acquisition of the financial support for the project. • Development and design of methodology. • Management and coordination responsibility for the planning and execution of research activities. • Provision of study materials and guidance with equipment, laboratory animals, instrumentation, computing resources, and other analysis tools. • Critical review, commentary and revision of concept article.
Prof Allan Kalueff	Advisor	1. Pharmacology Department and Neuroscience Program, Tulane University, New Orleans, USA	• Guidance on zebrafish use in neuropsychiatric research. • Critical review of the methodologies prior to beginning the study. • Critical review, commentary and revision of concept article.

Table 1-2: *Research team involved in the project (Continued).*

Name	***Role***	***Affiliations***	***Contributions***
		2. School of Pharmacy, Southwest University, Chongqing, China 3. Institute of Chemical Technology and Natural Sciences, Ural Federal University, Ekaterinburg, Russia	
Dr Stephan Steyn	Advisor	1. Department of Pharmacology, School of Pharmacy, NWU, Potchefstroom, South Africa 2. Centre of Excellence for Pharmaceutical Sciences, NWU, Potchefstroom, South Africa	• Application of statistical and computational techniques to analyse study data. • Critical review, commentary and revision of concept article.

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2. LITERATURE REVIEW

2.1. Post-traumatic stress disorder (PTSD)

2.1.1. Phenomenology of PTSD

A Post-traumatic stress disorder (PTSD) is a chronic psychiatric disorder caused by experiencing or witnessing an actual or perceived, life-threatening or traumatic event or series of events (see figure 2-1), such as violence or combat (First *et al.*, 2015, Hoffman *et al.*, 2011, Kang *et al.*, 2003). Various types of traumatic event lead to PTSD development, including either psychologically or physically damaging events, or events of either a visual or an aural nature (Asalgoo *et al.*, 2015). Dysregulated stress, fear and anxiety in response to emotional stimuli related to trauma underlies the development of PTSD (Stevens *et al.*, 2013, Mezzomo *et al.*, 2019). In this regard, stress represents the response to physical and psychological challenges, while fear is the cognitive response evoked by imminent danger. Finally, anxiety develops as an emotional response triggered by fear of subsequent potentially threatening situations (Lang *et al.*, 2000, Mezzomo *et al.*, 2019, Sylvers *et al.*, 2011). Therefore, failure to extinguish fear responses ultimately leads to the development of anxiety-related disorders such as PTSD (Radulovic *et al.*, 2019). Furthermore, the risk of developing PTSD is directly correlated to the intensity and duration of the trauma (Kang *et al.*, 2003). Of particular note is that only a subpopulation of individuals who survive a traumatic or life-threatening event will develop PTSD (Lisieski *et al.*, 2018, Morris and Rao, 2013, Nemeroff *et al.*, 2006, Stein *et al.*, 2008). This suggests that exposure to stress is not the only determinant in developing PTSD. These findings advocate that a window of opportunity exists wherein the development of PTSD can be halted by appropriate and well-placed treatment as soon as possible following trauma exposure. Moreover, vulnerability to PTSD development is exacerbated by complex interactions between a variety of factors, such as pre-existing risk factors, mediators of stress regulation and reactivity, and moderating factors, as presented in figure 2-1 (Morris and Rao, 2013).

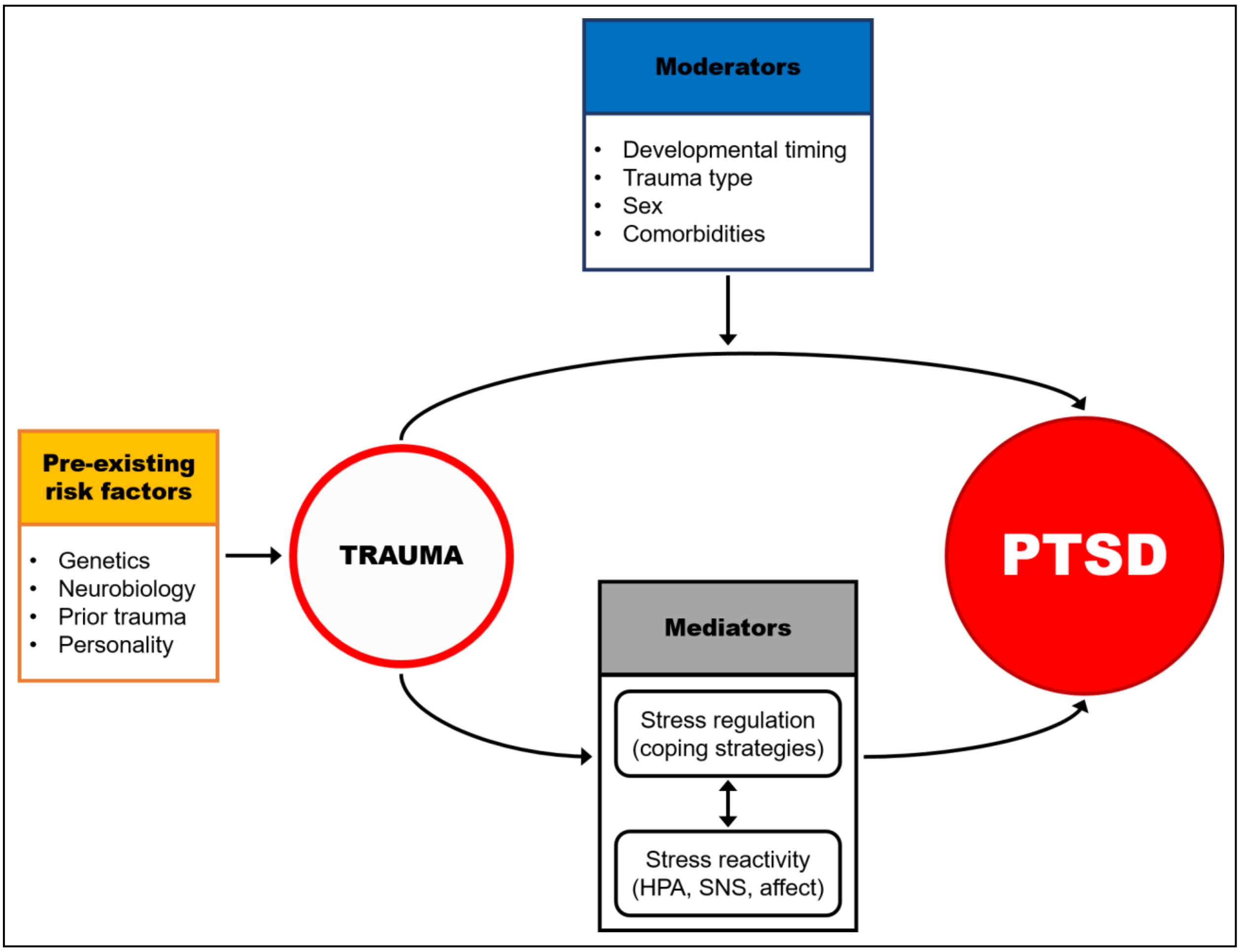

Figure 2-1: *Vulnerability factors for post-traumatic stress disorder (PTSD) development (adapted from Morris and Rao (2013). HPA – hypothalamic-pituitary-adrenergic. SNS – sympathetic nervous system.*

Pre-existing risk factors implicated in PTSD include genetic factors, neurobiological systems, prior trauma and personality (Morris and Rao, 2013). In this regard, genetic studies in twins have revealed that a smaller hippocampus, as has been noted in some PTSD patients, may predict an added risk of developing PTSD post exposure to trauma (Gilbertson *et al.*, 2002). Furthermore, a history of trauma and environmental adversity during early childhood further increases the risk of PTSD later in life (Koenen *et al.*, 2007, Stein *et al.*, 2008). Accordingly, prior trauma is related to lower peritraumatic cortisol levels and subsequent PTSD development (Ehring *et al.*, 2008).

The acute response to trauma is comprised of stress regulation and stress reactivity (Morris and Rao, 2013). In this regard, stress regulation consists of coping strategies in response to trauma, which are determined by an individual's cognitive appraisal (Morris and Rao, 2013). Coping refers to the conscious, volitional effort to regulate emotion, cognition, behaviour, physiology and the environment in response to stress (Compas *et al.*, 2001). Coping responses include primary control engagement (efforts to influence the situation or modulating one's emotional reaction to it), secondary control engagement (efforts to adapt to the situation) and disengagement (efforts to avoid the situation) (Compas *et al.*, 2001, Morris *et al.*, 2012). Additionally, most individuals tend to utilise multiple different coping strategies simultaneously and to adjust their coping strategies over time as contextual factors change (Morris and Rao, 2013, Norris *et al.*, 2002). Therefore, coping is considered a psychological predictor of subsequent PTSD development and it is postulated that coping strategies are as much an expression of PTSD symptoms as they are efforts to manage trauma (Morris and Rao, 2013, Spurrell and McFarlane, 1993). Adaptive coping strategies include social support and acceptance (Morris and Rao, 2013). Additionally, sources of resilience, such as self-esteem, optimism, hope and perceived social-support, are reciprocally correlated with PTSD symptoms and have been found to promote adjustment after experiencing a traumatic event (Besser *et al.*, 2014, Caramillo *et al.*, 2015, Kwon, 2002). On the other hand, maladaptive coping strategies include social support-seeking, thought suppression, avoidance, denial and wishful thinking (Morris and Rao, 2013). Coping related risk factors for developing PTSD also include a lack of fear response regulation or an overactive fear response, sensitivity to anxiety, rumination of a traumatic event and internalisation of stress (Caramillo *et al.*, 2015, McLaughlin and Hatzenbuehler, 2009, Qiao *et al.*, 2013).

Stress reactivity, on the other hand, consists of hypothalamic-pituitary-adrenergic (HPA) function, sympathetic nervous system (SNS) activity, and affect (Morris and Rao, 2013). HPA hypoactivity and SNS hyperactivity acutely after trauma exposure are considered biological predictors of PTSD onset (Morris and Rao, 2013). This suggests a sensitised neuroendocrine system primed to respond more rapidly and vigorously to perceived threat (Morris and Rao, 2013, Yehuda *et al.*, 1996). Regarding HPA function, PTSD patients generally have lower daily cortisol output compared to individuals never exposed to trauma (Morris *et al.*, 2012). Concerning SNS activity, however, PTSD patients present with increased central and peripheral noradrenergic activity (Morris and Rao, 2013). For a description of the role of the HPA axis and SNS in the pathophysiology of PTSD see section 2.1.5.

Bidirectional interactions exist between stress regulation and stress reactivity responses in predicting PTSD development (Morris and Rao, 2013). In this regard, primary control engagement is reciprocally associated with cortisol levels following trauma exposure, whereas secondary control engagement is directly associated with cortisol levels (Morris and Rao, 2013).

Moderating factors for PTSD development include developmental timing, type of trauma, sex and comorbid disorders (Morris and Rao, 2013). In this regard, lower peritraumatic cortisol levels in adults are associated with the subsequent development of PTSD symptoms, whereas in children higher peritraumatic cortisol levels are associated with PTSD (Pervanidou *et al.*, 2007). These contradicting findings between adults and children might be explained by the interactions between trauma and neurodevelopment (De Bellis *et al.*, 1999). Furthermore, there is clear evidence suggesting that women are more likely to develop PTSD and tend to express symptoms differently than men, with women reporting greater distress, sleep disturbances and anhedonia compared to men (Carragher *et al.*, 2016, Hourani *et al.*, 2015, Tolin and Foa, 2008). Accordingly, women are reported to have longer recovery periods in comparison to men, which might be ascribable to elevated cortisol being a protective factor against PTSD symptoms in men, since women have overall lower cortisol levels than men (Caramillo *et al.*, 2015, Meewisse *et al.*, 2007, Van Cauter *et al.*, 1996, Ward, 2016). Additionally, the primary female sex hormone, oestrogen, plays a role in modulating the stress response (Cover *et al.*, 2014). Although, as mentioned, women have an increased risk of PTSD, men show significantly higher rates of maladaptive coping behaviour (Carmassi *et al.*, 2014b).

PTSD is typically characterized by four symptom clusters: 1) re-experiencing or reliving the trauma in the form of intrusive memories, flashbacks, nightmares or panic attacks, typically accompanied by overwhelming emotions when reminded of the trauma; 2) avoidance of thoughts and memories of the trauma, or avoidance of places, people and situations reminiscent of the trauma; 3) negative thoughts, moods and beliefs, and changes in cognition; and 4) persistent perceptions of heightened current threat, indicated by hypervigilance or enhanced startle reaction (Asalgoo *et al.*, 2015, First *et al.*, 2015, Hendrickson and Raskind, 2016, Hoffman *et al.*, 2011). These symptoms typically last for at least a month and lead to distress and significant impairments in personal, family, social, educational or occupational life, or other important areas of functioning (First *et al.*, 2015, Hoffman *et al.*, 2011, Lisieski *et al.*, 2018). It is also worth noting that PTSD symptoms may fluctuate substantially over time, with symptoms being most severe during highly stressful periods (Asalgoo *et al.*, 2015).

Psychological symptoms of post-war mental alterations were first observed as early as 2000 BC by the ancient Egyptians (Asalgoo *et al.*, 2015). Ancient Greeks also described PTSD-like disturbances among soldiers after the battle of Marathon in 450 BC (Asalgoo *et al.*, 2015). PSTD became especially widespread during and after World Wars I (1914-1918) and II (1939-1945) (Asalgoo *et al.*, 2015). In 1975, survivors of the Vietnam War presented with PTSD-like symptoms, such as fear, panic and increased anxiety (Asalgoo *et al.*, 2015). Although, the term PTSD was only officially classified as a mental disorder in 1980 (Asalgoo *et al.*, 2015).

2.1.2. Epidemiology of PTSD

Lifetime prevalence of PTSD varies across different regions of the world and is higher in countries emerging from conflict (Atwoli *et al.*, 2015). Lifetime prevalence is similar in the adult population of South Africa (2.3%), Spain (2.2%) and Italy (2.4%), whereas the lowest prevalence is reported in China (0.3%) and Japan (1.3%), and the highest prevalence is reported in the USA (7.8%) and Northern Ireland (8.8%) (Atwoli *et al.*, 2013, Carmassi *et al.*, 2014a, Carriere, 2014, Ferry *et al.*, 2014, Kawakami *et al.*, 2014, Kessler *et al.*, 1995, Olaya *et al.*, 2015). These variations in PTSD statistics may be explained either by different trauma exposure rates or the use of different definitions and methodologies in the determination of prevalence, although the USA has arguably the most accurate PTSD statistics (Carriere, 2014). Kessler *et al.* (1995) reported a lifetime PTSD prevalence of 7.8% in the adult population of the USA, where women (10.4%) were twice as likely as men (5%) to develop PTSD. Furthermore, the main causes of PTSD in the USA are reported to include criminal victimisation, car accidents and childhood abuse (physical, sexual and emotional) (Kessler *et al.*, 1995, Nemeroff *et al.*, 2006).

In South Africa the largest relative burden of PTSD in the adult population is the witnessing of violent events (50%) (see figure 2-2) (Atwoli *et al.*, 2013). Other common events associated with the PTSD burden in South Africa include accidents, death of loved ones and physical violence (Atwoli *et al.*, 2013).

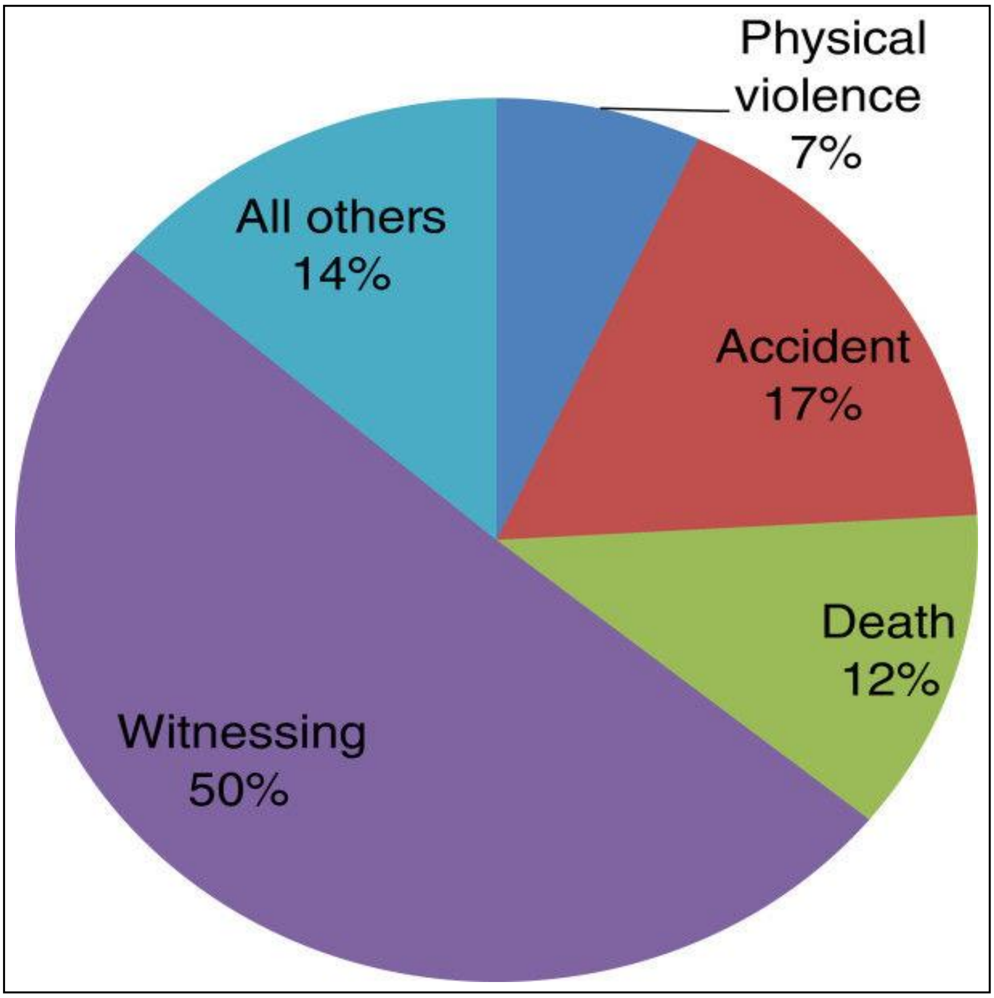

Figure 2-2: *Relative PTSD burden associated with witnessing traumatic events, accidents, death, physical violence and other traumatic events in the South African adult population (Atwoli et al., 2013).*

The conditional prevalence (prevalence among those specifically exposed to trauma, as opposed to the overall prevalence) of PTSD after trauma exposure in the adult population of South Africa is 3.5%, whereas the cross-national conditional prevalence of PTSD after trauma exposure is 9.2% (Atwoli *et al.*, 2015, Atwoli *et al.*, 2013). This conditional risk of PTSD after trauma exposure might be attributable to inter-individual differences in coping strategies, as vulnerability to PTSD is determined by a combination of pre-existing risk factors, stress reactivity and, importantly, coping strategies (see figure 2-1) (Morris and Rao, 2013).

2.1.3. Diagnosis of PTSD

The DSM-5 categorises PTSD as a trauma- and stressor-related disorder, with clearly defined diagnostic criteria (American Psychiatric Association, 2013). In keeping with this, the DSM-5 diagnostic criteria for PTSD are as follows (American Psychiatric Association, 2013):

A. Exposure to a traumatic event in one of the following manners:
    1. Directly experiencing a traumatic event.
    2. Witnessing a traumatic event.
    3. Learning about a traumatic event which happened to a loved one.
    4. Prolonged exposure to aversive details of traumatic events.

B. One of the following intrusion symptoms associated with the traumatic event, starting **after trauma exposure**:
    1. Intrusive memories of the traumatic event.
    2. Nightmares related to the traumatic event.
    3. Dissociation and reliving the traumatic event.
    4. Psychological distress in response to reminders of the traumatic event.

C. Avoidance of reminders of the traumatic event, starting **after trauma exposure** in one of the following manners:
    1. Avoidance of memories, thoughts or feelings related to the traumatic event.
    2. Avoidance of or efforts to avoid external reminders related to the traumatic event.

D. Negative alterations in cognition and mood, starting **after trauma exposure** in one of the following manners:
    1. Inability to recall important aspects of the traumatic event.
    2. Negative beliefs or expectations.
    3. Distorted memories about the traumatic event.
    4. Negative emotional state.
    5. Anhedonia.
    6. Detachment.
    7. Inability to experience positive emotions.

E. Alterations in arousal and reactivity, starting **after trauma exposure** in one of the following manners:
    1. Irritability, anger and aggression.
    2. Reckless or self-destructive behaviour.
    3. Hypervigilance.
    4. Exaggerated startle response.
    5. Concentration problems.
    6. Sleep disturbances.

A patient will be diagnosed with PTSD when (American Psychiatric Association, 2013):

I. Duration of Criteria B, C, D and E lasts for at least one month.

II. Criteria B, C, D and E cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

III. Criteria B, C, D and E cannot be attributed to the physiological effects of a substance or another medical condition.

Symptoms typically start within the first three months after experiencing trauma, although there are cases where there is a delay of months, or even years, after a trauma experience before the diagnostic criteria for PTSD is met (American Psychiatric Association, 2013). Furthermore, individuals present with different symptom patterns, where some patients will predominantly present with fear, re-experiencing, emotional and behavioural symptoms, whereas others predominantly display symptoms of anhedonia, dysphoric mood alterations and cognitive abnormalities (American Psychiatric Association, 2013). In other patients arousal and reactive externalising are the predominant symptoms, while some have predominantly dissociative symptoms (American Psychiatric Association, 2013). There are also some cases where patients display combinations of different symptom patterns (American Psychiatric Association, 2013).

2.1.4. Comorbid illnesses with PTSD

Symptoms such as abnormalities in mood, arousal, cognition and memory link PTSD to a variety of comorbid neuropsychiatric disorders, including major depressive disorder, anxiety disorders, substance use disorder, chronic fatigue syndrome, suicidal ideation and psychosis (Anderson *et al.*, 2014, Elhai *et al.*, 2011, Gallagher and Brown, 2015, Kang *et al.*, 2003, Rojas *et al.*, 2014, Zoellner *et al.*, 2014). Additionally, the presentation of depression in a PTSD patient often results in a treatment resistant form of depression (Brand and Harvey, 2017a). Long-term PTSD also increases the risk of physical illnesses and early death (Asalgoo *et al.*, 2015). In this regard, PTSD is associated with comorbidities such as cardiovascular, respiratory, gastrointestinal, autoimmune and chronic pain conditions (Fishbain *et al.*, 2017, Pace and Heim, 2011, Sareen *et al.*, 2007).

2.1.5. Pathophysiology of PTSD

The pathophysiology of PTSD development is largely unknown and requires further study (Sherin and Nemeroff, 2011, Stewart *et al.*, 2014c). Nevertheless, the stress response plays an important role in adaptation to and protection against stressors, and is manifested by a short-term activation of the HPA axis, which results in the release of stress hormones and other mediators, including neurotransmitters and cytokines (Harvey, 2005, McEwen, 2004). This process of maintaining stability through change is called allostasis (McEwen, 2004). Allostatic load, on the other hand, refers to a maladaptive response to stressors, leading to an imbalance of stress hormones and failure to terminate the activation of the HPA axis (Harvey, 2005, McEwen, 2004). Allostatic load results in structural and functional brain changes and may predispose an individual to the development of neuropsychiatric disorders, such as PTSD (Harvey, 2005, McEwen, 2004).

The primary stress hormone is cortisol (corticosterone in rodents) released into the bloodstream by the adrenal cortex. Its primary function is to mobilise energy stores (including glucose and glycogen), to suppress immune responses and, in the end, to contain stress-induced responses once the danger has passed by terminating the HPA axis stress response at the pituitary gland and hypothalamus (Hypothal) (Oosthuizen *et al.*, 2005).

In this regard, acute stress increases the release of cortisol that in turn activates glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in the hippocampus, amygdala, Hypothal and pituitary gland (Lisieski *et al.*, 2018, Oosthuizen *et al.*, 2005). Hypothalamic and pituitary GR activation limits the persistence of the HPA axis stress response through negative feedback inhibition (Lisieski *et al.*, 2018). Additionally, stress-elicited release of glutamate activates hippocampal N-methyl-D-aspartate (NMDA) receptors, which cooperatively facilitate learning and the consolidation of trauma-related memories (Oosthuizen *et al.*, 2005). On the other hand, gamma-aminobutyric acid (GABA) release after acute stress and negative feedback inhibition of cortisol at the HPA axis are collectively responsible for restoring normal homeostasis, which prevent the abnormal consolidation of fear memories (Oosthuizen *et al.*, 2005). Furthermore, noradrenaline (NA) and adrenaline are released from the adrenal medulla almost instantaneously after stress exposure as part of the SNS response (Morris and Rao, 2013). The subsequent release of cortisol initially amplifies the SNS response and then contains it through negative feedback inhibition (Morris and Rao, 2013). Therefore, these mechanisms are critical for a normal acute stress response and recovery (see figure 2-3) (Morris and Rao, 2013, Oosthuizen *et al.*, 2005). However, in PTSD patients these mechanisms are disrupted so that the stress response continues indefinitely, developing into a chronic stress response and hence contributing to allostatic load (Oosthuizen *et al.*, 2005). Moreover, continuous GR and NMDA receptor activation over a prolonged period results in the excessive consolidation of traumatic memories, as well as damage to neuronal cells, resulting, for instance, in atrophy of the hippocampus, the latter in response to glutamate-mediated release of nitric oxide (NO) (Maura *et al.*, 2000, Oosthuizen *et al.*, 2005).

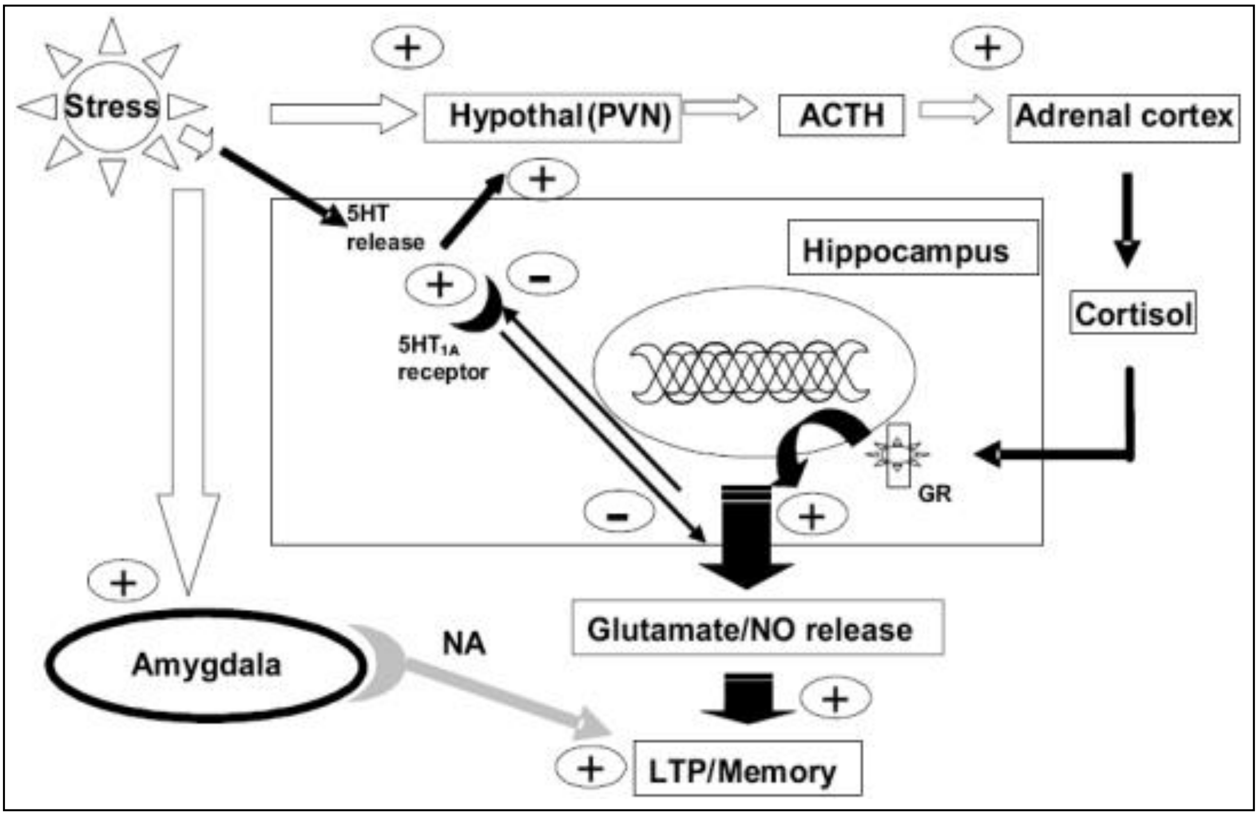

Figure 2-3: *Neuroanatomy of memory and stress (Oosthuizen et al., 2005). Hypothal – hypothalamus. PVN – paraventricular nucleus. ACTH – corticotropin. 5HT – serotonin. GR – glucocorticoid receptors. NO – nitric oxide. NA – noradrenaline. LTP – long-term potentiation. Refer to the text for detail.*

2.1.5.1. Hypothalamic-pituitary-adrenergic (HPA) axis

PTSD patients typically present with hypocortisolism, as well as increased cerebral catecholamine levels, which differs substantially from the normal fight-or-flight stress response where cortisol and catecholamines are increased in parallel (Asalgoo *et al.*, 2015, Caramillo *et al.*, 2015, Heim *et al.*, 2000). These manifestations implicate a dysfunctional HPA axis in the pathophysiology of PTSD, as the HPA axis is integral in the regulation of stress hormones (see figure 2-1) (Hageman *et al.*, 2001, Skelton *et al.*, 2012).

The HPA axis responds to stress by releasing vasopressin and corticotropin-releasing factor (CRF) from paraventricular nucleus (PVN) neurons in the Hypothal, liberating corticotropin (ACTH) release from the pituitary anterior lobe and the subsequent secretion of cortisol from the adrenal gland (see figure 2-4) (Asalgoo *et al.*, 2015, Rivier and Plotsky, 1986, Yehuda, 2002). Cortisol in turn activates GR to limit the persistence of the HPA axis stress response through negative feedback inhibition to the hypothalamic release of CRF and the pituitary release of ACTH so that systemic homeostasis returns. Additionally, the PVN has reciprocal interactions with the hippocampus and amygdala, both stimulating and being regulated by these brain regions (Lisieski *et al.*, 2018, Penzo *et al.*, 2015, Tsigos and Chrousos, 2002). Thereon, the hippocampus and the amygdala participate in both feedback inhibition and feed-forward activation of the HPA axis stress response via glucocorticoid signalling (Herman *et al.*, 2012, Liberzon *et al.*, 1999, Lisieski *et al.*, 2018, Wang *et al.*, 2009).

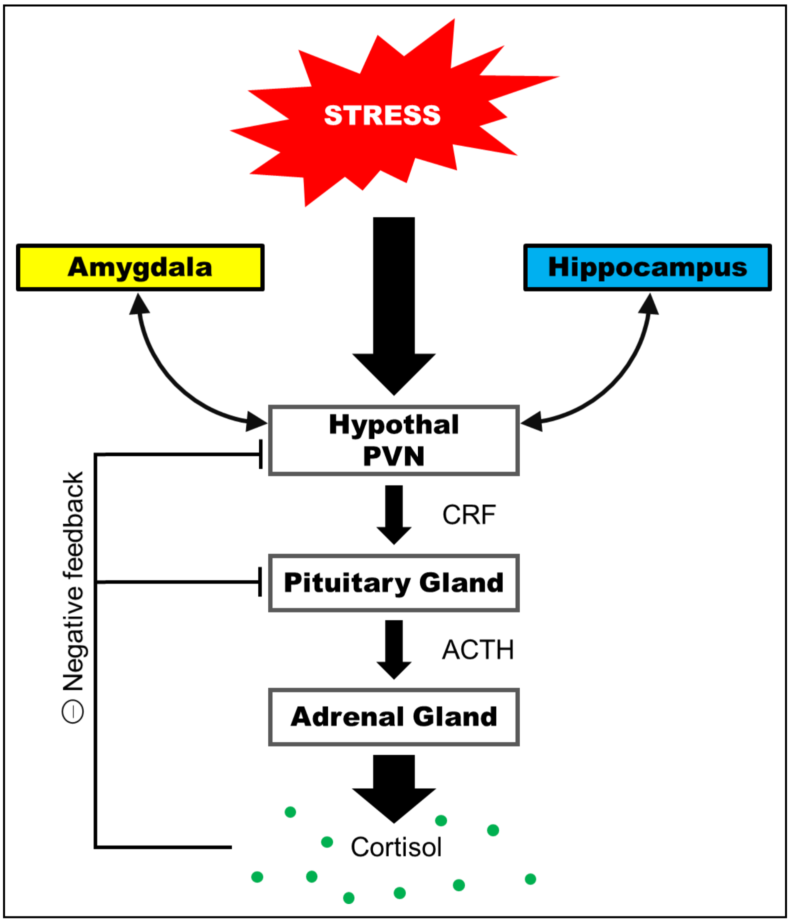

Figure 2-4: *The HPA axis stress response (own figure based on the text). PVN – paraventricular nucleus. CRF – corticotropin-releasing factor. ACTH – corticotropin.*

According to the developmental traumatology theory, hypocortisolism may be the result of hypersensitisation of the HPA axis in response to a stressor (Morris and Rao, 2013, Oosthuizen *et al.*, 2005, Yehuda *et al.*, 2000). This theory states that an initial period of HPA hyperactivity is followed by enhanced negative feedback inhibition of the pituitary gland and an adaptive downregulation of anterior pituitary CRF receptors in response to central stimulation of GR, eventually resulting in diminished basal HPA activity (Asalgoo *et al.*, 2015, De Bellis *et al.*, 1999, Lisieski *et al.*, 2018, Morris and Rao, 2013). Due to the increase in fear caused by a hypersensitised HPA axis in PTSD patients, non-adaptive learning paths are formed, so that PTSD can be regarded as a neuroendocrine disorder (Asalgoo *et al.*, 2015).

A possible mechanism for HPA axis hypersensitivity in PTSD involves changes in the function and density of corticosteroid receptors (particularly GR and MR) (Lisieski *et al.*, 2018). GR and

MR density is largely determined by stress and mediates the neurobiological response to cortisol (Lisieski *et al.*, 2018, Oitzl *et al.*, 1995). In this regard, a high GR:MR ratio leads to enhanced responsiveness to stress-induced cortisol release, including negative feedback inhibition of the HPA axis (Oitzl *et al.*, 1995). Furthermore, evidence indicates that increased glucocorticoid signalling contributes to memory impairment associated with PTSD by modulating the over-consolidation of aversive memories, and is also implicated in the expression of symptoms such as hypervigilance, anxiety and emotional distress (de Quervain *et al.*, 2017, Lisieski *et al.*, 2018, Raglan *et al.*, 2017). Therefore, corticosteroid signalling, and in particular the GR:MR ratio, have been suggested as useful biomarkers of PTSD (Daskalakis *et al.*, 2016, Lisieski *et al.*, 2018).

2.1.5.2. Central nervous system (CNS)

2.1.5.2.1. Neuroanatomy of PTSD

PTSD patients present with significant neurobiological and neurochemical alterations (Asalgoo *et al.*, 2015). The main cerebral areas involved in PTSD pathophysiology are the amygdala, hippocampus and prefrontal cortex (PFC) (see figure 2-5) (Caramillo *et al.*, 2015, Thomaes *et al.*, 2014), which collectively incorporate the fear regulation centres of the brain. Both structural and functional changes in these cerebral loci are implicated in PTSD development (Asalgoo *et al.*, 2015, Newport and Nemeroff, 2000). It is hypothesised that PTSD is characterised by a hyperresponsive amygdala and a hyporesponsive PFC and hippocampus, with the PFC and hippocampus failing to exert inhibitory control over the amygdala (Asalgoo *et al.*, 2015, Shin *et al.*, 2006).

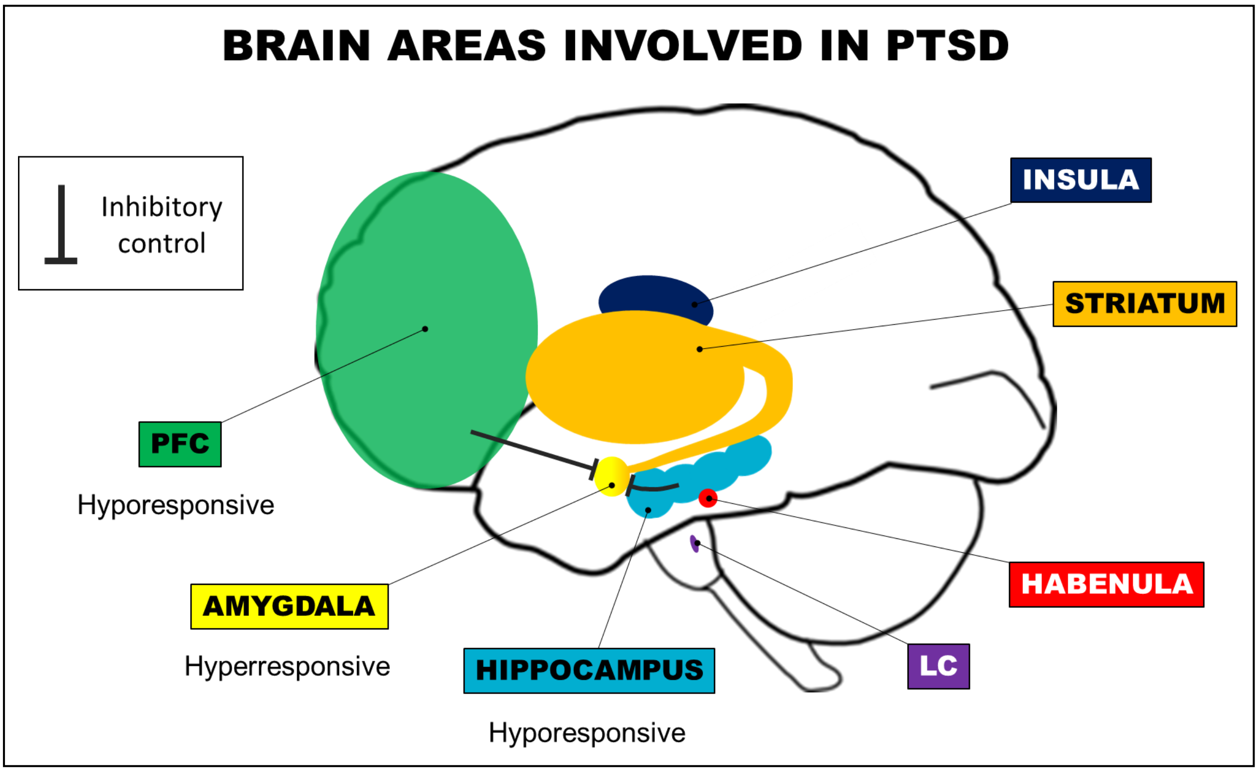

Figure 2-5: *Lateral median plane view of the main brain areas involved in PTSD pathophysiology (own figure based on the text). PFC – prefrontal cortex. LC – locus coeruleus.*

The PFC plays a critical role in decision-making, attention, working memory and emotional processing (Dixon *et al.*, 2017). Of importance, the PFC also regulates fear learning, expression and extinction (Gilmartin *et al.*, 2014). In this regard, the PFC is reduced in volume in PTSD, resulting in reduced functionality of fear circuitry in the brain (De Bellis *et al.*, 2002, Milad and Quirk, 2002, Shin *et al.*, 2006). The PFC is especially important in exerting top-down inhibitory control over the amygdala to inhibit fear expression (Giustino and Maren, 2015, Zotev *et al.*, 2013). Hence, any loss of PFC function will weaken its regulatory control over the amygdala (Arnsten *et al.*, 2015). Furthermore, reduced PFC activation in PTSD contributes to impaired emotional regulation and fear extinction recall (Helpman *et al.*, 2016, Lisieski *et al.*, 2018). Additionally, there is a reciprocal correlation between PFC activation (as it attempts to exert control) and PTSD symptom severity (Shin *et al.*, 2006). In fact, Vasterling *et al.* (1998) found that PTSD patients had cognitive deficits similar to patients with frontal lobe injury. PFC deficits may also be related to disinhibited monoaminergic neurotransmission following stress and may lead to the development of flashbacks, exaggerated startle response and other fear-related behaviour (Campeau *et al.*, 2011, Caramillo *et al.*, 2015, Etkin and Wager, 2007, Newport and Nemeroff, 2000).

The amygdala is the interface between perceiving a threatening stimulus and the biochemical and behavioural responses to the threat, with PTSD being frequently linked to increased activity in the amygdala (Stidd *et al.*, 2013, Yehuda, 2002). Neuro-imaging studies have repeatedly shown that amygdala hyperresponsivity to threat-related stimuli in PTSD patients predicts a poor treatment response (Bryant *et al.*, 2008, Etkin and Wager, 2007, Sartory *et al.*, 2013). Moreover, PTSD is a disorder of memory, presenting with excessive fear (traumatic) memories but disrupted explicit (factual) memories (Pittenger, 2013). In this regard, the amygdala is involved in the formation and recollection of emotional memories, especially fear-related memories (Caramillo *et al.*, 2015).

The hippocampus is a subcortical structure critical for learning and memory (Lisieski *et al.*, 2018, Squire, 1992). Accordingly, the hippocampus is involved in conditioning fear memories to context in order to prime the appropriate behaviour, with the ability to recall memories (Asalgoo *et al.*, 2015, Corcoran and Maren, 2001, Shin *et al.*, 2006, Smith and Bulkin, 2014). Moreover, the hippocampus interacts with the amygdala during the consolidation of fear memories and the modulation of emotional arousal (Fastenrath *et al.*, 2014, Hermans *et al.*, 2017, Shin *et al.*, 2006). The hippocampus also interacts with the PFC to regulate memory (Cohen, 2011). Thus, the hippocampus is implicated in the memory-related symptoms of PTSD, including re-experiencing, enhanced negative emotional memories, deficits in working and verbal memory, and impaired context-dependant memory processing (Garfinkel *et al.*, 2014, Lisieski *et al.*, 2018, Megías *et al.*, 2007, Scott *et al.*, 2015). The posterior hippocampus performs primarily information processing and cognitive functions, whereas the primary function of the anterior hippocampus is to regulate the stress response and affect (Fanselow and Dong, 2010). In this regard, abnormalities of the hippocampus present in PTSD correlates with PTSD symptom severity, with the hippocampus being reduced in volume in PTSD, resulting in obsessive recall of traumatic memories (Asalgoo *et al.*, 2015, Chen and Etkin, 2013, Dunkley *et al.*, 2014). Additionally, deficits in verbal memory recall are associated with hippocampal atrophy (Bremner *et al.*, 1995, Caramillo *et al.*, 2015). On the other hand, greater hippocampal volume correlates with improved clinical response to PTSD treatment (Levy-Gigi *et al.*, 2013).

Deficits in the aforementioned brain regions have been linked to dysregulation of the HPA axis, as well as neurotoxic effects of transmitters such as glutamate (Jing *et al.*, 2015, Lu and Richardson, 2014). Dysregulation of the HPA, along with deficits in the PFC, amygdala and hippocampus, are subsequently implicated in fear learning in PTSD patients (Asalgoo *et al.*, 2015).

Other brain regions have also been implicated in PTSD (see figure 2-5) (Lisieski *et al.*, 2018). For example, dysfunction of the habenula has been implicated in the pathophysiology of PTSD since the habenula is partially responsible for the modulation of fear behaviours in response to stress (Okamoto *et al.*, 2012). Moreover, the locus coeruleus (LC) has been linked to PTSD and in fact plays a role in mediating fear-related behavioural responses (Samuels and Szabadi, 2008). The LC is also involved in physiological functions such as arousal, startle response, cognition and stress reactivity (McCall *et al.*, 2015, Olson *et al.*, 2011, Snyder *et al.*, 2012). Increased noradrenergic output in PTSD patients further argues the involvement of LC dysfunction in PTSD, especially since the LC mediates NA release (Aston-Jones and Cohen, 2005, Lisieski *et al.*, 2018, Nieuwenhuis *et al.*, 2005). Furthermore, altered insula activity is involved in PTSD development, with insular functions including affective processing and interoception (Lisieski *et al.*, 2018, Stark *et al.*, 2015, Uddin *et al.*, 2014). Finally, neuro-imaging studies have shown that striatal dysfunction is related to affective and cognitive symptoms of PTSD, as well as reduced reward anticipation and hedonic responses (Felmingham *et al.*, 2014, Nawijn *et al.*, 2015). Striatal dysfunction also links PTSD with behaviours such as substance abuse, compulsions and risk-taking (Hsieh *et al.*, 2016, James *et al.*, 2014, Lisieski *et al.*, 2018).

Traumatic stress also significantly influences neuronal structures in the brain, resulting in pathological changes in the activity of these neurons and their dendritic spines (Lisieski *et al.*, 2018, Maras and Baram, 2012, Stewart *et al.*, 2014a, Yuste and Bonhoeffer, 2001). Reduced dendritic branching in the hippocampus has been identified in rodent models of PTSD, explaining reduced hippocampal volume in both pre-clinical and clinical PTSD studies (Golub *et al.*, 2011, Lisieski *et al.*, 2018, Woon *et al.*, 2010). Moreover, post-mortem PFC from PTSD patients display dysregulated cell survival and apoptosis genes, indicating abnormal apoptosis in individuals with PTSD (Lisieski *et al.*, 2018, Su *et al.*, 2008).

2.1.5.2.2. Neurochemistry of PTSD

2.1.5.2.2.1. Monoamines

Noradrenaline: The overall increase in peripheral SNS activity in PTSD patients is evidence of increased NA and adrenaline signalling (Hendrickson and Raskind, 2016). In this regard, hypocortisolism results in insufficient cortisol levels to suppress the sympathetic stress response caused by increased NA release (Oosthuizen *et al.*, 2005). The subsequent innervation of the hippocampus by afferent NA neurons from the amygdala is responsible for excessive long-term potentiation (LTP), consolidation and retrieval of emotional memories, and enhanced fear conditioning, via prolonged NA responses in the brain (Morris and Rao, 2013, Oosthuizen *et al.*, 2005, Pacak *et al.*, 1995). Accordingly, pre-clinical evidence from our laboratory has shown that NA levels in the hippocampus were raised 7 days after acute trauma in rodent models of PTSD (Harvey *et al.*, 2006). NA also plays a role in anxiety and high synaptic NA concentrations, in conjunction with hypersensitised adrenergic heteroreceptors, lead to high tonic firing of NA neurons in the LC (see figure 2-6), which subsequently generates PTSD symptoms such as hyperarousal, hypervigilance, intrusive symptoms, chronic fear, exaggerated startle response, increased avoidance of trauma-related cues and attention deficits, as well as anxiety-induced pupil dilation (Asalgoo *et al.*, 2015, Aston-Jones and Cohen, 2005, Blier, 2001, Cascardi *et al.*, 2015, Hendrickson and Raskind, 2016, Howells *et al.*, 2012, Michopoulos *et al.*, 2015, Pietrzak *et al.*, 2013, Southwick *et al.*, 1999). The mechanism for increased synaptic NA concentrations in PTSD patients is suggested to be a decrease in the NA transporter (NAT) density in the LC

(Hendrickson and Raskind, 2016). Moreover, the number of alleles of a single nucleotide polymorphism in the promotor region of the NA transporter gene is strongly associated with arousal symptoms of PTSD (Pietrzak *et al.*, 2015). Sustained heightened central nervous system (CNS) NA activity during rapid eye movement and deep sleep is also implicated in the pathophysiology of PTSD, with PTSD patients presenting with increased heart rate during sleep (Bertram *et al.*, 2014, Hendrickson and Raskind, 2016, Mellman *et al.*, 2004, Vanderheyden *et al.*, 2014). Finally, NA is involved in neuroinflammation, as evidenced by the association between NA and interleukin 6 signalling (Giustino *et al.*, 2016).

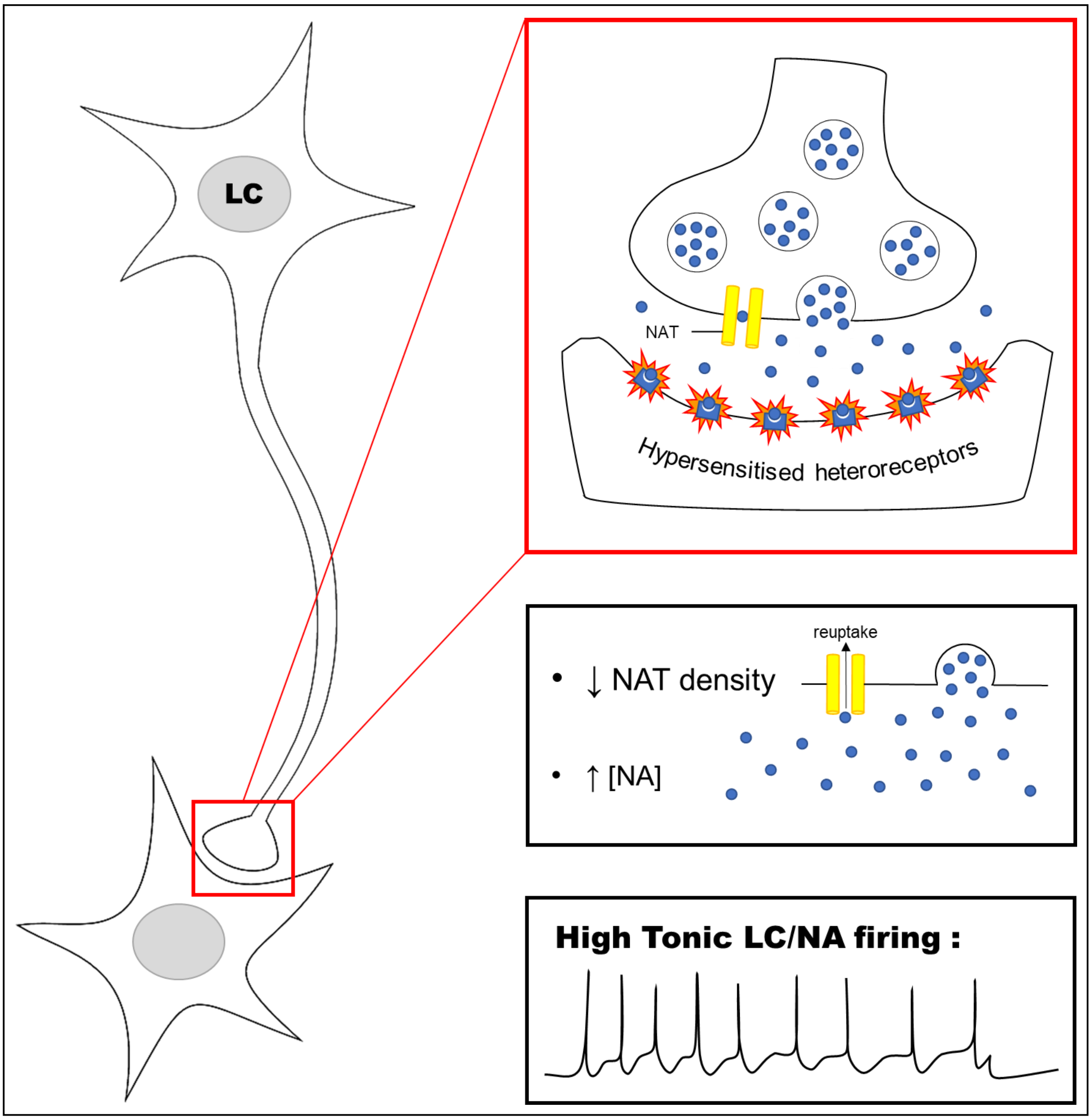

Figure 2-6: *High tonic firing of NA neurons in the LC (own figure based on the text).*

Adrenaline: As mentioned, adrenaline is released from the adrenal medulla after stress exposure to provide a rapid fight-or-flight response (Asalgoo *et al.*, 2015, Morris and Rao, 2013). Considering this, animal models of PTSD exhibit a trauma-induced increase in adrenaline levels (Martinho *et al.*, 2020). In clinical PTSD, memory recall of the trauma increases adrenaline levels, resulting in restlessness and insomnia (Asalgoo *et al.*, 2015). Furthermore, increased adrenaline levels cause substantial neurochemical and neurophysiological changes in the brain, which ultimately lead to a persistent sense of fear in PTSD patients (Asalgoo *et al.*, 2015). In this regard, high levels of adrenaline released during stress disturb the normal functioning of the hippocampus, meaning that traumatic memories cannot be correctly processed and will persist, which subsequently cause symptoms such as nightmares (Asalgoo *et al.*, 2015). Additionally, hypothalamic functions are supressed in response to high levels of SNS stress hormones (Asalgoo *et al.*, 2015).

Serotonin: Regarding serotonergic mechanisms implicated in PTSD, serotonin (5HT) is involved in the regulation of mood, anxiety, aggression and irritability; as well as emotional, social and reward-related behaviours (Asalgoo *et al.*, 2015, Lisieski *et al.*, 2018, Puglisi-Allegra and Andolina, 2015). Moreover, 5HT signalling interacts with cortisol and CRF signalling to regulate stress coping (Asalgoo *et al.*, 2015, Lisieski *et al.*, 2018, Puglisi-Allegra and Andolina, 2015). From this it follows that dysregulated 5HT release or signalling may explain many PTSD behavioural alterations (Lisieski *et al.*, 2018). In this regard, 5HT receptors are firmly implicated in memory and stress responses associated with PTSD, especially $5HT_{1A}$ and $5HT_{2C}$ receptors (Meneses, 2015, Wu *et al.*, 2017). Accordingly, $5HT_{1A}$ receptors are involved in contextual fear conditioning, with long-term stress exposure downregulating $5HT_{1A}$ receptors in animal models (Homberg, 2012, Stiedl *et al.*, 2000, Wu *et al.*, 2017). On the other hand, $5HT_{2C}$ receptors enhance glutamatergic neurotransmission and subsequently facilitate the consolidation and retrieval of fear memory (Homberg, 2012). Our laboratory has done substantial pre-clinical work regarding 5HT signalling in stress and restress models of PTSD, where rodents were exposed to acute stress and restressed 7 days later. The rodents in these experiments exhibited increased receptor density and decreased receptor affinity for HT_{1A} receptors and increased receptor affinity for $5HT_{2A}$ receptors on day 7 post acute stress (Harvey *et al.*, 2003). These changes were associated with deficits in spatial memory and depressed plasma corticosterone levels (Harvey *et al.*, 2003). Additionally, these experiments showed an early decrease in hippocampal 5HT levels following restress (Harvey *et al.*, 2006). This reiterates pre-clinical findings of reduced hippocampal 5HT in the rodent brain following chronic restraint stress (Torres *et al.*, 2002). Concerning the interaction between cortisol signalling and 5HT signalling, it is the belief that hypocortisolism in PTSD may underlie the decrease in hippocampal 5HT levels, which in turn leads to the expression of anxiety (Harvey *et al.*, 2006). Apropos of clinical PTSD, patients present with abnormally low 5HT levels (Asalgoo *et al.*, 2015). Furthermore, the serotonergic system is sensitised in PTSD patients (Hendrickson and Raskind, 2016). Interestingly, 5HT is both anxiogenic and anxiolytic, depending on the activation of different receptor-subtypes (Nowicki *et al.*, 2014, Zangrossi Jr and Graeff, 2014). For example, facilitation of $5HT_{1A}$ receptors has anxiolytic effects, while facilitation of $5HT_{2C}$ receptors augments anxiety. As such, $5HT_{2C}$ receptor antagonists have shown efficacy in animal models of anxiety (Alex and Pehek, 2007, Kennett *et al.*, 1997, Regenass *et al.*, 2018).

Dopamine (DA): DA plays a role in motivation, reward, affect, learning and movement, and is suggested to play a role in PTSD by mediating behaviours such as anhedonia, substance abuse, and fear learning and extinction (Abraham *et al.*, 2014, Lisieski *et al.*, 2018, Nawijn *et al.*, 2015, Norman *et al.*, 2012, Wise, 2004). Thereon, pre-clinical findings from our laboratory have shown that hippocampal concentrations of DA were raised 7 days after acute trauma in rodent models of PTSD (Harvey *et al.*, 2006). Clinical findings of DA dysfunction in PTSD include decreased cerebrospinal fluid concentrations of DA metabolites following reminders of the traumatic event, increased striatal DA transporter density, and polymorphisms in genes encoding DA transporters and receptors (Banerjee *et al.*, 2017, Geracioti Jr *et al.*, 2013, Hoexter *et al.*, 2012).

Importantly, monoamine oxidase (MAO) is a flavin-adenine dinucleotide enzyme with the function of breaking down monoamines, including DA, 5HT, NA and adrenaline, and thus the two mammalian isoenzymes, MAO-A and MAO-B, also serve as severity markers in PTSD (Bortolato and Shih, 2011, Perkovic *et al.*, 2016, Quadros *et al.*, 2018).

2.1.5.2.2.2. Acetylcholine and amino acid transmitters

In view of the fact that acetylcholine mediates excitatory (glutamate) and inhibitory (GABA, glycine) neurotransmission in the CNS (Lisieski *et al.*, 2018), acetylcholine neurotransmission presents a promising target for the treatment of both central and peripheral manifestations of PTSD (Lisieski *et al.*, 2018). As such, several behavioural symptoms of PTSD, such as dissociation, sleep disturbances, and disrupted fear learning and extinction processes, are linked to the imbalance of acetylcholine-mediated excitatory and inhibitory neurotransmission (Lisieski *et al.*, 2018, Meyerhoff *et al.*, 2014, Steckler and Risbrough, 2012).

Reduced $GABA_A$ receptor binding throughout the cerebral cortex, hippocampus and thalamus in PTSD supports that GABA receptors are dysregulated after traumatic stress (Geuze *et al.*, 2008, Lisieski *et al.*, 2018). It has also been demonstrated that both acute and chronic stress alter GABA signalling in PVN neurons of the Hypothal (Maguire, 2014). These changes in inhibitory neurotransmission contributes to dysregulated CRF secretion (Maguire, 2014). With regard to glutamate neurotransmission, increased peripheral glutamate triggers pro-inflammatory processes and excitotoxicity leading to reductions in synaptic connectivity in the hippocampus and PFC, brain areas critical to emotional regulation, fear conditioning, and stress response (Averill *et al.*, 2017). Thus, research on how inhibitory and excitatory neurotransmission are related to behavioural PTSD symptoms may present novel pharmacotherapeutic targets (Lisieski *et al.*, 2018). Of note, however, is that available evidence does not support the use of benzodiazepines, which are essentially GABA-mimetics, as treatments for PTSD (Bandelow *et al.*, 2008, Dell'osso and Lader, 2013). In fact, studies have shown that benzodiazepines are not only ineffective but possibly detrimental (Dell'osso and Lader, 2013). In this respect, early benzodiazepine treatment disrupts the normal HPA axis stress response, thereby increasing vulnerability to subsequent stress (Dell'osso and Lader, 2013, Matar *et al.*, 2009).

2.1.5.2.2.3. Neuropeptides

Various neuropeptides have been implicated in the stress response, including neuropeptide Y and oxytocin (Lisieski *et al.*, 2018). Neuropeptide Y is widely distributed throughout the brain and has been linked with arousal, fear conditioning and traumatic stress responses (Enman *et al.*, 2015b, Lisieski *et al.*, 2018, Verma *et al.*, 2009). In this regard, neuropeptide Y reduces the secretion of NA and thus lowers anxiety levels, while neuropeptide Y concentrations are decreased in PTSD patients (Asalgoo *et al.*, 2015, Skelton *et al.*, 2012). Additionally, polymorphisms in genes encoding neuropeptide Y and its receptors are implicated in PTSD (Lisieski *et al.*, 2018). Thus, the neuropeptide Y system is considered a promising pharmacotherapeutic target for PTSD (Enman *et al.*, 2015b).

Oxytocin contributes considerably to social behaviour and emotional regulation (Carter, 2014). As such, it is suggested that oxytocin may promote effective psychotherapy of PTSD (Koch *et al.*, 2014). It has also been observed that acute oxytocin administration in recently trauma-exposed individuals is effective as an early intervention to prevent the development of PTSD symptoms (Frijling, 2017). Furthermore, polymorphisms in oxytocin receptor genes contribute to susceptibility to PTSD development (Feldman *et al.*, 2014).

2.1.5.2.2.4. Cannabinoid system

The cannabinoid system is dysregulated in PTSD (Lisieski *et al.*, 2018). Endogenous cannabinoids such as anandamide and 2-arachidonoylglycerol are lipid-based neuromodulators that modulate the release and action of various neurotransmitters in different areas of the brain, this via retrograde signalling (Di Marzo *et al.*, 1998, Hanus *et al.*, 1993, Lisieski *et al.*, 2018). In other terms, endocannabinoids are synthesised in the postsynaptic neuronal compartment and act in a retrograde manner (reversely) across the synapse to bind to presynaptically expressed cannabinoid receptors (Younts and Castillo, 2014) (see figure 2-7). With the recent expeditious expansion of cannabinoid research and pharmacology, the cannabinoid system has become an opportune target for novel PTSD treatment (Lisieski *et al.*, 2018). Pre-clinical evidence shows that PTSD-like symptoms are associated with altered cannabinoid signalling (Lisieski *et al.*, 2018). Considering this, experiments on rodents found that cannabinoid signalling interacts with glucocorticoid signalling in the amygdala to modulate stress-induced intrusive memories (Ganon-

Elazar and Akirav, 2013, Ramot and Akirav, 2012). Moreover, clinical PTSD patients report greater cannabis use as a method of coping with PTSD symptoms (Bonn-Miller *et al.*, 2014).

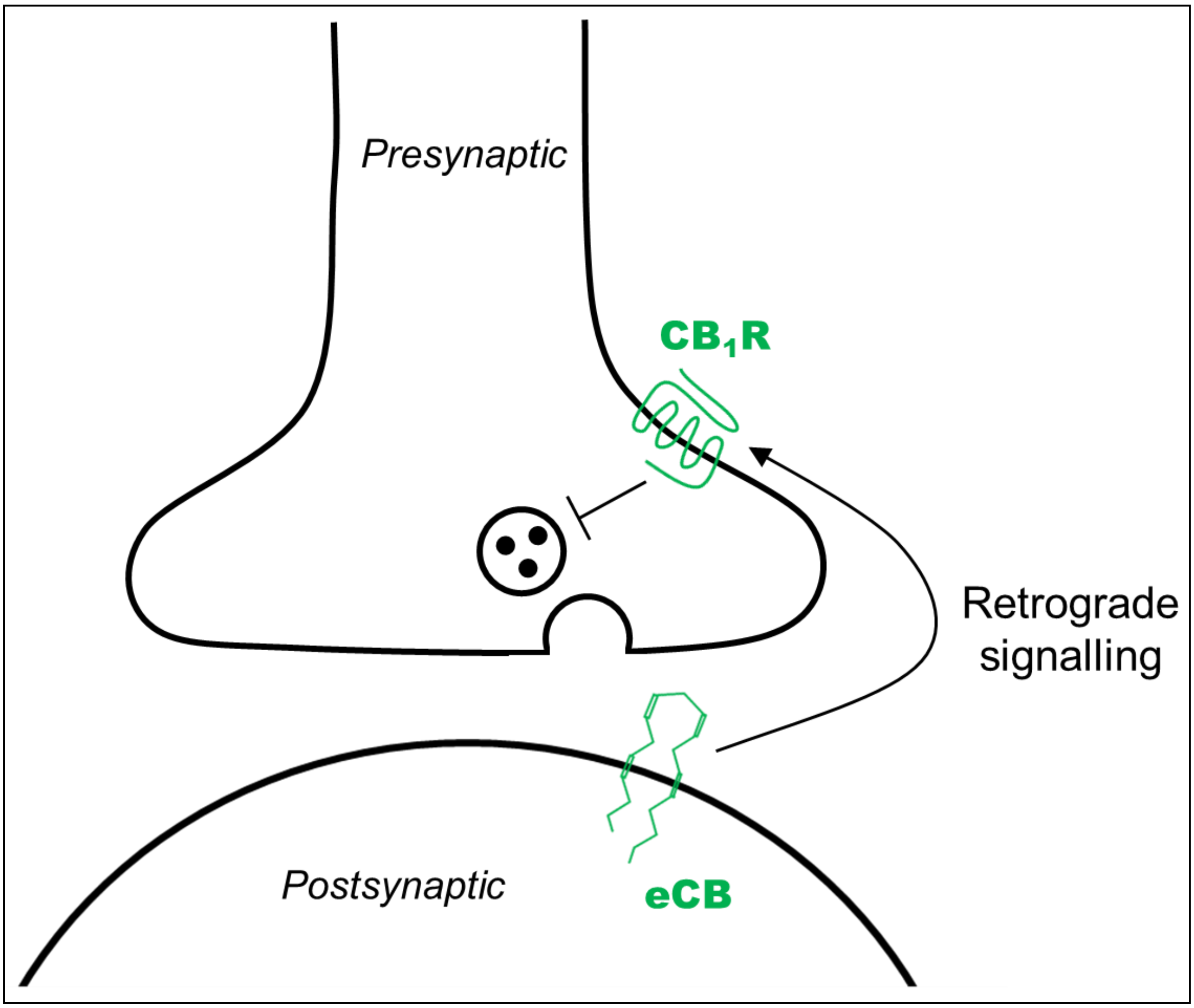

Figure 2-7: *Retrograde signalling by endogenous cannabinoids (adapted from Miller and Yeh (2017). CB_1R – cannabinoid type 1 receptor. eCB – endocannabinoid.*

2.1.5.2.3. Peripheral nervous system

Regarding the role of the SNS in the pathophysiology of PTSD, increased CRF levels in PTSD patients augment SNS activity, leading to increased fear and startle response (Asalgoo *et al.*, 2015, Kalin *et al.*, 2000, Hendrickson and Raskind, 2016). Increased peripheral SNS activity in PTSD patients is evidenced by increased basal heart rate and blood pressure (Hendrickson and Raskind, 2016, Pole, 2007). Additionally, greater rates of hypertension are reported in PTSD patients (Kibler *et al.*, 2009). Given the reciprocal inhibition between the SNS and parasympathetic nervous system (PSNS), parasympathetic activity is decreased in PTSD patients, as evidenced by attenuated baroreceptor reflex sensitivity and lower respiratory sinus arrythmia (Hendrickson and Raskind, 2016, Hughes *et al.*, 2006, Woodward *et al.*, 2009).

2.1.5.2.4. Inflammatory mediators

Cortisol plays a critical role in the regulation of inflammation and reducing immunopathology (Gola *et al.*, 2013). Given hypocortisolism in PTSD, it is not surprising that abnormalities in inflammation and immune activation are reported in PTSD patients (Gola *et al.*, 2013). Concomitant oxidative stress has also been implicated in a number of neuropsychiatric disorders (Salim, 2017). Thereon, the mechanism for oxidative stress in anxiety disorders such as PTSD involves psychological stress disrupting redox homeostasis in the brain, which leads to diminished antioxidant enzyme activity and increased oxidative stress (Salim, 2017). In this regard, oxidative stress alters normal cellular function, as well as neurotransmission (Hovatta *et al.*, 2010). Studies in animals have also demonstrated the role of NO mediated inflammation in PTSD (Harvey *et al.*, 2005, Harvey *et al.*, 2004b). In fact, increased cytokines up-regulate NO and reactive oxygen species production, which may cause cell death and tissue damage (Hu *et al.*, 1998, Wilson *et al.*, 2013). This subsequently leads to the production of more cytokines, ultimately resulting in a positive feedback loop (see figure 2-8) (Wilson *et al.*, 2013). As such, the possible neuroinflammatory component in PTSD is evident by elevated pro-inflammatory cytokines, such as interleukin 6, in the brain (Gola *et al.*, 2013, Wilson *et al.*, 2013). Moreover, it has been observed that pro-inflammatory biomarkers are significantly elevated in individuals with current symptomatic PTSD compared to those in recovery, indicating that psychological recovery from PTSD results in a return to baseline levels.

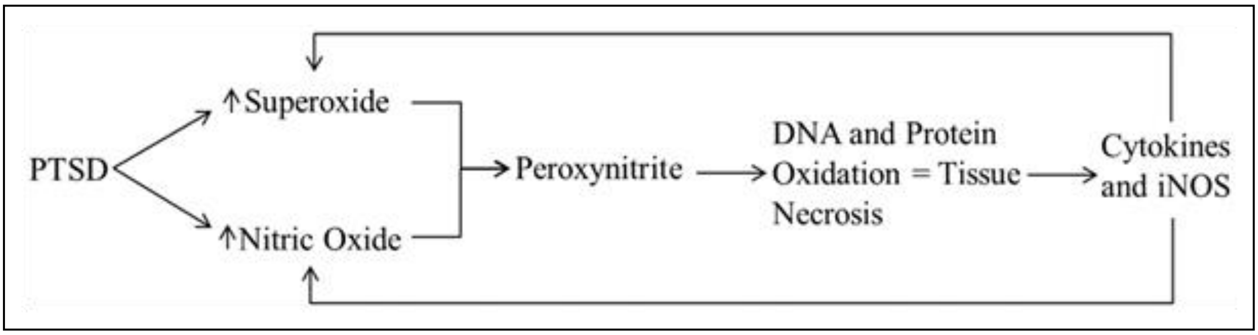

Figure 2-8: *Positive feedback loop of pro-inflammatory cytokine production in PTSD (Wilson et al., 2013). DNA - deoxyribonucleic acid. iNOS – inducible NO synthase.*

2.1.5.2.5. Circadian rhythms

Evidence for altered circadian rhythm in PTSD has also been demonstrated and as such circadian clock genes may represent genetic risk factors in PTSD (Dell'Osso *et al.*, 2014, Landgraf *et al.*, 2014, Morris *et al.*, 2012, Thompson *et al.*, 2014). In line with disrupted circadian rhythms, PTSD is characterised by sleep disturbances (Dell'Osso *et al.*, 2014, Germain *et al.*, 2005) as well as altered circadian hormones, such as melatonin (Feldman *et al.*, 2014, Gannon, 2014, Kalsbeek *et al.*, 2010, Ordyan *et al.*, 2016, Ramirez *et al.*, 2017, Seyffert and Gettys, 2013). Similarly, monoamines such as 5HT, NA and DA, and neuropeptides such as vasopressin and oxytocin, are also subject to circadian rhythms (Albrecht and Stork, 2017, Baratta *et al.*, 2016, Hendrickson and Raskind, 2016, Kawai *et al.*, 2018). Circadian rhythms are predominantly regulated by a dual action involving $5HT_{2C}$ and melatonin 1 and 2 receptors located in the retina, cerebellum, hippocampus and suprachiasmatic nucleus of the hypothalamus (Dubocovich *et al.*, 2003, Gahr, 2014, Zlotos, 2005). A recent study in an animal model of PTSD has demonstrated that circadian related genes play a role in the neurobiological response to emotional trauma (Koresh *et al.*, 2012). Importantly, resynchronisation of these responses is possible with agomelatine (AGM), a dual $5HT_{2C}$ receptor antagonist and melatonin 1 and 2 receptor agonist (Koresh *et al.*, 2012). Another circadian biomarker of PTSD, salivary alpha amylase, is correlated with PTSD symptom severity, particularly hyperarousal and intrusive symptoms (Hendrickson and Raskind, 2016, Keeshin *et al.*, 2015). Finally, worth noting is that circadian clock deviations result in dysregulation of the HPA axis and disrupted cortisol release (Landgraf *et al.*, 2014, Pinna *et al.*, 2014).

2.1.6. Time-dependant sensitisation (TDS)

2.1.6.1. The TDS concept

It is broadly suggested that PTSD patients present with abnormal fear learning processes culminating in dysregulated fear responses to perceived threats (Lisieski *et al.*, 2018, Parsons and Ressler, 2013). However, further study is required to determine what factors predispose trauma-exposed individuals to maladaptive disruptions of fear learning (Lisieski *et al.*, 2018) and how the condition perpetuates and worsens over time in the *absence* of trauma. It is somehow the sensory and contextual cues of the original trauma that, if severe enough and if susceptibility exists, prompts the illness to a worse prognosis over time. In this regard, time-dependent sensitisation (TDS) can serve as a robust pre-clinical translational model to assess the temporal behavioural and neurobiological adaptations involved in fear learning and memory abnormalities that contribute to PTSD (Bowers and Ressler, 2015, Lisieski *et al.*, 2018, Souza *et al.*, 2017).

While the concept of TDS is observable in humans with PTSD, it is not easily studied or quantifiable. However, TDS is a well-validated stress-restress animal model of PTSD implementing an initial severe (life-threatening) trauma followed by repeated contextual reminders of the original stress exposure to promote long-term changes in the CNS, which induces PTSD-like alterations in locomotor activity and behaviour over time (Harvey *et al.*, 2004b, Uys *et al.*, 2003, Wright *et al.*, 2013). Restress can be equated to reliving past traumatic events in the form of flashbacks, causing the re-release of cortisol, which is then often associated with adrenal insufficiency as the HPA axis fails to adapt (Harvey *et al.*, 2006, Harvey *et al.*, 2003, Van der Kolk, 1994). TDS evokes escalating fear responsiveness and cognitive deficits via the sensitisation of neurobiological mechanisms and is characterised by enhanced negative feedback inhibition emerging after a consolidation period following stress exposure (Harvey *et al.*, 2003, Lisieski *et al.*, 2018). The inhibition of the HPA axis triggered by TDS subsequently results in hypocortisolism

(Harvey *et al.*, 2003). Importantly, the altered neuroendocrine stress response in PTSD patients, as well as contextual reminders, are associated with ephemeral changes in the HPA axis and regional brain monoamines resulting in escalating behavioural symptoms over time, such as anxiety and memory deficits, ultimately causing long-term dysfunction (Harvey *et al.*, 2006, Lisieski *et al.*, 2018, O'Donnell *et al.*, 2007, Yehuda *et al.*, 2006). Therefore, the specificity of the neurobiological phenotypes and the time-dependant nature of TDS-induced alterations relates the TDS model to the phenomenology and clinical presentation of PTSD (Lisieski *et al.*, 2018, Yamamoto *et al.*, 2009).

2.1.6.2. The behavioural manifestations of TDS

Several symptoms defined as diagnostic criteria for PTSD by the DSM-5 have been extensively studied in animal models of TDS, including exaggerated startle response, sleep disturbances and cognitive alterations (American Psychiatric Association, 2013, Lisieski *et al.*, 2018). In this regard, startle response is used to index anxiety and hypervigilance in pre-clinical models, and to indicate sensitivity to perceived threats (Grillon, 2002, Lisieski *et al.*, 2018). Startle response has been confirmed to be elevated in the TDS model and has been used for the evaluation of PTSD treatment in reducing TDS effects (Berlant, 2006, Khan and Liberzon, 2004, Serova *et al.*, 2013). Moreover, sleep disruptions in the TDS model are indicated by increased wakefulness and rapid eye movement sleep (Nedelcovych *et al.*, 2015, Vanderheyden *et al.*, 2013). These sleep abnormalities significantly correlate with impaired fear retention following TDS stress (Vanderheyden *et al.*, 2015). Furthermore, TDS induces cognitive alterations, including attention, memory and behavioural flexibility impairments (Harvey *et al.*, 2003, Lisieski *et al.*, 2018, Piao *et al.*, 2017). This suggests that TDS alters LC and PFC activity, as these brain regions are involved in the aforementioned cognitive processes (Aston-Jones and Cohen, 2005, George *et al.*, 2013, Knox *et al.*, 2016, Logue and Gould, 2014).

Other characteristics of PTSD, including enhanced anxiety-like behaviour in response to trauma-associated cues, have been reported in the TDS model (Harvey *et al.*, 2006, Lee *et al.*, 2016a, Toledano and Gisquet-Verrier, 2014, Yehuda *et al.*, 2001). Additionally, TDS stress has also been shown to result in affect and reward dysfunction, including decreased sucrose preference, as well as increased learned helplessness to trauma-associated cues (Enman *et al.*, 2015a, Le Dorze and Gisquet-Verrier, 2016, Lisieski *et al.*, 2018).

TDS studies used to examine pre-existing risk factors indicate that increased initial anxiety prior to stress exposure predicts a larger response to trauma-associated cues after stress exposure (Lisieski *et al.*, 2018). Additionally, individual differences in vulnerability to trauma are evaluated by sorting TDS-exposed animals into susceptible and resilient groups using applicable behavioural assays (Lisieski *et al.*, 2018). However, longitudinal studies are necessary to determine the inter-individual differences in traumatic stress response between susceptible and resilient animals, as well as the neurobiological mechanisms underlying these differences (Lisieski *et al.*, 2018).

TDS can further be used to assess the effects of sex, age and life history on traumatic stress response (Lisieski *et al.*, 2018). In this regard, it has been demonstrated that TDS stress induces less fear extinction retention deficits in female rodents than in male rodents, though it does alter GR density in the posterior hippocampus of both female and male rodents (Keller *et al.*, 2015). Furthermore, neonatal isolation has been shown to increase anxiety and contextual fear in animals subjected to TDS stress later in life (Imanaka *et al.*, 2006). However, further research investigating the effects of age, sex and life history on neurobiological alterations post-TDS is advocated (Lisieski *et al.*, 2018).

TDS has also been used to study PTSD comorbidities, including substance use disorder and chronic pain (Lisieski *et al.*, 2018), as well as treatment resistant depression (Brand and Harvey, 2017a, b). To examine the neurobiological link between PTSD and substance use disorder, studies have combined the TDS model with drug sensitisation models (Lisieski *et al.*, 2018). In this regard, TDS stress altered drug sensitisation to various abuse substances (Lisieski *et al.*, 2018). The relationship between TDS and substance use disorder has subsequently been attributed to noradrenergic, glutamatergic, dopaminergic and cannabinoid alterations in the striatum (Enman *et al.*, 2015a, Matchynski-Franks *et al.*, 2016, Toledano *et al.*, 2013). Regarding chronic pain, TDS stress causes long-lasting hyperalgesia through mechanisms such as increased LC reactivity to aversive stimuli (George *et al.*, 2013, He *et al.*, 2013, Lisieski *et al.*, 2018).

2.1.6.3. The neurobiology of TDS

The effects of TDS stress on cellular alterations in the brain have been increasingly investigated (Lisieski *et al.*, 2018). TDS studies used to analyse structural changes in neurons have found dendritic hypertrophy in the amygdala, as well as neuronal damage in the amygdala and hippocampus, with these changes being implicated in fear conditioning and somatic alterations, respectively (Cui *et al.*, 2008, Han *et al.*, 2015a, Lisieski *et al.*, 2018). Moreover, there have been several reports of changes in apoptosis biomarkers in the brain tissue of TDS-exposed animals (Lisieski *et al.*, 2018). Considering this, apoptosis may be the cause of hippocampal volume reduction in TDS models (Lisieski *et al.*, 2018). Alternatively, a reduction in biomarkers for axonal growth has also been exhibited in TDS rodents, suggesting that axonal size shrinkage may underlie the loss in hippocampal volume (Golub *et al.*, 2011). More research on cell cycle regulation, neurogenesis and apoptosis post-TDS is needed to determine the role of these processes on behavioural alterations, as well as how it is linked to human neurobiology (Lisieski *et al.*, 2018). Additionally, the regional specificity of TDS-induced neuronal apoptosis requires further examination (Lisieski *et al.*, 2018).

TDS studies have additionally been used to link stress-induced behaviour to neurobiological alterations in HPA axis function, as well as GR and MR density in the brain (Lisieski *et al.*, 2018). In this regard, TDS stress enhances rapid negative feedback inhibition of corticosterone in rodents, corresponding with negative feedback inhibition of cortisol documented in clinical PTSD (Ganon-Elazar and Akirav, 2012, Harvey *et al.*, 2006, Perrine *et al.*, 2016). TDS stress has also been demonstrated to upregulate GR in brain regions involved in emotion, arousal and memory, including the hippocampus, PFC and LC (Eagle *et al.*, 2013, George *et al.*, 2015, Li *et al.*, 2011). On the other hand, TDS stress appears to downregulate MR in the hippocampus and LC (Li *et al.*, 2011, Liberzon *et al.*, 1999). Therefore, the altered GR:MR ratio contributes to the neuroendocrine dysfunction following TDS stress (Lisieski *et al.*, 2018). Accordingly, GR antagonists prevent the potentiation of contextual fear induced by TDS (Kohda *et al.*, 2007). Nevertheless, corticosteroid receptor density in the HPA axis and limbic system of TDS-exposed animals require further examination (Lisieski *et al.*, 2018).

Brand *et al.* (2008) described increased cortical and hippocampal muscarinic receptor density 7 days after TDS, although receptor affinity remained unaltered. They concluded that cholinergic pathways may mediate especially associative memory in PTSD (Brand *et al.*, 2008). Considering this, nicotinic acetylcholine receptor agonism reverses hyperalgesia produced by TDS stress (Sun *et al.*, 2017). Moreover, as acetylcholine plays a role in excitatory and inhibitory neurotransmission (Lisieski *et al.*, 2018), this may have relevance for increased glutamate levels in the cerebrospinal fluid of patients with PTSD (Feng *et al.*, 2015).

Regarding amino acid transmission, decreased hippocampal GABA levels have been reported in rats subjected to TDS (Harvey *et al.*, 2004b), supporting the idea of loss of inhibitory neurotransmission in the brain. Additionally, contextual fear conditioning in the TDS model triggered a significant upregulation in hippocampal glycine transporter density, along with decreased extracellular glycine levels in the hippocampus (Iwamoto *et al.*, 2007, Yamamoto *et al.*, 2010). Simultaneously, TDS has also been found to reduce glutamate NMDA receptor density in the hippocampus of TDS rats, suggesting increased excitatory glutamatergic activity in this brain region (Harvey et al., 2004), which the authors suggest underlies increased excitotoxic damage and atrophy of the hippocampus in PTSD. In support of this, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, topiramate, which demonstrates potent anti-kindling effects, abrogated single prolonged stress-induced startle responses in rats (Khan and Liberzon, 2004).

As has been alluded to earlier, the monoamines NA, 5HT and DA are widely regarded to play a major role in the pattern of behavioural manifestations following acute and chronic stress. NA, 5HT and DA are severely disrupted by TDS stress (Lisieski *et al.*, 2018). In this regard, TDS stress dysregulates 5HT signalling and receptor density across various brain regions (Lisieski *et al.*, 2018). Importantly, our group earlier noted that acute severe emotional trauma and subsequent contextual reminders (i.e. stress/restress) engender various cortisol and regional brain monoamine responses over time, that in turn diversely impact aversive behaviour (Harvey *et al.*, 2006). In an earlier study, our group found that TDS stress induces dysregulation of 5HT pathways important in memory and emotion, such as increased density of and reduced ligand affinity for hippocampal $5HT_{1A}$ receptors and elevated ligand affinity for PFC $5HT_{2A}$ receptors, with evidence for a contributory role in the cognitive deficits of PTSD (Harvey *et al.*, 2003). Moreover, TDS stress attenuates the density of hippocampal $5HT_3$ receptors, which in turn reduces neurogenesis and antidepressant effects of $5HT_3$ receptors (Kondo *et al.*, 2015, Lisieski *et al.*, 2018). In keeping with this, $5HT_3$ receptor agonist administration immediately following TDS stress is reported to promote contextual fear extinction (Wu *et al.*, 2017). Furthermore, various studies demonstrate that selective 5HT reuptake inhibitors (SSRI’s; including FLX, paroxetine and escitalopram) improve the neurobiological and behavioural alterations induced by TDS (Han *et al.*, 2015b, Lin *et al.*, 2016, Lisieski *et al.*, 2018, Perrine *et al.*, 2016), while depleting 5HT has the opposite effect (Harvey *et al.*, 2004a). Decreased DA levels and D_2 receptor density in the striatum have also been observed following TDS stress, along with corresponding increased striatal levels of DA transporter density (Enman *et al.*, 2015a). TDS stress may also impair fear extinction learning by decreasing synaptic DA in the PFC (Gilmartin *et al.*, 2014, Lin *et al.*, 2016). However, further research into the mechanisms of TDS-induced dopaminergic alterations leading to affective and motivational symptoms of PTSD, as well as substance use disorder comorbidity associated with these symptoms, is advocated and may present a promising target for novel PTSD therapeutics (Lisieski *et al.*, 2018).

Cannabinoid receptor agonism has been demonstrated to prevent contextual fear extinction deficits induced by TDS (Ganon-Elazar and Akirav, 2013). As such, additional examination of the cannabinoid system using the TDS model is necessary to explore the appropriateness of the cannabinoid system as a potential pharmacotherapeutic target for PTSD, as well as to explain the prevalence of cannabis use and abuse among PTSD patients (Lisieski *et al.*, 2018).

Animal models of TDS indicate that neuropeptide Y interacts with monoamines and the HPA axis to induce behavioural alterations (Lisieski *et al.*, 2018). TDS triggers downregulation of neuropeptide Y receptor density and upregulation of CRF receptor density in the LC, as well as elevated CRF levels (Sabban *et al.*, 2015a). These alterations, along with TDS-induced increase of hippocampal GR and the behavioural resulting alterations, can be prevented by administering neuropeptide Y immediately after stress exposure (Laukova *et al.*, 2014, Lisieski *et al.*, 2018, Sabban *et al.*, 2015b). Moreover, TDS studies have also demonstrated that TDS triggers increased oxytocin receptor density in the amygdala, Hypothal and hippocampus (Lisieski *et al.*, 2018). In this regard, further investigation of the mechanisms involved in TDS-induced oxytocinergic alterations may assist in determining the potential of oxytocin administration as an additive treatment of PTSD (Lisieski *et al.*, 2018).

Finally, since GR signalling has a regulatory role in immune responses, it is noteworthy that the abovementioned GR alterations lead to abnormalities in inflammation (Silverman and Sternberg, 2012). Accordingly, TDS enhances inflammation in the hippocampus, as evidenced by elevated interleukin 6 levels and inducible NO synthase (NOS) expression (Lisieski *et al.*, 2018, Liu *et al.*, 2016, Peng *et al.*, 2013). Interestingly, there appears to be temporal conversion of NO production over time from constitutive NOS (acute stress) to inducible NOS (chronic stress) activation (Harvey *et al.*, 2004b), thus describing PTSD as a progressive inflammatory condition (Oosthuizen *et al.*, 2005). Treatment with the non-steroidal anti-inflammatory drug, ibuprofen, has been effective in reversing these TDS-induced increases in hippocampal pro-inflammatory biomarkers (Lee *et al.*, 2016b), while chronic elevated NOS activity is reversed by blocking glucocorticoid synthesis (Harvey *et al.*, 2004b).

2.1.7. Fear conditioning

Pavlovian conditional procedures are used to study associative learning in both human and animal models (Blaser and Vira, 2014). In this regard, an essential component of PTSD, and indeed TDS, is fear conditioning, a form of Pavlovian conditioning where a neutral conditioned stimulus is paired with an aversive unconditioned stimulus (Uys *et al.*, 2003).

After repeated pairings, animals learn that the conditioned stimulus predicts the aversive stimulus (Maren, 2001, Uys *et al.*, 2003). The conditioned stimulus then evokes a conditioned fear response in the *absence* of the aversive stimulus, engendering fear behaviours such as freezing and autonomic alterations such as increased respiration and heart rate (Maren, 2001, Uys *et al.*, 2003). As mentioned (see section 2.1.1), the hippocampus plays a prominent role in conditioning fear memories to context (Corcoran and Maren, 2001). This fear conditioning process consists of five phases: 1) the acquisition of a fear memory; 2) the expression of conditioned fear after a consolidation period; 3) the extinction of the conditioned fear response; 4) the retrieval of this extinction; and finally, 5) the reactivation of an extinguished fear response through spontaneous recovery, reinstatement and renewal (Lisieski *et al.*, 2018).

Fear conditioning can be divided into two categories: 1) trauma-cue fear conditioning and 2) *de novo* fear conditioning (Lisieski *et al.*, 2018). Trauma-cue fear conditioning refers to a neutral stimulus paired with the initial trauma being used to reactivate the traumatic memories (Lisieski *et al.*, 2018). Accordingly, PTSD is often characterised by hyperresponsivity to and avoidance of cues related to the traumatic stressor (American Psychiatric Association, 2013). In the context of trauma-cue fear conditioning in rodents, single prolonged stress, an animal model of PTSD similar to TDS but without reminders, enhances associative sensory aversion learning 7 days after stressor-taste pairing, this with and without re-stress (Brand *et al.*, 2008). This demonstrates that sensory experience associated with the initial trauma may perpetuate the disorder in the absence of the original emotional event, not unlike that in PTSD. Additionally, trauma-cue fear conditioning has also been widely researched in humans and has consequently been proposed as a potential biomarker to predict and index treatment outcomes in PTSD patients (Blanchard *et al.*, 2002, Lisieski *et al.*, 2018, Norrholm *et al.*, 2016, Shvil *et al.*, 2013).

De novo fear conditioning, on the other hand, refers to a neutral stimulus being paired with a novel aversive stimulus, with this process taking place after the initial trauma exposure (Lisieski *et al.*, 2018). In this regard, fear extinction and its retention are impaired in PTSD, possibly due to trauma-induced neurobiological alterations or pre-existing memory dysfunction that predispose individuals to PTSD development following trauma exposure (Guthrie and Bryant, 2006, Lisieski *et al.*, 2018, Milad *et al.*, 2008, Milad *et al.*, 2009). Additionally, *de novo* fear conditioning has been linked to hippocampal GR up-regulation, as well as hyporesponsivity of the PFC during fear extinction recall (Knox *et al.*, 2012, Knox *et al.*, 2016, Lisieski *et al.*, 2018). In the context of *de novo* fear conditioning in rodents, single prolonged stress disrupts extinction retrieval in rodents trained to associate auditory, visual and olfactory cues or environmental contexts with foot shock (Eskandarian *et al.*, 2013, Lin *et al.*, 2016, Perrine *et al.*, 2016). Similarly, in PTSD the contextual processing of aversive stimuli is impaired, leading to a reduced ability to learn and respond to safety signals during the fear extinction phase, as well as overgeneralisation of conditioned fear (Lisieski *et al.*, 2018). This was demonstrated in a clinical fear conditioning study where PTSD patients could not appropriately use contextual cues to differentiate between aversive and safe conditions (Garfinkel *et al.*, 2014). Furthermore, the ability of PTSD patients to distinguish between threat- and safety-related stimuli pre-treatment may predict their treatment responsiveness (Aikins *et al.*, 2011).

2.1.8. Treatment of PTSD

PTSD is often treated with a combination of both non-pharmacological and pharmacological treatments. Non-pharmacological treatments (psychotherapy) of PTSD include cognitive behavioural therapy and eye movement desensitisation and reprocessing therapy (Caramillo *et al.*, 2015). Cognitive behavioural therapy mimics features of fear extinction in order to change the manner in which PTSD patients think of traumatic memories to subsequently reduce the stress caused by these memories and to make these memories more manageable (Asalgoo *et al.*, 2015, Giustino *et al.*, 2016). Eye movement desensitisation and reprocessing therapy refers to a technique which uses recordings of eye movements to assist the brain in processing traumatic memories (Asalgoo *et al.*, 2015). However, psychotherapy is more useful as a preventative intervention given soon after trauma and is often unsuccessful over the long-term if PTSD symptoms persists for longer than a month (Asalgoo *et al.*, 2015, Giustino *et al.*, 2016).

Adjunctive to non-pharmacological treatment, the recommended first line pharmacological treatment for PTSD are SSRI's, including fluoxetine (FLX), paroxetine and sertraline, with their mechanism of action in PTSD treatment possibly related to the serotonergic regulation of the HPA axis (Baldwin *et al.*, 2014, Caramillo *et al.*, 2015, Lanfumey *et al.*, 2008). That said, the dorsal raphe nucleus, which is the main source of 5HT in the brain, is probably the primary target for SSRI treatment in PTSD (Lisieski *et al.*, 2018) These antidepressants are generally well-tolerated, with chronic treatment necessary to reach clinical improvement (Bernardy and Friedman, 2015). Thereafter, sustained treatment is obligatory, with discontinuation associated with relapse of PTSD symptoms (Martenyi *et al.*, 2002). The efficacy of dual 5HT and NA reuptake inhibitors (SNRI's) like venlafaxine as first line treatment for PTSD has also been confirmed (Bernardy and Friedman, 2015, Poundja *et al.*, 2012). It must be emphasized, however, that SSRI's and SNRIs only have moderate efficacy in the treatment of PTSD symptoms, highlighting the need for improved understanding of the underlying neurobiology of PTSD (Caramillo *et al.*, 2015, Lisieski *et al.*, 2018), and a need for new treatments.

Prior to the increase in the prescribing frequency of SSRI's and venlafaxine for PTSD treatment, tricyclic antidepressants (including imipramine, amitriptyline and desipramine) and MAO inhibitors (including phenelzine) were the recommended pharmacotherapy for PTSD (Bernardy and Friedman, 2015). These drugs continue to be prescribed as second line treatments, particularly in the treatment of PTSD patients with comorbid major depressive disorder (Albucher and Liberzon, 2002, Belkin and Schwartz, 2015, Bernardy and Friedman, 2015, Forbes *et al.*, 2010). Moreover, tricyclic antidepressants have shown efficacy in the treatment of PTSD patients with comorbid alcohol use disorder (Bernardy and Friedman, 2015, Petrakis *et al.*, 2012). The side effect profile of tricyclic antidepressants and MAO inhibitors are more complex than that of SSRI's, though and, thus require closer monitoring (Bernardy and Friedman, 2015). Additionally, pharmacotherapies that diminish hyperactive NA neurotransmission have shown success in the treatment of PTSD, including prazosin (alpha-1 heteroreceptor antagonist), clonidine and guanfacine (alpha-2 autoreceptor agonists), and propranolol (non-selective beta-antagonist) (Albucher and Liberzon, 2002, Belkin and Schwartz, 2015, Bernardy and Friedman, 2015, Connor *et al.*, 2013, Feder *et al.*, 2014, Giustino *et al.*, 2016, Hendrickson and Raskind, 2016, Henry *et al.*, 2007, Poundja *et al.*, 2012, Raskind *et al.*, 2003). Propranolol, however, is arguably most effective as a prophylactic measure immediately post-trauma when psychological stress is high, especially in conjunction with behavioural therapy, acting to facilitate immediate fear extinction and reduce fear throughout the extinction learning process (Giustino *et al.*, 2016, Henry *et al.*, 2007). It may also be possible that propranolol treatment immediately post-trauma results in weaker fear memories through interference with fear memory consolidation or decreased reactivation of abated fear response post-extinction (Giustino *et al.*, 2016). Alternatively, PTSD patients may benefit from treatment with pharmacotherapies that increase NA neurotransmission, including yohimbine (alpha-2 autoreceptor antagonist) (Giustino *et al.*, 2016, Morris and Bouton, 2007, Wangelin *et al.*, 2013). This paradox fits earlier animal work that describes the time-dependent change in monoamine involvement post-trauma (Harvey *et al.*, 2006).

Therefore, despite the clear evidence for the role of monoamines in PTSD, the use of monoamine active antidepressants, be it SSRI's, SNRI's, etc., is suboptimal, highlighting the causal role of other neuro-molecular pathways that work in concert with the monoamines. In fact, monotherapy with currently available PTSD medicine is particularly ineffective in PTSD patients with comorbidities, so that effective treatment needs to address PTSD and comorbidities (Bernardy and Friedman, 2015). Accordingly, anticonvulsants (including topiramate) are effective in reducing the symptoms of both PTSD and comorbid alcohol use disorder in PTSD patients (Albucher and Liberzon, 2002, Batki *et al.*, 2014, Belkin and Schwartz, 2015, Bernardy and Friedman, 2015). Furthermore, in addition to being effective in the treatment of PTSD, guanfacine has displayed great efficacy in relieving the symptoms of substance use disorder (Bernardy and Friedman, 2015, Fox *et al.*, 2012). Prazosin has also been shown to decrease the symptom severity of comorbid alcohol use disorder and sleep disturbances, while atypical antipsychotics (including risperidone and olanzapine) are also recommended adjunctive pharmacotherapies for sleep disturbances (Albucher and Liberzon, 2002, Belkin and Schwartz, 2015, Bernardy and Friedman, 2015, Feder *et al.*, 2014). Sleep disturbances are particularly resistant to psycho- and pharmacotherapy, and needs to be addressed comprehensively (Nappi *et al.*, 2012, Zayfert and DeViva, 2004). In this regard, exploring the role of altered circadian rhythms in PTSD seems relevant, as well as the therapeutic value of the circadian rhythm regulator, AGM (De Berardis *et al.*, 2013, De Berardis *et al.*, 2012). Other pharmacotherapies with some efficacy in the improvement of PTSD symptoms include glutamatergic antagonists (including ketamine), psychostimulants, cannabinoids, $GABA_B$ agonists, non-steroidal anti-inflammatory drugs, hydrocortisone, hydroxyzine, neuropeptide Y and oxytocin (Abrams *et al.*, 2013, Albucher and Liberzon, 2002, Belkin and Schwartz, 2015, Bernardy and Friedman, 2015, Feder *et al.*, 2014, Frijling, 2017, Ganon-Elazar and Akirav, 2012, Gao *et al.*, 2009, Hendrickson and Raskind, 2016, Raskind *et al.*, 2003, Schelling *et al.*, 2001, Serova *et al.*, 2013, Toledano and Gisquet-Verrier, 2014). Adjunctive treatment with natural herbal products such as saffron, which has shown efficacy in rodent models (Asalgoo *et al.*, 2015), may also be of value (Asalgoo *et al.*, 2015). However, before establishing the need for adjunctive treatments it is imperative that clinicians consider the time required to achieve a positive treatment response, as well as if the patient is compliant with treatment (Bernardy and Friedman, 2015).

Trauma-exposed individuals are differential in their response to treatment (Lisieski *et al.*, 2018). Various factors play a role in predicting treatment response, including severity of the stressful event, chronicity, symptom severity, previous treatments, time since trauma, childhood abuse, comorbidities, suicidality and resilience (Bernardy and Friedman, 2015, Laddis, 2011). In this regard, chronicity of PTSD, childhood abuse, comorbid major depressive disorder and suicidal urges all predict a poor treatment response in PTSD patients (Bernardy and Friedman, 2015, Laddis, 2011). PTSD patients with low symptom severity are more likely to achieve a greater treatment response (Bernardy and Friedman, 2015, Davidson *et al.*, 2012). Moreover, PTSD patients who have experienced a traumatic event recently are more responsive to treatment than those whose trauma occurred earlier (Bernardy and Friedman, 2015). A greater baseline resilience in PTSD patients also increases the likelihood of a positive treatment response (Davidson *et al.*, 2012).

2.2. Zebrafish

Although rodent models of PTSD have proved valid translational models for exploratory PTSD research, they are hampered by high cost and low throughput (Freudenberg *et al.*, 2018, Kafkafi *et al.*, 2018, Planchart *et al.*, 2016). However, zebrafish (*Danio rerio*) emerge as a useful model of PTSD owing to high homology in neuronal mechanisms and mediator systems with rodents and humans, especially that pertaining to the stress response (Kaslin and Panula, 2001, Stewart *et al.*, 2014c).

Zebrafish were first classified by a Scottish physician, Francis Hamilton, under the subgenus *Danio Cyprini* as the species *Cyprinus rerio* (Hamilton, 1822). This Latin name has undergone several changes and zebrafish are now referred to as *Danio rerio* (Parichy, 2015). Hamilton described zebrafish as beautiful fish with alternating blue and silver stripes lengthwise on each side (Hamilton, 1822). In the late 1960s, the father of modern zebrafish research, George Streisinger, chose to work on zebrafish because this species was readily available, easy to breed, and the transparent embryo was quick to develop (Chitramuthu, 2013, Parichy, 2015).

2.2.1. Zebrafish ecology

Zebrafish are a floodplain (low-lying water bodies) species, rather than a riverine species, and typically inhabit slow moving streams, shallow ponds, standing bodies of water, and still pools that form on the edges of streams during the monsoon season (Bhat *et al.*, 2015, Lawrence, 2007, Parichy, 2015, Spence *et al.*, 2008). Given that they co-occur with humans, zebrafish also inhabit rice fields, draining ditches and stock ponds (Parichy, 2015). Zebrafish are native to South Asia, naturally residing in India, Nepal, Bangladesh, and as far as Pakistan and Myanmar (see figure 2-9) (Arunachalam *et al.*, 2013, Spence *et al.*, 2008, Stewart *et al.*, 2014a). Additionally, zebrafish are resilient animals and have been reported in a range of conditions, including temperatures between 12ºC and 39ºC and pH levels of 5.9 to 9.8 (Arunachalam *et al.*, 2013, Engeszer *et al.*, 2007, Parichy, 2015).

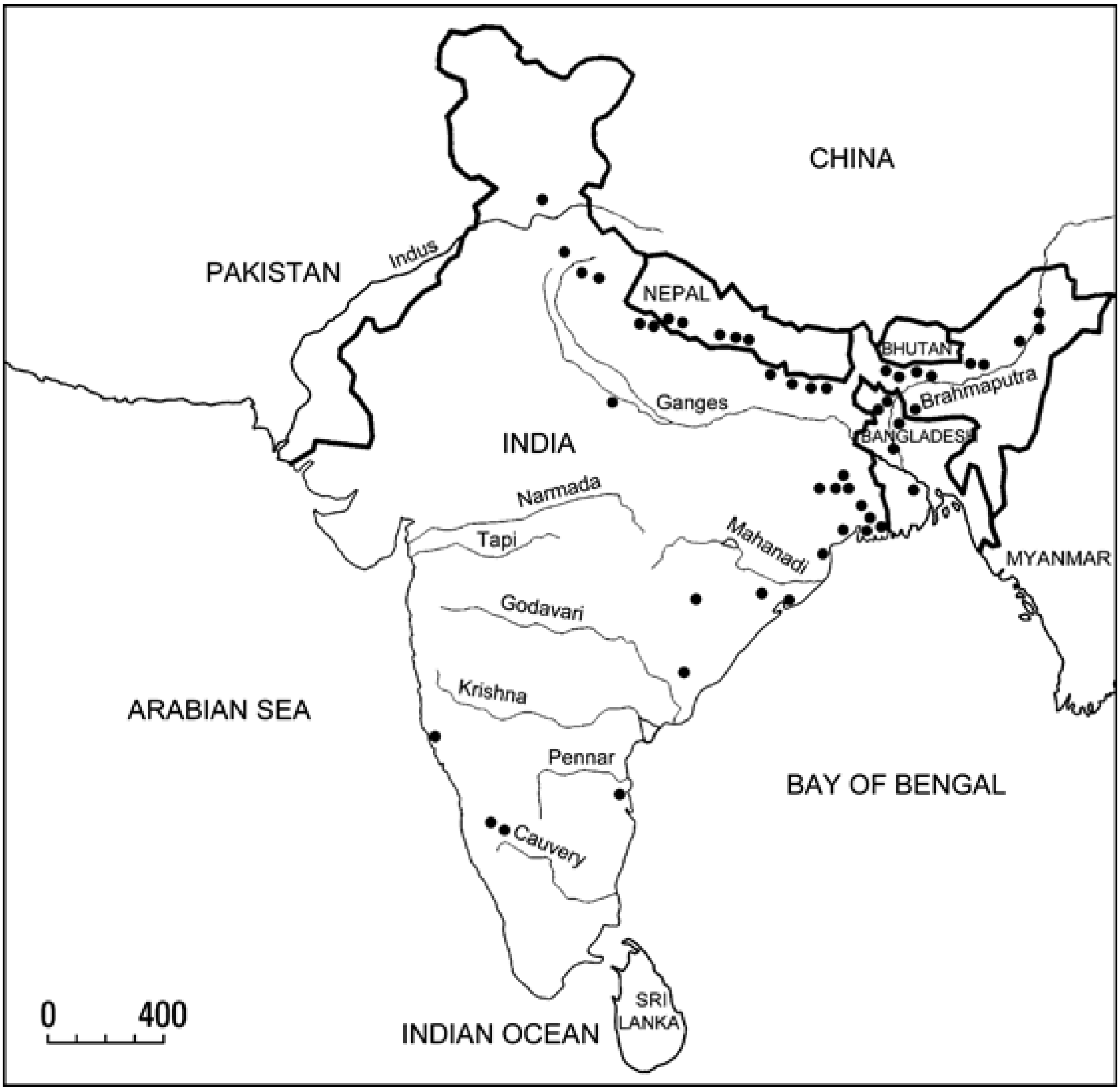

Figure 2-9: *Natural distribution of zebrafish (Spence et al., 2008).*

Zebrafish are omnivores with a natural diet consisting of zooplankton, insects, algae, and detritus (Arunachalam *et al.*, 2013, Spence *et al.*, 2008, Spence *et al.*, 2007). Although laboratory zebrafish strains receive an artificial diet, wild zebrafish and domesticated laboratory strains have similar intestinal bacteria, indicating the conservation of core gut microbiota essential for cell proliferation in the developing zebrafish intestine (Cheesman *et al.*, 2011, Roeselers *et al.*, 2011). Moreover, natural competitors for food and other resources include other minnows such as *Esomus*, *Puntius*, and larger *Danio* species (Parichy, 2015). Likely natural predators of zebrafish include other fish such as snakehead fish, knifefish, and catfish; birds such as kingfishers and herons; as well as aquatic dragonfly larvae that prey on zebrafish larvae (Engeszer *et al.*, 2007, Parichy, 2015).

Laboratory zebrafish strains breed all year round, although reproduction in the wild starts just before the onset of the monsoon season when ephemeral pools start to form (Parichy, 2015, Spence *et al.*, 2008, Spence *et al.*, 2007). These still pools facilitate waterborne pheromone function in oogenesis and courtship, and offer ideal breeding conditions, with an abundance of foodstuff and shelter against currents and predators, and facilitates waterborne pheromone function in oogenesis and courtship (Gerlach, 2006, Parichy, 2015, Van den Hurk *et al.*, 1987). Zebrafish spawning typically occurs at dawn, with oviposition sites dependant on the preference of the female and males defending such territories (Spence *et al.*, 2008). Spawning of zebrafish begins with the initial approach, followed by the male chasing the female, touching her side or tail with his snout, and the male then circling and quivering, the male pinning the female against an object, and finally oviposition (Kang *et al.*, 2013, Parichy, 2015, Sessa *et al.*, 2008). After the eggs are fertilised zebrafish grow rapidly until reaching adulthood at three months post-fertilisation, after which the growth rate starts to decrease, reaching maximum development by approximately eighteen months (Santos-Fandila *et al.*, 2015, Spence *et al.*, 2008). The zebrafish life cycle starts from the embryonic stage (0-3 days post-fertilisation), to the larval (3-29 days post-fertilisation), juvenile (1-3 months) and adult (>3 months) stages (see figure 2-10) (Stewart *et al.*, 2014a). Zebrafish reach reproductive maturity at 4-6 weeks post-fertilisation during the juvenile stage (Parichy, 2015).

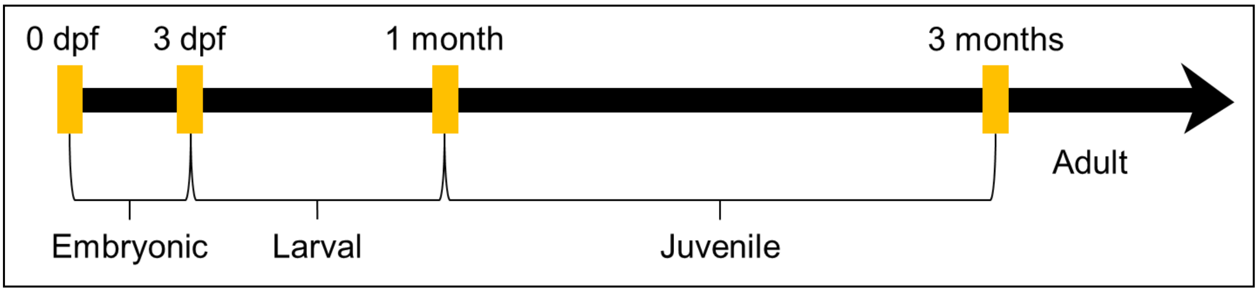

Figure 2-10: *The life cycle of zebrafish (own figure based on the text). dpf – days post-fertilisation.*

Zebrafish are highly social animals and spend most of their time swimming in shoals, an innate behaviour to provide protection against predators, improve foraging success, and increase access to mates (Miller and Gerlai, 2007, Parichy, 2015, Stewart *et al.*, 2014b, Stewart *et al.*, 2014c). Thereon, shoaling preference in zebrafish is influenced by factors such as group size, sex ratio, odour cues, kin recognition (responding to colour and stripe patterns), threat of predators, and light cycle (Engeszer *et al.*, 2008, Gerlach and Lysiak, 2006, Pritchard *et al.*, 2001, Ruhl *et al.*, 2009, Stewart *et al.*, 2014b). Conversely, zebrafish also display agonistic behaviour toward conspecifics to establish dominance and hierarchies (Oliveira *et al.*, 2011). To this end, Darwinian fitness in zebrafish is expressed as territorial claims to areas rich in resources such as food, shelter and mates (Oliveira *et al.*, 2011).

2.2.2. Rationale of using zebrafish as a translational model

Dynamic equilibrium in animals, both basal homeostasis and adaptive responses to stressors, is mediated by biochemical and physiological procedures (Chrousos, 2009, Miller and Raison, 2016). In turn, maladaptive changes in these homeostatic procedures following prolonged stress exposure may lead to the development of anxiety-related disorders, including PTSD (see section 2.1.1.) (Mocelin *et al.*, 2015, Quadros *et al.*, 2019). Current pre-clinical models for PTSD include Pavlovian fear conditioning, stress-enhanced fear learning, predator or predator cue exposure, physiological stressors (such as forced underwater submersion), restraint stress and TDS (Armario *et al.*, 2008, Clinchy *et al.*, 2013, Johnson *et al.*, 2012, Lisieski *et al.*, 2018, Rau and Fanselow, 2009, Richter-Levin, 1998). Most PTSD behavioural symptoms can be studied in animals, including exaggerated startle response, sleep disturbances, avoidance of trauma-related cues, and fear conditioning (Aikins *et al.*, 2017, Lisieski *et al.*, 2018). Other PTSD symptoms that are normally self-reported in humans but can be derived from behavioural experiments include alterations in anxiety-like behaviour and threat assessment, restriction of affect and reward, and attention and cognitive deficits (Lisieski *et al.*, 2018).

However, due to the increase in PTSD prevalence and the emerging complexity of PTSD pathophysiology there is a need for the development of new treatment strategies and, thus, novel experimental paradigms to discover new therapeutics (Stewart *et al.*, 2014a, Twenge, 2000). Moreover, high through-put screening (HTS) is the order of the day to identify novel compounds. In this regard, one approach is the use of a wider range of model species to enable translational analysis of conserved behavioural phenotypes (Kalueff *et al.*, 2007, Stewart *et al.*, 2014a). Because zebrafish exhibit evolutionary conserved homologies in neuronal mechanisms and mediator systems with rodents and humans, this species has emerged as a useful model in translational studies of complex neuropsychiatric disorders such as PTSD (Kalueff *et al.*, 2014b, Kaslin and Panula, 2001, Stewart *et al.*, 2014a, Stewart *et al.*, 2014c). For example, the structure of the zebrafish habenula, which as noted earlier is implicated in the stress response, is similar to that of the mammalian habenula (Okamoto *et al.*, 2012). Zebrafish also possess homologous gene sequences with humans (Dooley and Zon, 2000, Stewart *et al.*, 2014c). When comparing zebrafish and human protein-coding genes it is revealed that 71.4% of human genes have at least one zebrafish orthologue (Howe *et al.*, 2013). Other homologies between zebrafish and humans include amino acid order of proteins and, to a greater extent, their ligand-binding sites (Renier *et al.*, 2007, Stewart *et al.*, 2014c). Thereon, the zebrafish MAO isoform is ~70% analogous to mammalian MAO-A and MAO-B (Aldeco *et al.*, 2011). Moreover, zebrafish share vertebrate-specific physiological processes with mammals, such as organogenesis (Barbazuk *et al.*, 2000, Stewart *et al.*, 2012). Experimental evidence also indicates that zebrafish exhibit a range of complex affective, cognitive, and social behaviours, similar to those observed in rodents and humans (Kalueff *et al.*, 2013, Stewart *et al.*, 2013).

Notably, zebrafish exhibit interindividual differences, including biological variables, sex differences, and behavioural phenotypes, such as extroversion (boldness, aggression) and introversion (shyness, diffidence), which relates to the interindividual differences displayed in humans (Caramillo *et al.*, 2015, Moretz *et al.*, 2007, Norton *et al.*, 2011, Toms *et al.*, 2010). Such properties would be of value in studying stress-related disorders, given that humans and rodents also present with inherent risk versus resilience towards the development of a disorder such as PTSD (see section 1.1.) (Cohen *et al.*, 2003, Cohen *et al.*, 2004, Nemeroff *et al.*, 2006).

The zebrafish hypothalamic-pituitary-interrenal (HPI) axis is highly homologous to the human HPA axis and involves a similar hormone-driven stress response, starting with the secretion of CRF from the Hypothal, which stimulates the pituitary gland to release ACTH and, finally, the release of cortisol from the head kidney (the zebrafish homologue of the mammalian adrenal gland) (Cachat *et al.*, 2011b, Ghisleni *et al.*, 2012, Stewart *et al.*, 2014c, Tran *et al.*, 2014). Cortisol in turn activates GR to inform physiological functions, including glucose metabolism, immune response and behaviour (Bury and Sturm, 2007, Mezzomo *et al.*, 2019). These similarities to the human stress response make zebrafish an excellent model for research in altered cortisol regulation associated with PTSD (Alsop and Vijayan, 2009, Stewart *et al.*, 2014c). In this regard, mutant zebrafish with non-functional GR display elevated cortisol levels indicating resistance to GR signalling, resulting in higher stress responsivity with anxiety-like behaviour (Griffiths *et al.*, 2012, Ziv *et al.*, 2013).

Zebrafish models are ideal for HTS as they can easily be genetically and pharmacologically manipulated, are prolific breeders that fertilise externally, great numbers can economically be housed in a small area, and they require little maintenance (Gerlai, 2010, Collier and Echevarria, 2013). Additionally, zebrafish development takes place rapidly, which allows for a time- and cost-efficient model to study the developmental aspect critical to PTSD (Stewart *et al.*, 2014c). The simplicity of zebrafish CNS organisation further allows for improved isolation of shared core neurobiological and molecular pathways relevant to PTSD (Ablain and Zon, 2013, Stewart *et al.*, 2014a, Stewart *et al.*, 2014c). Collectively, this points to zebrafish possessing the necessary properties required for a comparative animal model for assessing PTSD and screening pharmacotherapies (Caramillo *et al.*, 2015, Stewart *et al.*, 2014c).

In summary, zebrafish represent an opportune model species for neurophenotyping, HTS, brain imaging and drug discovery studies (Stewart *et al.*, 2014a). In this regard, currently available public access to zebrafish biomedical databases include neurogenetic, pharmacology, and brain imaging databases (Stewart *et al.*, 2014a). Moreover, the advantages of using zebrafish in pre-clinical neuropsychiatric research include external fertilisation, rapid development, ease of genetic and pharmacological manipulation, fully characterised genome, physiological and neuronal circuitry homologies with rodents and humans, and time-, cost-, and space-effectiveness (Collier and Echevarria, 2013, Kalueff *et al.*, 2014b, Parker *et al.*, 2013, Stewart *et al.*, 2012).

Animal models play an imperative role in understanding pathophysiological processes underlying neuropsychiatric conditions such as PTSD and developing novel pharmacotherapies to address core symptoms (Kas *et al.*, 2014). The validity of such animal models must, however, be assessed using the following criteria: 1) face validity – the model must replicate behavioural symptoms associated with clinical PTSD; 2) construct validity – the model must replicate the underlying neurobiological mechanisms of clinical PTSD; and 3) predictive validity – the model must replicate the treatment response seen in clinical treatment of PTSD (Daskalakis *et al.*, 2013, McGonigle and Ruggeri, 2014). Zebrafish have been extensively validated with respect to the bio-behavioural response to stress and relevance for anxiety disorders, as will duly be discussed. While the TDS model has been extensively validated as an animal model of PTSD in rodents (Harvey *et al.*, 2003, Harvey *et al.*, 2004b, Harvey *et al.*, 2006, Liberzon *et al.*, 1997, Yamamoto *et al.*, 2009), very little work has been done in developing this model in ZF.

2.2.3. Zebrafish CNS

Hitherto neuromorphological studies indicate that, although the zebrafish brain is small compared to the mammalian brain, they do share structural characteristics (Kalueff *et al.*, 2014b, Stewart *et al.*, 2014a). The main areas that have been identified in the zebrafish brain include the olfactory bulbs, telencephalon, diencephalon, mesencephalon, rhombencephalon, habenula, optic tectum, cerebellum and medulla (see figure 2-11 and 2-12) (Gupta and Mullins, 2010, Jurisch-Yaksi *et al.*, 2020). In this regard, the lateral- and ventral telencephalic areas of the zebrafish brain are functionally similar to the mammalian hippocampus and striatum, respectively (Racagni *et al.*, 2011, Rodríguez *et al.*, 2005). The lateral pallium of the telencephalon controls memory processing, while the medial pallium of the dorsal telencephalic region is the anatomical homologue of the mammalian amygdala (Racagni *et al.*, 2011, Mezzomo *et al.*, 2019, Norton and Bally-Cuif, 2010). Thereon, these similarities between the zebrafish and mammalian brain indicate that zebrafish are ideal for the study of both normal neurobiology and pathological neurobiology related to PTSD (Kalueff *et al.*, 2014b, Stewart *et al.*, 2014a).

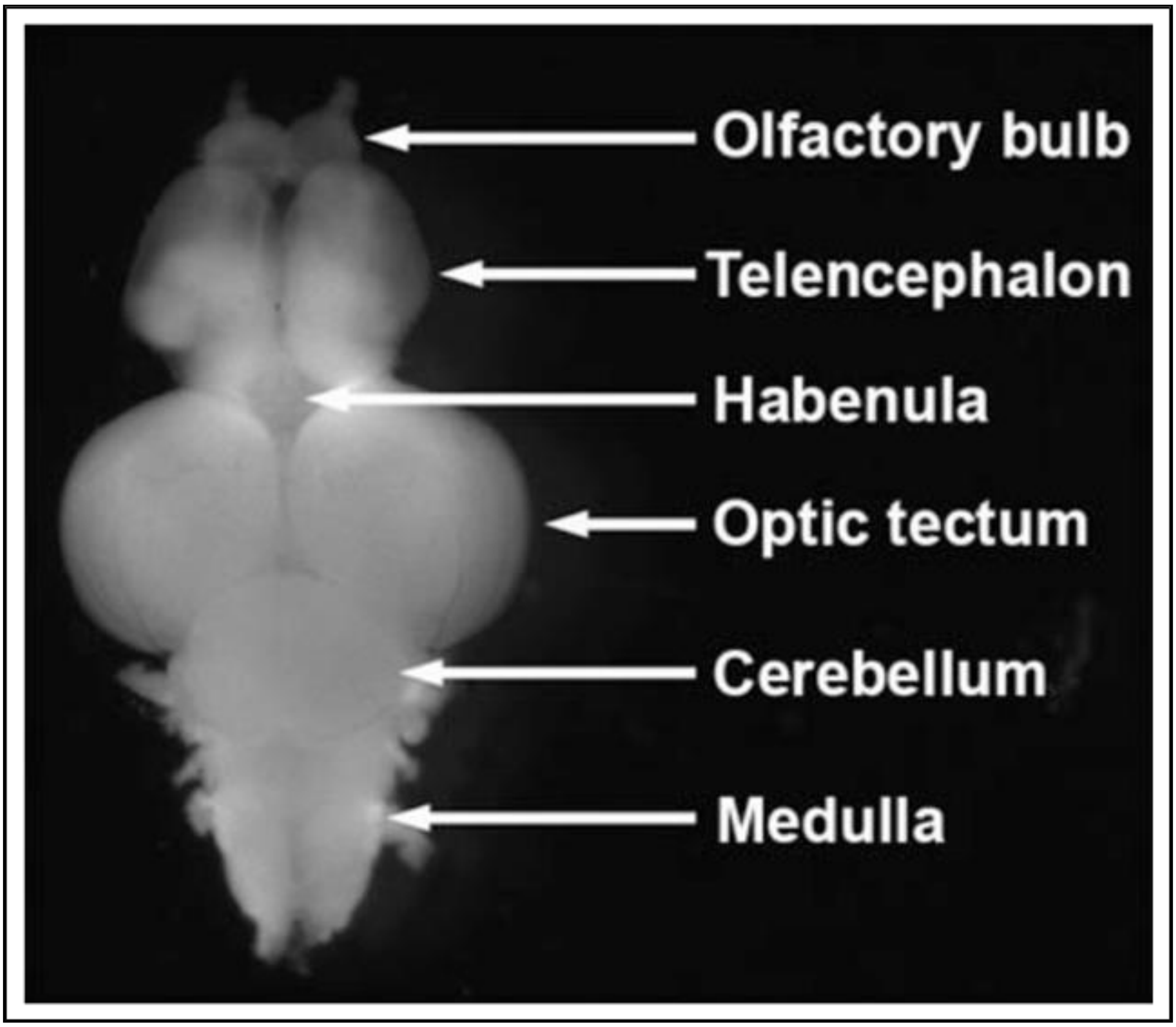

Figure 2-11: *Dorsal view of dissected adult zebrafish brain (Gupta and Mullins, 2010).*

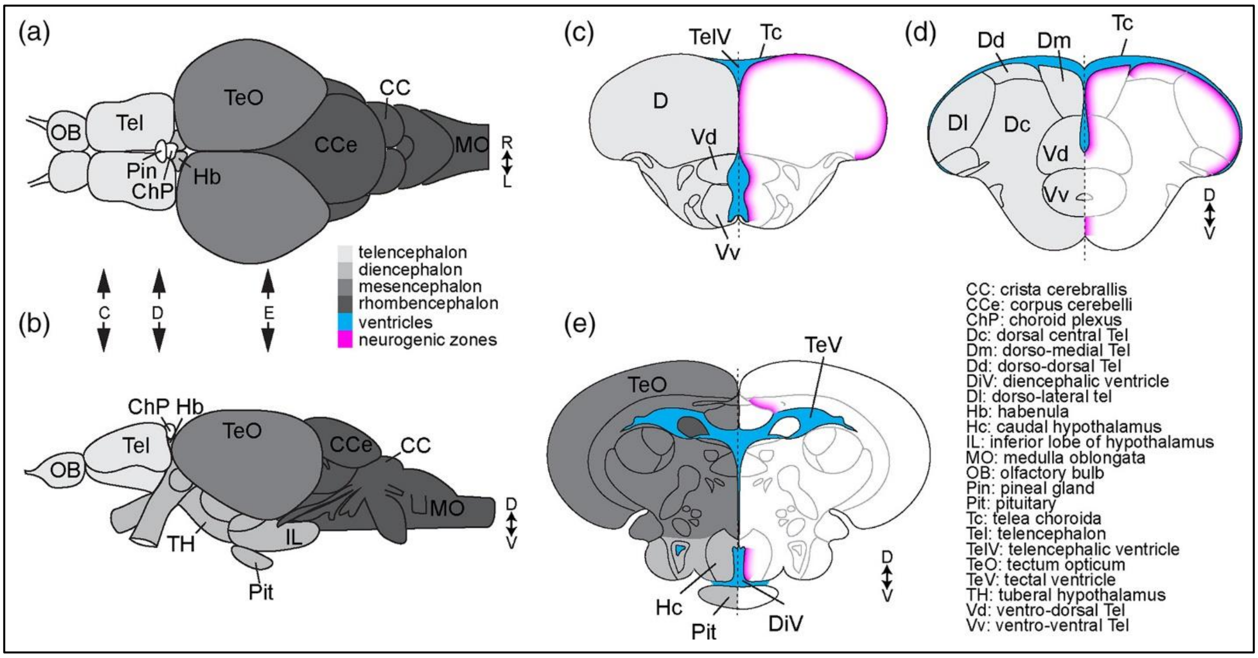

Figure 2-112: *Anatomy of adult zebrafish brain (Jurisch-Yaksi et al., 2020). (a) Dorsal, (b) lateral, and (c-e) transverse views. Transverse sections through the (c) anterior and (d) posterior telencephalon and (e) di-/mesencephalon.*

Psychiatric disorders are increasingly being recognised as disorders of neuronal circuitry (Racagni *et al.*, 2011, Ljunggren *et al.*, 2014, Stewart *et al.*, 2014a). Therefore, the conservation of neuronal circuitry in vertebrate evolution supports the use of zebrafish in translational neuropsychiatric research (Okamoto and Aizawa, 2013, Stewart *et al.*, 2014a). As such, the projection pathways of the lateral and medial subnuclei in the zebrafish dorsal habenula to the interpeduncular nucleus are important in the regulation of the anxiety circuitry, which in turn modulates fear responses (Agetsuma *et al.*, 2010, Stewart *et al.*, 2014a). Furthermore, the neuronal pathways from the habenula to the ventral- and dorsal telencephalon are also implicated in fear- and anxiety-like behaviour in zebrafish (Racagni *et al.*, 2011, Maximino *et al.*, 2013, Okamoto *et al.*, 2012, Stewart *et al.*, 2014a). Moreover, the ventral- and lateral telencephalon, together with the cerebellum, are thought to be critical in learning and memory in zebrafish (Rodríguez *et al.*, 2005). The modulatory actions of many neurotransmitter systems on neuronal circuits have also been established in the zebrafish brain, including the monoaminergic, cholinergic and neuropeptide systems (Panula *et al.*, 2010). These neurotransmitter systems in zebrafish correspond functionally to the mammalian homologues (Panula *et al.*, 2010).

2.2.4. Biomarkers of PTSD in zebrafish

A variety of biomarkers common in human PTSD are applicable to zebrafish. Of particular relevance would be whole-body cortisol (Barcellos *et al.*, 2007, Sink *et al.*, 2007, Stewart *et al.*, 2014c), circadian rhythm biomarkers (melatonin) and monoamines (5HT, NA, DA) (Demin *et al.*, 2017, Khan *et al.*, 2016). Accordingly, disordered circadian rhythms is a noted pathological feature of anxiety in zebrafish (Pinheiro-da-Silva *et al.*, 2018, Stankiewicz *et al.*, 2017). Thereon, similar to oxytocin in mammals, the zebrafish homologue, isotocin, plays a role in the regulation of circadian rhythms, as well as social behaviour (Braida *et al.*, 2012). The cholinergic system has also been evaluated in zebrafish by determining acetylcholine levels and activity of cholinergic transmission enzymes, such as choline acetyltransferase and acetylcholinesterase (Agostini *et al.*, 2018). Furthermore, many biomarkers of clinical PTSD associated with immune response and inflammation, such as interleukins 6 and 8, and serum amyloid A, share gene orders with zebrafish equivalents (Stewart *et al.*, 2014c). Additionally, the kynurenine pathway represents a relevant inflammation cascade that can be assayed in zebrafish (Majewski *et al.*, 2018, Moller *et al.*, 2015, Robinson *et al.*, 2013). Moreover, as distinctly evident in rodent and human stress-related conditions (Brand *et al.*, 2015), restraint stress also evokes increased oxidative stress in the zebrafish brain (Dal Santo *et al.*, 2014, Piato *et al.*, 2011, Stewart *et al.*, 2014c). Genetic predictors of PTSD development in humans, for example catechol-O-methyltransferase, have also been successfully identified in zebrafish (Caramillo *et al.*, 2015, Pavlidis *et al.*, 2011). Finally, given that PTSD patients present with increased sympathetic tone, sympathetic cardiac responses (including altered heart rate) have been reported in zebrafish, supporting the use of zebrafish models to investigate physiological biomarkers of PTSD (Hageman *et al.*, 2001, Mann *et al.*, 2010, Stewart *et al.*, 2014c).

2.2.5. Zebrafish behaviour

Abnormal human stress responses, such as fear underlying PTSD, are caused by dysfunctional neurobiological mechanisms that originally evolved to promote predator avoidance in nature (Gerlai, 2010, Stewart *et al.*, 2014c). In this regard, zebrafish display significant anti-predatory responses in reaction to direct or simulated stimuli, as well as exposure to predator-related cues, such as conspecific alarm substance (CAS) (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart *et al.*, 2014c). Speedie and Gerlai (2008) found that CAS exposure is effective in inducing fear responses and anti-predatory behaviour in zebrafish and that it may be useful in the analyses of fear and anxiety (see section 2.2.6.). The robust fear- and anxiety-like behaviours displayed by zebrafish in their aquarium or test tank include increased scototaxis (dark preference), geotaxis (bottom dwelling), thigmotaxis (preference for peripheral regions), diving, freezing, jumping, erratic movements and hyperventilation (Blaser and Vira, 2014, Caramillo *et al.*, 2015, Egan *et al.*, 2009, Quadros *et al.*, 2018, Stewart *et al.*, 2014a). These behavioural phenotypes are similar to those of rodents and humans (Caramillo *et al.*, 2015). It is worth noting that the analyses of anxiety behaviours such as diving and geotaxis are only attainable through the addition of a vertical plane in the 3-dimensional reconstruction of zebrafish swimming, as explained later (Stewart *et al.*, 2014a).

As mentioned, zebrafish typically swim in shoals (Miller and Gerlai, 2007, Stewart *et al.*, 2014b, Stewart *et al.*, 2014c). With that, shoaling is characterised by short inter-fish distance, smaller group diameter, and synchronised swimming (at the same speed and in the same direction) (Stewart *et al.*, 2014b). On the other hand, looser and larger groups, reduced synchronisation and a higher number of zebrafish leaving the group indicates disrupted shoaling (Stewart *et al.*, 2014b). As such, social behaviour can be measured in a group of zebrafish using 3-point video tracking (Green *et al.*, 2012). Furthermore, in a social preference assay, a target zebrafish given a choice between an empty and a conspecific area, it typically spends significantly more time in proximity to conspecifics, similar to social interaction tests in rodents (Kas *et al.*, 2014, Stewart *et al.*, 2014b). In keeping with this, given the choice between a single conspecific and a group of conspecifics, a target zebrafish tends to spend more time near the group (Stewart *et al.*, 2014b). Collectively, this points to the utility posed by zebrafish models for the study of both normal and pathological social behaviour associated with PTSD (Stewart *et al.*, 2014b, Stewart *et al.*, 2014c).

Similar to rodents, zebrafish also exhibit higher cognitive functions and decision-making strategies which are observable in a variety of behavioural assays (Oliveira, 2013, Stewart and Kalueff, 2012). In this regard, the effects of stress on memory in zebrafish are assessed using a submerged plus-maze, where the amount of time spent in the correct arm, as well as the number of entries into the correct arm, are recorded (Gaikwad *et al.*, 2011). Here zebrafish are trained to associate a food reward with a visual cue and a spatial location (Gaikwad *et al.* (2011). Zebrafish exposed to CAS showed reduced cue and spatial memory (Gaikwad *et al.*, 2011), which mirrors the memory and learning deficits displayed by patients with PTSD (Caramillo *et al.*, 2015).

Learning capabilities of zebrafish also include acquisition and extinction of Pavlovian associations (Salas *et al.*, 2006). Accordingly, zebrafish possess the ability to contextualise fear by pairing a neutral conditioned stimulus with an aversive unconditioned stimulus, to then elicit fear behaviours after numerous pairings when presented with the conditioned stimulus alone (Agetsuma *et al.*, 2010, Ogawa *et al.*, 2014). Thereon, fear conditioning in zebrafish has been established in various trials by pairing conditioned stimuli, such as visual, auditory, olfactory, or tactile cues, with unconditioned stimuli, such as shock, touch or CAS (alarm pheromone), to elicit robust and replicable behaviours (Aizenberg and Schuman, 2011, Blank *et al.*, 2009, Blaser and Vira, 2014, Hall and Suboski, 1995).

Ideally, associative learning paradigms in zebrafish should incorporate the following (Blaser and Vira, 2014):

1. Control over conditioned stimuli (intensity, duration and frequency).
2. Unconditioned stimuli that are not excessively susceptible to habituation or saturation.
3. Behavioural responses to conditioned stimuli amenable to objective (automated/ computerised) measurement to eliminate experimenter bias and improve the reliability of the results.
4. Analyses of behaviour in both acquisition and testing to distinguish between normal and conditioned behaviours.
5. Efficient assays apropos of time and number of animals required for statistical reliability to accommodate HTS.

The six basic sensory modalities of zebrafish, *viz.* taste, tactile (lateral line system), olfaction, equilibrium (vestibular system), vision and audition, are highly amenable to various experimental procedures (Blaser and Vira, 2014, Moorman, 2001). For this reason, zebrafish display a wide range of clearly discernible and complex emotional behaviours triggered by a diversity of environmental stimuli, which mimics behaviours exhibited by rodents and humans (Blaser *et al.*, 2010, Caramillo *et al.*, 2015, Kalueff *et al.*, 2013, Stewart *et al.*, 2014a, Stewart *et al.*, 2014c). The natural behaviours exhibited by zebrafish remain highly sensitive in laboratory zebrafish (Stewart *et al.*, 2014a).

High-throughput behavioural experiments, similar to anxiety paradigms in rodents, have been extensively validated in zebrafish, including the novel tank test, light/dark test, CAS exposure, and pharmacogenic anxiety (Stewart *et al.*, 2014a). However, the standardisation of these behavioural assays remains a concern (Stewart *et al.*, 2014a), which is essential for consistency and cross-laboratory comparability (Stewart *et al.*, 2014a). Hence, standardised assays are advocated, while supplementary custom-tailored tests may be added to analyse any unique behaviours, if need be (Stewart *et al.*, 2014a).

Automated video tracking software is especially useful for the visualisation and quantification of swimming behaviour in zebrafish, such as locomotor activity, expressed as spatial measurements (distance, speed, velocity, meandering), (Ahmad *et al.*, 2012, Cachat *et al.*, 2013). But there are other advantages for the use of zebrafish over rodent models in translational studies. One is that the locomotion of zebrafish is 3-dimensional, whereas rodents only move in a horizontal 2-dimensional plane (Cachat *et al.*, 2011a, Stewart *et al.*, 2014a). Thus, a two-camera set-up permits 3-dimensional reconstructions of zebrafish locomotion by combining 2-dimensional swimming traces (top- and side view) into 3-dimensional X,Y,Z-coordinates using integration software (Cachat *et al.*, 2011a, Stewart *et al.*, 2014a). This allows zebrafish behaviours to be visualised and quantified in 3 dimensions, therefore enabling comprehensive analyses of behavioural endpoints and motor patterns (Cachat *et al.*, 2011a, Stewart *et al.*, 2014a).

Taken together, the upsurge in behavioural assays and the advancements in video tracking and quantification of zebrafish behaviours allow for comprehensive characterization of an animal phenotype (Gerlai, 2002, Stewart *et al.*, 2014a). However, behavioural phenotypes cannot be identified using only a single behavioural assay, so that test batteries are required (Crawley, 2008, McIlwain *et al.*, 2001). Such a battery of tests should be from least to most invasive to minimise the impact of prior tests on behavioural responses (McIlwain *et al.*, 2001). Test batteries will enable the analyses of several different behavioural domains (including anxiety, cognition, affect and social behaviour) implicated in a range of neuropsychiatric disorders (Stewart *et al.*, 2014a). This cross-domain approach will lend higher construct validity to models of neuropsychiatric disorders (such as PTSD) because it links multiple co-expressed phenotypes into a single system (Kalueff *et al.*, 2008).

2.2.6. Conspecific alarm substance (CAS)

A commonly used animal model of PTSD is the predator exposure model (Uys et al., 2003). As mentioned earlier, zebrafish display significant anti-predatory responses in reaction to exposure to predator-related cues, such as CAS (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart et al., 2014c). CAS is a pheromone-like mixture produced by specialized epidermal club cells and is released in the water upon skin damage of zebrafish (Maximino et al., 2018, Speedie and Gerlai, 2008). Upon release CAS is detected through olfaction by neighbouring zebrafish and alarm reactions are elicited (Speedie and Gerlai, 2008, Waldman, 1982). In this regard, the peripheral olfactory organs of zebrafish are in direct contact with the surrounding environment (Wang and Gallagher, 2013). Zebrafish have a well-characterised olfactory system, with the olfactory epithelium containing three distinct types of olfactory sensory neurons, namely ciliated, microvillous, and crypt cells (Wang and Gallagher, 2013, Yoshihara, 2008). The pathway through which CAS evokes alarm reactions involves the projection of the olfactory sensory neuron axons to the mitral cells in the olfactory bulb, which in turn project to the right habenula in the brain (Decarvalho *et al.*, 2013).

CAS-exposed zebrafish display exacerbated anxiety and fear behaviours, as well as increased aggression, shoaling and prolonged avoidance behaviour (Canzian *et al.*, 2017, Egan *et al.*, 2009, Maximino *et al.*, 2018, Quadros *et al.*, 2018, Speedie and Gerlai, 2008). Thereon, observable behaviours, such as erratic movement, can be quantified using computerised video-tracking-based methods (see section 2.2.5.) (Maximino *et al.*, 2018, Speedie and Gerlai, 2008). Additionally, a linear relationship exists between CAS concentration and the frequency and duration of alarm reactions, due to high concentrations being more detectable by neighbouring zebrafish (Speedie and Gerlai, 2008). From this dose-dependent response to CAS exposure it has been derived that zebrafish likely exhibit a stronger alarm response when an injured zebrafish is nearby and thus a higher concentration of CAS is detected by the neighbouring zebrafish, compared to a diminished alarm response to a smaller concentration of CAS, when an injured zebrafish is further away and the predator is unlikely to be nearby (Speedie and Gerlai, 2008). The quantifiability of this dose-dependent response to CAS makes it susceptible to drug screening (Speedie and Gerlai, 2008). Furthermore, CAS increases oxidative stress biomarkers in the brain (indicated by increased catalase and glutathione S-transferase activities and non-protein sulfhydryl levels), as well as decreasing MAO and increasing blood glucose, haemoglobin, adrenaline, NA and extracellular 5HT in the brain, which can be blocked with FLX pre-treatment (Maximino *et al.*, 2014, Quadros *et al.*, 2018, Quadros *et al.*, 2019). Importantly, CAS exposure also increases whole-body cortisol levels, a process that has also been shown to display time-dependent changes post-stress exposure, not unlike that noted in rodent models of PTSD (Harvey *et al.*, 2006, Oliveira *et al.*, 2014, Tudorache *et al.*, 2013).

2.2.7. Sensitivity to anti-PTSD drugs

Behavioural phenotypic analyses (see section 2.2.4.) enable time-, cost-, and space-efficient HTS of pharmacotherapies in zebrafish models of complex neuropsychiatric disorders (Kalueff *et al.*, 2014b, Stewart *et al.*, 2014a). In this regard, the behaviours displayed by zebrafish are susceptible to a wide range of pharmacological manipulations (Cachat *et al.*, 2011a). Accordingly, zebrafish exhibit a high sensitivity to various pharmacotherapies used in the treatment of PTSD, including first line treatments such as SSRI's, and second- and third line treatments such as tricyclic antidepressants, MAO inhibitors, propranolol, anticonvulsants, and atypical antipsychotics (Baraban *et al.*, 2013, Berghmans *et al.*, 2007, Lee *et al.*, 2013, Mitchell and Moon, 2016, Sackerman *et al.*, 2010, Stewart *et al.*, 2011). The pharmacological profiles of these agents in zebrafish demonstrate striking parallels with rodent and human data (Kalueff *et al.*, 2014b). Noteworthy drug responses demonstrated by zebrafish include anxiolytic actions following treatment with SSRI's, tricyclic antidepressants, MAO inhibitors, and propranolol (Egan *et al.*, 2009, Mitchell and Moon, 2016, Sackerman *et al.*, 2010, Stewart *et al.*, 2011, Wong *et al.*, 2010). Such responses include a reduction in bottom dwelling, freezing, and erratic movements in the novel tank test, and emphasises the potential of zebrafish models in the screening of drugs for PTSD treatment (Egan *et al.*, 2009, Mitchell and Moon, 2016, Sackerman *et al.*, 2010, Stewart *et al.*, 2011, Stewart *et al.*, 2014c, Wong *et al.*, 2010).

2.2.8. Limitations of zebrafish model

Although humans and zebrafish share certain brain structures, there are areas of the human CNS that are not well developed in zebrafish, while other structures are difficult to map to their counterparts or altogether not present in the zebrafish brain (Kalueff *et al.*, 2014a). Of particular concern is the lack of an expanded PFC, which is one of the primary cerebral areas involved in the pathophysiology of PTSD in humans (Caramillo *et al.*, 2015). However, as mentioned, homologues of the mammalian amygdala and hippocampus have been identified (see section 2.2.3.) (Mezzomo *et al.*, 2019, Norton and Bally-Cuif, 2010, Rodríguez *et al.*, 2005). Another concern is that zebrafish lose fertility with inbreeding and, thus, there are not many well-characterised zebrafish strains (Kalueff *et al.*, 2014b). Accordingly, many zebrafish are outbred or have an unclear breeding history (Kalueff *et al.*, 2014b). This may negatively impact the reproducibility of zebrafish studies, especially since animals from divergent breeding lines behave slightly differently (Voelkl *et al.*, 2020).

Species differences exist in the blood-brain barrier between zebrafish and humans, which may affect the permeability of certain drugs (Kalueff *et al.*, 2014a). Furthermore, it may prove difficult to translate drug doses from humans and rodents to zebrafish (Kalueff *et al.*, 2014a). Additionally, water-insoluble drugs also present a problem in zebrafish studies, since these drugs cannot be administered via water immersion, necessitating more invasive methods of administration (Kalueff *et al.*, 2014a). Of note, however, is that alternative vehicles, such as dimethyl sulfoxide, can be used to solubilise various water-insoluble drugs (Kalueff *et al.*, 2014a).

2.3. Summary

PTSD is a disorder that develops following exposure to an actual or perceived life-threatening or traumatic event or series of events (First *et al.*, 2015, Hoffman *et al.*, 2011, Kang *et al.*, 2003). The DSM-5 classifies PTSD as a trauma- and stressor-related disorder, with symptoms usually emerging within the first 3 months after the trauma (American Psychiatric Association, 2013). PTSD predominantly manifests in adverse changes in anxiogenesis, with accompanying decline in working and emotional memory (Caramillo *et al.*, 2015, Honzel *et al.*, 2014). Accordingly, PTSD is characterised by the over-consolidation of traumatic memories and a reduction of explicit memories (Bentz *et al.*, 2013, Bowers and Ressler, 2015, Parsons and Ressler, 2013, Pittenger, 2013). Moreover, diagnosis of PTSD typically includes negative thoughts and moods, persistent perceptions of heightened current threat, re-experiencing or reliving the trauma, persistent avoidance (First *et al.*, 2015, Hoffman *et al.*, 2011). These symptoms last for at least a month and lead to significant problems in personal, family, social, school or work life, or other important areas of functioning (First *et al.*, 2015, Hoffman *et al.*, 2011).

PTSD has a poorly understood pathophysiology and, thus, further studies investigating PTSD development are essential (Sherin and Nemeroff, 2011, Stewart *et al.*, 2014c). Additionally, the current pharmacological treatments for PTSD are sub-optimal due to the limited research portfolio of novel pharmacotherapies for PTSD (Kelmendi *et al.*, 2016, Krystal *et al.*, 2017, Lisieski *et al.*, 2018). These limitations necessitate novel animal models that can mimic the human condition. Currently, PTSD research strongly relies on mammalian models of PTSD, especially rodent models (Stewart *et al.*, 2014c). However, these models are hampered by high cost, low throughput, and long breeding periods, as well as being time consuming (Caramillo *et al.*, 2015, Freudenberg *et al.*, 2018, Kafkafi *et al.*, 2018, Planchart *et al.*, 2016). Therefore, alternative models such as the zebrafish, need consideration.

Zebrafish possess neuronal mechanisms and mediator systems with high homology to mammals, thereby emerging as a promising model of complex neuropsychiatric disorders (Kaslin and Panula, 2001, Stewart *et al.*, 2014c). The zebrafish habenula, implicated in the modulation of fear behaviours, is analogous to that of mammals; and the fish HPI axis is homologous to the human HPA axis, exhibiting similar cortisol driven stress responses (Cachat *et al.*, 2013, Okamoto *et al.*, 2012, Stewart *et al.*, 2014c). Such characteristics make zebrafish an excellent model for investigation into alteration in cortisol regulation with application to PTSD research (Alsop and Vijayan, 2009, Stewart *et al.*, 2014c). Furthermore, zebrafish models are particularly appropriate for HTS as they are easy to maintain and breed in the laboratory and large numbers can be housed in small fish tanks (Gerlai, 2010).

An example of an animal model of PTSD includes exposure to a predator (Uys *et al.*, 2003). In this regard, zebrafish respond to exposure to predator-related stimuli such as CAS with robust anti-predatory reactions (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart *et al.*, 2014c). Since CAS treatment is effective in eliciting fear reactions in zebrafish, it offers utility for translational fear and anxiety research in these animals (Speedie and Gerlai, 2008). Moreover, zebrafish possess the ability to contextualise fear (Ogawa *et al.*, 2014). Indeed, fear conditioning is a form of Pavlovian conditioning where a sensory cue is paired with an aversive event and, as noted earlier, is an essential component of PTSD (Uys *et al.*, 2003). Zebrafish also display a range of fear- and anxiety-like behaviours that are quantifiable (Caramillo *et al.*, 2015, Egan *et al.*, 2009) and resemble stress-evoked rodent and clinical stereotypies (Caramillo *et al.*, 2015, Stewart *et al.*, 2014b, Stewart *et al.*, 2014c, Yang *et al.*, 2020). The purpose of this study was therefore to establish a novel translational zebrafish model of PTSD by assessing the anxiety-like behavioural responses following CAS exposure. Thereon, akin to stress-restress models of PTSD in rodents (Brand *et al.*, 2008, Harvey *et al.*, 2005, Harvey *et al.*, 2006, Harvey *et al.*, 2003, Harvey *et al.*, 2004b), CAS was paired with contextual reminders to evoke fear conditioning in zebrafish, and to determine whether fearful behaviour persists in the absence of CAS.

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3. MANUSCRIPT A

Article to be submitted to *Journal of Neuroscience Methods* titled:

"Conspecific alarm substance-induced fear conditioning in zebrafish: A model potentially relevant for post-traumatic stress disorder"

Important information

- The text was written in British English; however, the American spelling was used for the keywords for indexing purposes.
- The *Journal of Neuroscience Methods* 'Author Information Pack' is included in Addendum A.
- All co-authors provided consent for the research article to be assessed as part of the dissertation of *Heslie Loots*, submitted in fulfilment of the requirements for the degree Master of Science in Pharmacology. The letters of consent are included in Addendum B.

Conspecific alarm substance-induced fear conditioning in zebrafish: A model potentially relevant for post-traumatic stress disorder

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Keywords:

Alarm substance; Fear conditioning; Zebrafish; Predator exposure model; Post-traumatic stress disorder; Anxiety

Highlights

- Alarm substance induces sustained fear/anxiety behaviour in zebrafish.
- Pairing alarm substance with contextual reminders evokes fear conditioning in fish.
- Fear conditioning can be used to model post-traumatic stress in this animal model.

Abstract

Post-traumatic stress disorder (PTSD) is caused by traumatic experiences and is often associated with dysregulated fear conditioning. Zebrafish exhibit genetic, physiological and neuronal circuitry homologies with humans and have emerged as a useful species to study neuropsychiatric disorders such as PTSD. Conspecific alarm substance (CAS) is a pheromone-like exudate released by zebrafish upon skin damage, eliciting fear-like reactions in conspecifics. In developing a novel model of PTSD in zebrafish, we assessed behavioural responses to CAS in zebrafish following exposure and contextual re-experience. Zebrafish were randomly divided into four groups, including ‘vehicle/no cue’, ‘CAS/no cue’, ‘vehicle/cue’ and ‘CAS/cue’, and exposed to these treatments for 6 min on day 1, following a 1-h habituation period. Behavioural observation commenced immediately after stress or sham exposure. On day 2, zebrafish were returned to the same tanks (used for the initial exposure) without CAS. Anxiety-like behaviour was tested in the tanks on both days by measuring time in top zone, the number of top zone entries, meandering and immobility. Overall, CAS-exposed zebrafish displayed overt anxiety-like behaviour immediately after CAS exposure, which was sustained on day 2 (following a contextual reminder) under both cued and non-cued conditions. The observed fear conditioning and its persistence following contextual reminder even without the primary stressor indicate that CAS exposure induces PTSD-like behavioural changes in zebrafish. However, further validation in respect of PTSD biology and treatment is necessary.

1. Introduction

Post-traumatic stress disorder (PTSD) is a severely debilitating chronic mental illness caused by an actual or perceived life-threatening or traumatic event or series of events (First *et al.*, 2015, Hoffman *et al.*, 2011, Kang *et al.*, 2003). The DSM-5 categorizes PTSD as a trauma- and stressor-related disorder, with symptoms generally emerging within 3 months after experiencing trauma (American Psychiatric Association, 2013). PTSD typically presents with negative thoughts, moods and beliefs (e.g., detachment, guilt, fear, anger and anhedonia), persistent perceptions of heightened current threat (hypervigilance or enhanced startle reaction), re-experiencing or reliving the trauma (in the form of intrusive memories, flashbacks or nightmares), avoidance of thoughts and memories of the trauma, as well as avoidance of places, people and situations reminiscent of the trauma (First *et al.*, 2015, Hoffman *et al.*, 2011). These symptoms typically last for at least a month and lead to significant impairments in personal, family, social, educational and occupational life, or other important areas of functioning (First *et al.*, 2015, Hoffman *et al.*, 2011). Given these symptoms, PTSD is widely described as a disorder of affective memory, characterised by over-consolidated fear- and reduced explicit memory (Bentz *et al.*, 2013, Bowers and Ressler, 2015, Parsons and Ressler, 2013).

Despite our understanding of the causes of PTSD, its current pharmacological treatments remain sub-optimal (Kelmendi *et al.*, 2016) due to poorly understood neurobiological mechanisms and the limited research portfolio of novel pharmacotherapy for PTSD (Krystal *et al.*, 2017, Lisieski *et al.*, 2018). Rodent models of PTSD, such as the predator exposure model (PEM) (Cohen *et al.*, 2003, Cohen *et al.*, 2004) and time-dependent sensitisation (TDS) (Harvey *et al.*, 2005, Harvey *et al.*, 2006, Harvey *et al.*, 2003, Harvey *et al.*, 2004), have proved valid translational models for exploratory PTSD research. However, they are hampered by high cost, low throughput and failure to be replicated and reproduced (Freudenberg *et al.*, 2018, Kafkafi *et al.*, 2018, Planchart *et al.*, 2016). Zebrafish (*Danio rerio*) exhibit high homology in neuronal mechanisms and mediator systems with rodents and humans, thus emerging as a useful model of complex neuropsychiatric disorders, including PTSD (Kaslin and Panula, 2001, Stewart *et al.*, 2014b). For example, the zebrafish habenula, implicated in the stress response, is similar to that of mammals (Okamoto *et al.*, 2012), while the zebrafish hypothalamic-pituitary-interrenal (HPI) axis parallels the human hypothalamic-pituitary-adrenergic (HPA) axis, both sharing similar glucocorticoid-driven stress responses (Cachat *et al.*, 2013, Stewart *et al.*, 2014b). These attributes make zebrafish an excellent model of altered cortisol regulation associated with stress-related disorders, such as PTSD (Alsop and Vijayan, 2009, Stewart *et al.*, 2014b). However, contextual fear learning, which is a common factor in PTSD pathogenesis (Morrison and Ressler, 2014), has not been well studied in zebrafish (Stewart *et al.*, 2014b, Yang *et al.*, 2020).

Zebrafish display a wide range of clearly discernible and complex behaviours induced by various stimuli (Blaser *et al.*, 2010, Caramillo *et al.*, 2015, Stewart *et al.*, 2014b), and are also able to contextualize fear (Ogawa *et al.*, 2014). Zebrafish fear/anxiety-like behaviours include increased scototaxis (dark preference), geotaxis (bottom dwelling), thigmotaxis (preference for peripheral regions), freezing (immobility), and erratic movements (Caramillo *et al.*, 2015, Egan *et al.*, 2009), generally similar to those of rodents and humans subjected to stress and trauma (Stewart *et al.*, 2014a, Stewart *et al.*, 2014b, Yang *et al.*, 2020). However, the presentation and diagnosis of PTSD depends on the type and duration of the stressor (American Psychiatric Association, 2013) and its progression over time, which must be considered when modelling this disorder in animals.

The PEM in rodents is a commonly used animal model of PTSD (Uys *et al.*, 2003). Likewise, zebrafish display robust anti-predatory responses to predator-related cues, such as predator image (Gerlai, 2013) or conspecific alarm substance (CAS) (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart *et al.*, 2014b). CAS is a pheromone-like substance released in the water upon skin damage of zebrafish and detected through olfaction by conspecifics, hence alarming them (Speedie and Gerlai, 2008, Waldman, 1982).

Here, we develop a novel translational zebrafish model of PTSD by assessing the anxiety-like behavioural responses following CAS exposure. Furthermore, similar in construct to rodent models of PTSD that utilise a contextual reminder to perpetuate fear-induced behaviour in the absence of the initiating stressor (Brand *et al.*, 2008, Harvey *et al.*, 2005, Harvey *et al.*, 2006, Harvey *et al.*, 2003, Harvey *et al.*, 2004), we investigated the impact of re-experience in zebrafish using a contextual reminder that has previously been paired with CAS. We hypothesise that CAS (with/without a visual cue) will evoke a robust anxiogenic response on day 1 of exposure, and that this evoked anxiety will extinguish by day 2 post-exposure in animals *not* presented with a visual cue on that day, but will be sustained in fish co-exposed to CAS and vision cue, recapitulating fear conditioned learning seen in clinical PTSD.

2. Methods

2.1. Animals

A total of 72 adult zebrafish (3-8 months old; both sexes, ~1:1 male:female ratio), of the wild type outbred long fin strain, were obtained from the National Aquatic Bioassay Facility of the Unit for Environmental Sciences and Management, North West University (NWU), Potchefstroom, South Africa. After being transported to the National Aquatic Bioassay Facility, animals were habituated to the National Aquatic Bioassay Facility for at least 2 weeks, and to a behavioural testing facility within the Unit for 24-h prior to testing. Zebrafish were housed in 15-L tanks (2-3 fish per 5-L tank water) under constantly controlled conditions, including water pH of 7, temperature of 28 ± 2°C, and aeration of 7.2 mg O_2/L, under a 12-h light-dark cycle (lights on at 06:00). The animals were fed with commercially available artificial diet (ZM-400 fish food, Zebrafish Management Ltd, United Kingdom) twice daily (at 8:30 and 16:00). All experiments and procedures fully complied with national legislation, international guidelines, as well as the principles of the 4R's of ethical experimentation (replacement, reduction, refinement, and responsibility), and were approved by the NWU AnimCare committee (NHREC registration AREC-130913-015; approval number NWU-00169-18-A5).

2.2. Study design

A total of 32 zebrafish were used as donors for the preparation of CAS. For the behavioural tests the remaining 40 fish were randomly divided into 4 groups (*n* = 10 per group), each exposed to a different condition, as depicted in the flowchart of the study design in **Fig. 1**. Sample sizes were based on previously published stress-studies in zebrafish (Demin *et al.*, 2017, Meshalkina *et al.*, 2018, Volgin *et al.*, 2018, Wang *et al.*, 2020). For conditioning on day 1, two groups were exposed to the vehicle (VEH, distilled water), and two groups were exposed to CAS (see preparation of CAS below). Groups 'VEH/no cue' and 'CAS/no cue' were exposed without a specific visual cue, with only external cues (i.e. the curtains around this area, the cameras, the structure to which the cameras were fixed) acting as spatial reminders. Groups 'VEH/cue' and 'CAS/cue' were exposed in the presence of the visual cues (black and white stripes (Ziani *et al.*, 2018). For re-experience on day 2 (24-h after conditioning), fish were returned to their testing tanks for 6 min and grouped as 'VEH/no cue' and 'CAS/no cue' in the absence of the visual cue, and 'VEH/cue' and 'CAS/cue' in the presence of the visual cue, thus allowing the conditioned stimulus to be assessed. Duration and testing conditions of the experiments are described further below.

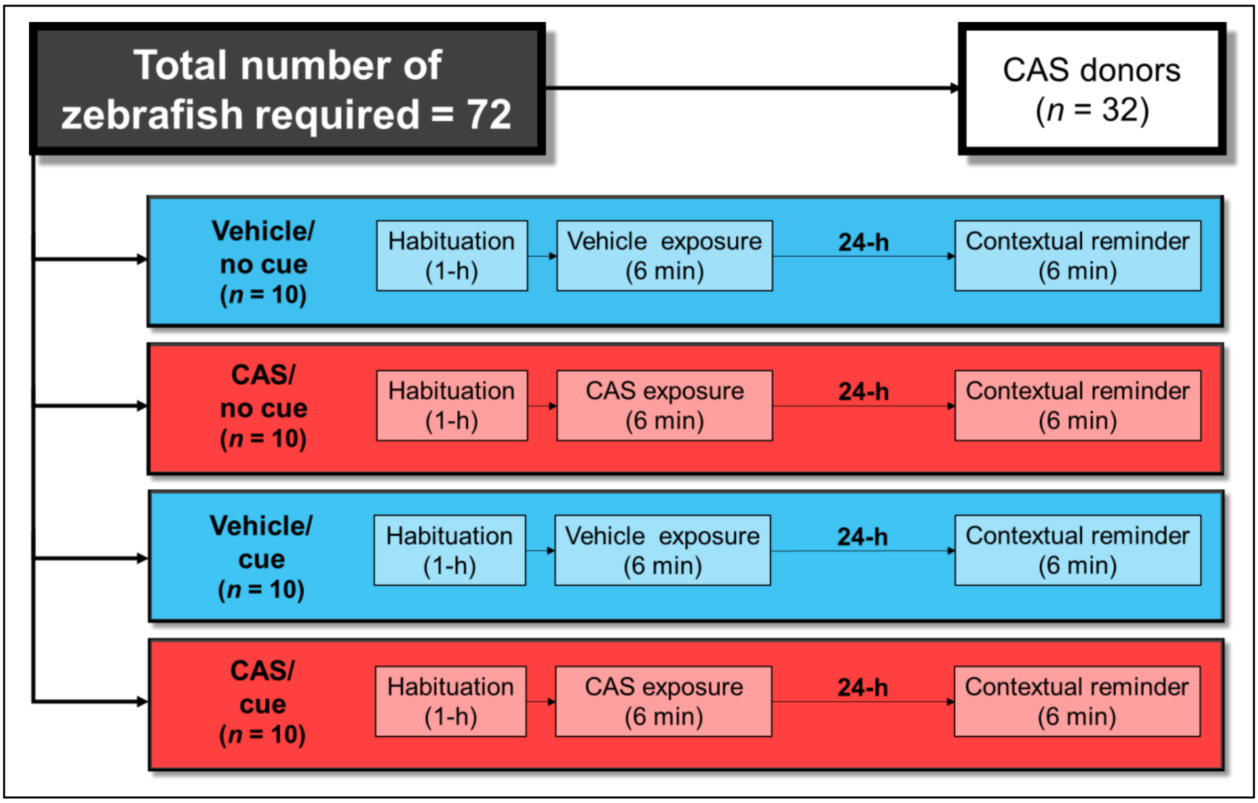

Figure 1: *Study design and timelines of experimental procedures. CAS – conspecific alarm substance.*

2.3. Preparation of Conspecific Alarm Substance (CAS)

CAS was derived from the damaged skin of donor fish, as adapted from the method of Faustino *et al.* (2017) and Speedie and Gerlai (2008). In short, a single donor Zf was collected from the holding tank and received a blow to the head followed by euthanasia by breaking the spinal cord with tweezers. The Zf was then placed in a petri dish, where after 15 shallow cuts were made on each side of the Zf trunk using a surgical scalpel. The cuts on one side of the trunk of the donor Zf was washed with 5 mL of distilled water, where after the Zf was turned over to wash the cuts on the other side with a further 5 mL of distilled water. The 10 mL of collected CAS solution was stored at -20ºC until the commencement of experiments on the following day (Faustino *et al.*, 2017). The solution was allowed to pass through the thawing process 1-h before the commencement of experiments (Faustino *et al.*, 2017). Controls were exposed to the VEH (distilled water).

2.4. Behavioural tests

Stress response in zebrafish was measured in small sized trapezoid test tanks (15 height × 28 top × 22 bottom × 7 width cm), according to Cachat *et al.* (2010). The test tanks were made of matt Perspex on the inside in order to visually block the view to the outside, reducing the influence of the surrounding area and to prevent a mirror effect. Immediately after CAS/VEH exposure, the lateral walls and floor of each test tank were covered with either plain white laminated paper (no cue) or laminated paper in a pattern of alternating black- and white-coloured stripes spaced 2 cm apart (visual cue), as in Ziani *et al.* (2018).

A digital camera (Basler monochrome GigE, 1/1.8” CMOS sensor) was mounted 150 cm in front of the test tanks. All sessions were subsequently video-taped. EthoVision XT 14 video-tracking software was used to analyse zebrafish behaviour, assessing frequency in the top zone (number of entries), time spent in top zone (s), duration immobile (s), and mean meandering (deg/cm), based on the centre-body point location. Two infrared backlights were placed behind the test tanks (110 x 110 cm horizontally and 42 x 80 cm vertically), in order to provide good visibility and contrast during recordings. An infrared filter was attached to the lens of the camera.

Zebrafish behaviour was recorded with the camera mounted in front of the test tanks during both the conditioning (day 1) and re-experience (day 2), with two test tanks recorded in parallel. The test tanks were divided virtually into 2 equal horizontal sections (bottom and top zones) using EthoVision XT 14. The position within the test tank – bottom (indicative of greater anxiety) or top (indicative of less anxiety) – was considered as a general index of anxiety. Frequency (number of entries) and total duration (s) in the top of the test tank was calculated with EthoVision XT 14 software (Noldus IT, Wageningen, Netherlands). Bottom dwelling was assessed as a position of relative safety (Bencan *et al.*, 2009, Caramillo *et al.*, 2015), with reduced top exploration regarded as an anxiogenic-like response to threat (Kalueff *et al.*, 2013).

Locomotor activity, including total duration of immobility (s) and mean meandering (degrees/cm; the degree of turning over distance travelled) was also assessed, according to Egan *et al.* (2009). With immobility (freezing) and meandering a typical response to acute fear-inducing stressors (Kalueff *et al.*, 2013), these alarm reactions were also scored as measurements of anxiety. Immobility was regarded as the total cessation of movement (except for gills and eyes), while meandering was defined as movement without a definite direction (Kalueff *et al.*, 2013). All behaviours recorded fully adhered to the zebrafish behavioural catalogue (Kalueff *et al.*, 2013).

2.4.4. Fear conditioning (Day 1) and re-experience (contextual reminder; Day 2)

Similar to Canzian *et al.* (2017) and Ziani *et al.* (2018), zebrafish were exposed to VEH/CAS in the absence/presence of a co-presenting visual cue, namely black and white stripes, thus producing a stressor-context pairing. Conditioning on day 1 and re-experience on day 2 were performed in the same 2-L trapezoid test tanks. On day 1, fish were individually placed in the test tanks, and recorded following a 1-h habituation period. This was done to eliminate the effect of novelty of the test tank on provoking anxiety, enabling only CAS exposure to act as an aversive experience. During the habituation period the fish were individually transferred from the housing tank to the test tank and allowed to explore freely for 1-h. Immediately thereafter, the lateral walls and floor of each test tank were covered with either plain white laminated paper (groups

'VEH/no cue' and 'CAS/no cue') or laminated paper with the visual cue (groups 'VEH/cue' and 'CAS/cue'). Either 3.5 ml/L of the CAS solution (stressor; groups 'CAS/no cue' and 'CAS/cue') or distilled water (control; groups 'VEH/no cue' and 'VEH/cue') was then delivered to the test tanks using a 5 mL syringe. The exposure lasted for 6 min, after which the animals were returned to the housing tanks. The testing tanks were thoroughly cleaned after each exposure to prevent cross contamination. On day 2, behavioural responses of zebrafish were analysed again by returning fish to the test tanks, this taking place 24-h after conditioning. Behavioural analysis started immediately after the fish were returned to the test tanks. Groups designated as 'VEH/no cue' and 'CAS/no cue' were returned to the test tanks without the visual cues, while groups designated as 'VEH/cue' and 'CAS/cue' were returned to the test tanks with the visual cues. These groups were all tested in the absence of VEH/CAS for 6 min, thus allowing the conditioned stimulus to take effect. Zebrafish were then captured and immediately frozen in liquid nitrogen for 30 s and stored at −80ºC for possible future studies. The latter procedure is the standard form of euthanasia in zebrafish for studies evaluating the stress response (Barcellos *et al.*, 2007, De Abreu *et al.*, 2014, Tudorache *et al.*, 2013).

2.5. Statistical analysis

IBM® SPSS® Statistics (version 26) and GraphPad Prism (version 8) was used for statistical analysis, assisted by the Statistical Consultation Service of the NWU.

Differences in behavioural data between the stressor groups on day 1 (conditioning) and day 2 (re-experience) were analysed using normal two-way analyses of variance (ANOVAs, factors: stressor and time), followed by Bonferroni post-hoc testing. Of note, none of the current data sets passed the Shapiro-Wilk test for normality. Still, normal two-way ANOVAs were employed as univariate analyses, such as ANOVAs, are considered somewhat robust to distributional deviations (Leard Statistics, 2017, Maxwell and Delaney, 2004, Nimon, 2012). Moreover, parametric analyses are generally sufficient in two factorial designs regardless of distributional deviations, as recently shown in a study from our laboratory (Steyn *et al.*, 2020). Moreover, bootstrap estimators (1 000 replicates) were calculated and reported in all group comparisons to improve the robustness of these results. All graphs are expressed as mean ± standard error of the mean, with $p < 0.05$ accepted as significant. Of note, partial eta squared (η_p^2) were used to calculate effect magnitude of ANOVA results to determine the most robust main effect. Only large effect magnitude indicators ($\eta_p^2 \geq 0.14$ (Ellis, 2010) were accepted as significant. All experiments were performed during this study as planned, and all analyses and endpoints assessed were included in the final report without removing outliers.

Behavioural analyses were performed by automated video tracking software (EthoVision XT 14) with statistical analyses of data performed using GraphPad Prism. Automated digital tracking prevents experimenter bias from influencing the results of the experiments. The experimental design and its description here, as well as data analysis and presenting, adhered to the ARRIVE guidelines for reporting animal research and the PREPARE guidelines for planning animal research and testing.

3. Results

The graphs in **Fig. 2-5** show the effect of CAS *vs*. VEH exposure on the respective behavioural parameters, with fewer entries (**Fig. 2**) and less time (**Fig. 3**) in the top zone, as well as higher immobility (**Fig. 4**) and meandering (**Fig. 5**), representing behavioural surrogates for anxiety.

3.1. Fear-related responses

There was no significant interaction between time and stressor with regard to frequency in the top zone ($F_{3,72} = 1.86$, $p = 0.14$, $\eta_p^2 = 0.07$, **Fig. 2**). Nevertheless, the main effect of stressor had a significant effect on frequency in the top zone ($F_{3,72} = 12.49$, $p \leq 0.0005$, $\eta_p^2 = 0.34$). Overall, CAS (irrespective of time and cue) significantly decreased frequency in the top zone (**Fig. 2**).

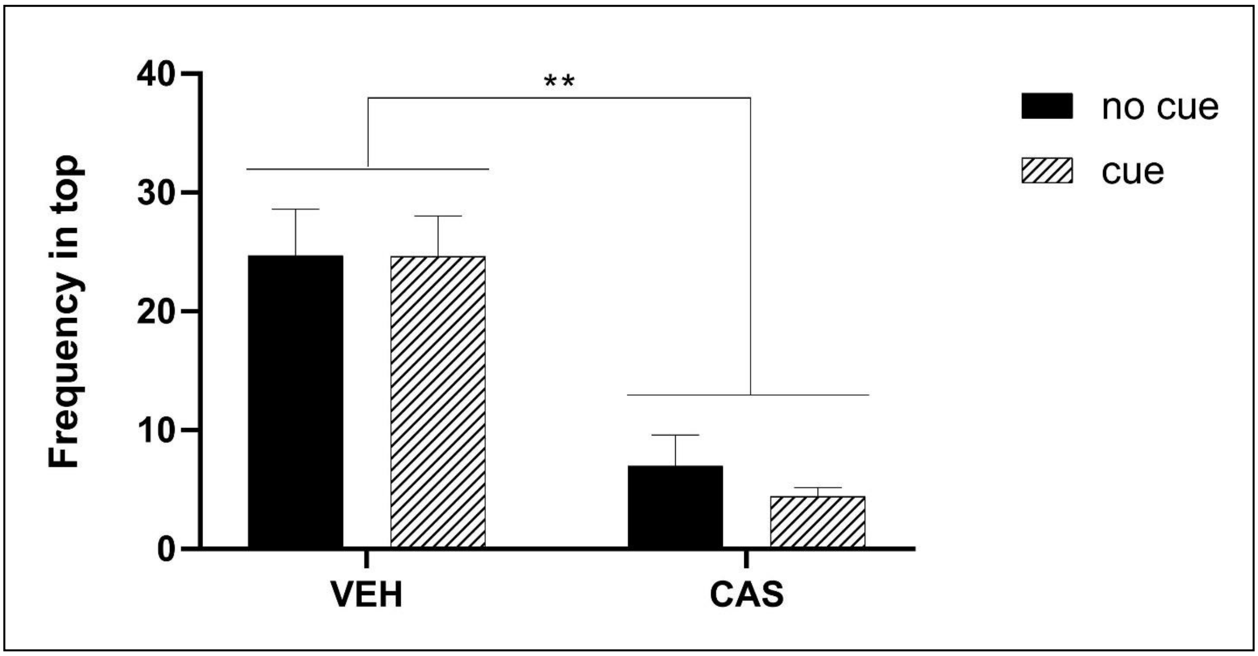

Figure 2: *Main effect of stressor exposure on frequency in the top zone irrespective of the influence of time. Data presented as mean ± standard error of the mean. The frequency in the top zone were significantly lower in the CAS/no cue and CAS/cue groups compared to the VEH/no cue group (p = 0.003, p = 0.001, respectively). Similarly, frequency in the top zone were significantly lower in CAS/no cue and CAS/cue groups vs. VEH/cue group (p = 0.001, p = 0.001, respectively).*

There was no significant interaction between time and stressor with regard to duration in the top zone ($F_{3,72}$ = 0.77, p = 0.51, η_p^2 = 0.03; **Fig. 3**). Nevertheless, both time ($F_{1,72}$ = 5.04, p = 0.03, η_p^2 = 0.07) and stressor ($F_{3,72}$ = 4.80, p = 0.004, η_p^2 = 0.17) significantly influenced duration in the top zone, with the latter accepted as most robust (based on effect magnitude). Overall, CAS (irrespective of time and cue) significantly reduced time spent in the top zone (**Fig. 3**).

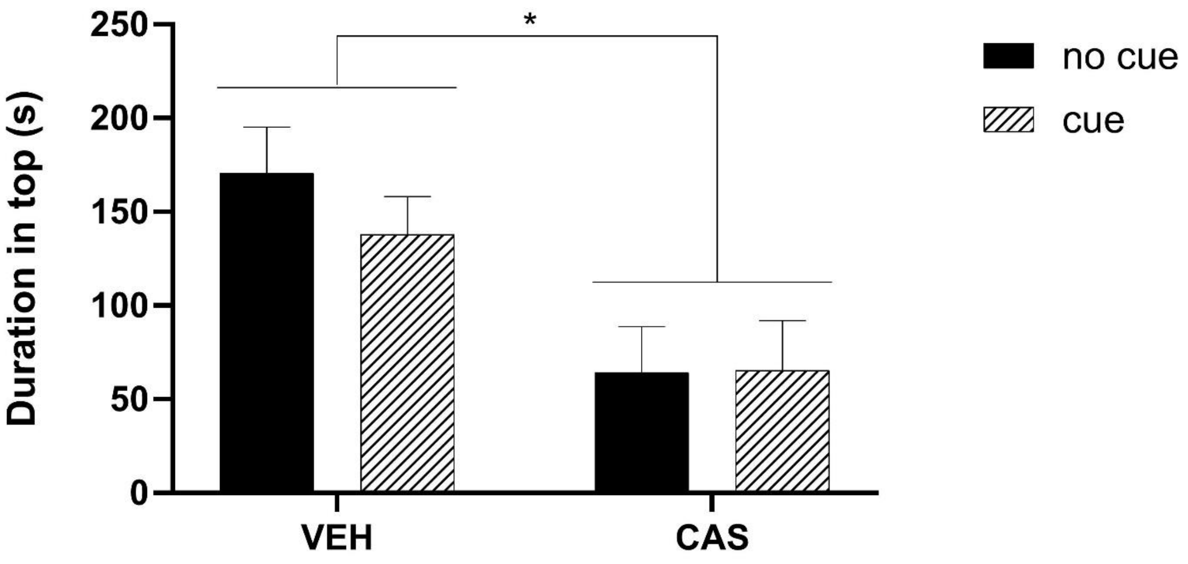

Figure 3: *Main effect of stressor exposure on duration in the top zone (s) irrespective of the influence of time. Data presented as mean ± standard error of the mean. The time spent in the top zone were significantly less in the CAS/no cue and CAS/cue groups compared to the VEH/no cue group (p = 0.005, p = 0.005, respectively). Similarly, time spent in the top zone was significantly less in CAS/no cue and CAS/cue groups vs. VEH/cue group (p = 0.03, p = 0.03, respectively).*

There was no significant interaction between time and stressor with regard to duration of immobility ($F_{3,72} = 1.26$, $p = 0.30$, $\eta_p^2 = 0.05$; **Fig. 4**). Nevertheless, the main effect of stressor had a significant effect on immobility ($F_{3,72} = 11.51$, $p \leq 0.0005$, $\eta_p^2 = 0.32$). Overall, CAS (irrespective of time and cue) significantly increased duration of immobility (**Fig. 4**).

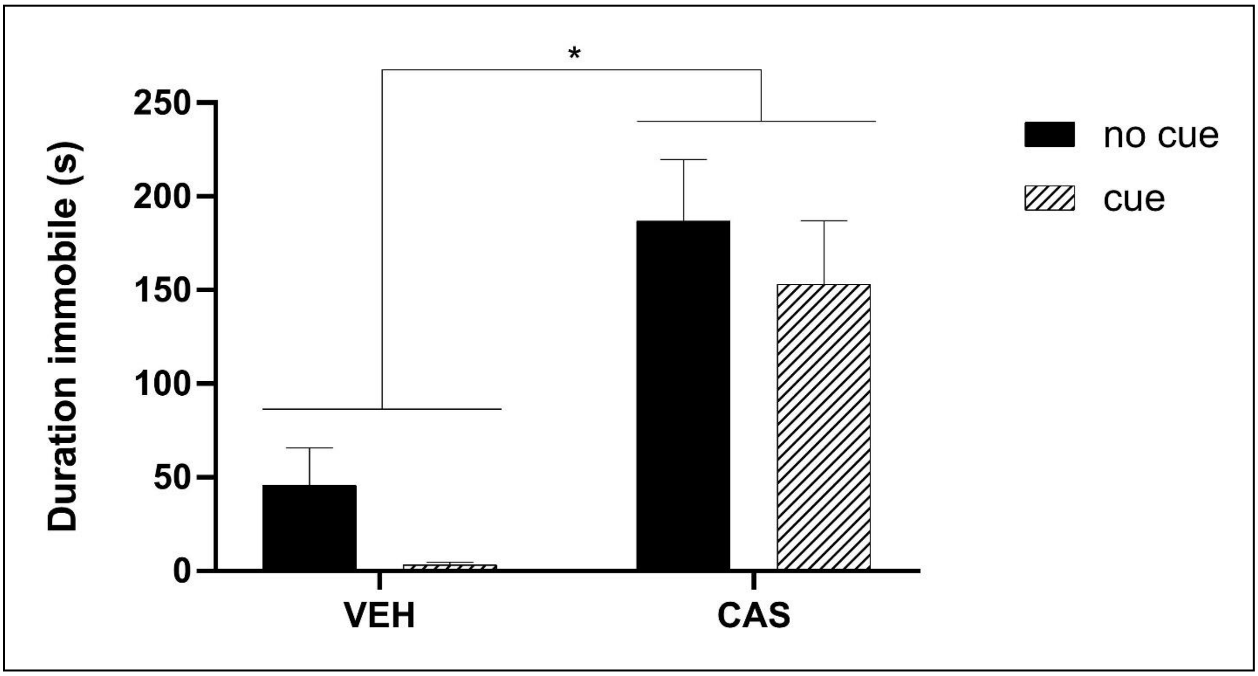

Figure 4: *Main effect of stressor exposure on duration of immobility (s) irrespective of the influence of time. Data presented as mean ± standard error of the mean. Immobility was significantly increased in the CAS/no cue and CAS/cue groups compared to the VEH/no cue group (p = 0.004, p = 0.011, respectively). Similarly, immobility was significantly increased in the CAS/no cue and CAS/cue groups vs. VEH/cue group (p = 0.001, p = 0.001, respectively).*

There was no significant interaction between time and stressor with regard to mean meander ($F_{3,72} = 2.66$, $p = 0.054$, $\eta_p^2 = 0.10$; **Fig. 5**). Nevertheless, the main effect of stressor had a significant effect on meandering ($F_{3,72} = 8.76$, $p \leq 0.0005$, $\eta_p^2 = 0.27$). Overall, CAS (irrespective of time and cue) significantly increased meandering (**Fig. 5**).

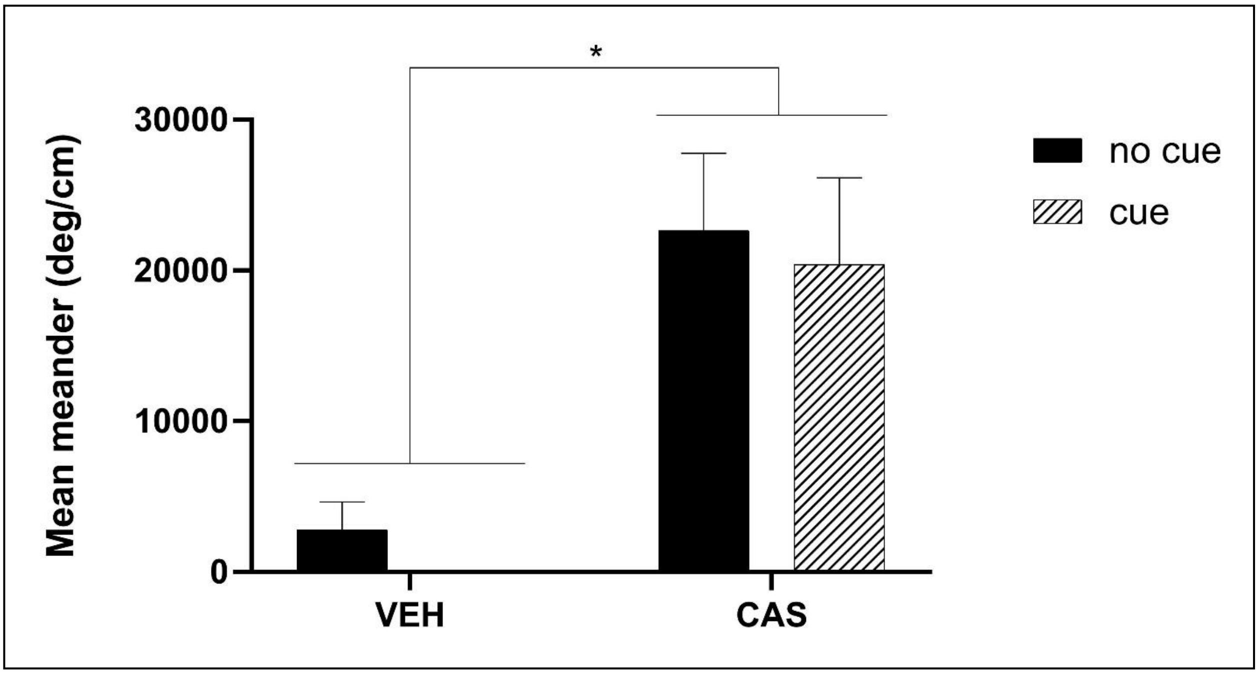

Figure 5: *Main effect of stressor exposure on mean meander (deg/cm) irrespective of the influence of time. Data presented as mean ± standard error of the mean. The mean meander was significantly increased in the CAS/no cue and CAS/cue groups compared to the VEH/no cue group (p = 0.003, p = 0.014, respectively). Similarly, mean meander was significantly increased in the CAS/no cue and CAS/cue groups vs. VEH/cue group (p = 0.002, p = 0.006, respectively).*

4. Discussion

The present study demonstrated that CAS evokes a significant anxiogenic response in zebrafish irrespective of time. Therefore, CAS increased fear/anxiety behaviour on both day 1 of exposure, and on day 2 in zebrafish previously exposed to CAS presented with the contextual reminder. This 're-experiencing' phenomenon is indicative of PTSD-like fear contextualisation, where a conditioned stimulus (e.g., a visual cue and a spatial location) is paired with an aversive unconditioned stimulus (e.g., CAS).

A central aspect of this study is that a putative animal model of PTSD should not only evoke the typical anxiety-related behavioural responses noted in human patients but should result in sustained fear responses to trauma-related cues in the *absence* of the original stressor. In this regard, a key clinical characteristic in PTSD is sensory/emotional hypermnesia, which is characterised as contextual reminders of the original trauma sustaining fear responses, even to the point of sensitising patients and worsening the disorder over time (Desmedt *et al.*, 2015, Parsons and Ressler, 2013). Therefore, we hypothesised that a spatial location *per se* would not be robust enough as a reminder to induce fear-related behaviour 24-h after conditioning (CAS exposure), and that it would require pairing a visual cue with CAS to serve as a robust contextual reminder. However, our results disproved this hypothesis, since there was no significant difference in the behaviour displayed between the CAS-exposed groups that received the visual cue versus those that did not. In contrast, we show (**Fig. 2-5**) that exposure to CAS, and not the presence of the visual cue, is the more significant stressor irrespective of time, with CAS *vs*. VEH being the common difference between the groups rather than cue *vs*. no cue. In other words, returning the fish to the context in which it was originally exposed to CAS 24-h after conditioning, whether with or without a visual cue, evokes a fear-like response in the absence of the aversive stimulus. This indicates that a spatial location where CAS was introduced (i.e. external cues that can be observed by the fish from the testing area, such as curtains, cameras, the structure to which the cameras are fixed) is an adequate reminder, and no pairing of a spatial reminder with a visual cue is needed to induce fear conditioning in CAS-exposed zebrafish. Furthermore, there was no significant difference in the behaviour displayed by the VEH group that received the visual cue and the VEH group that did not, indicating that the visual cue alone is not a stressor for zebrafish.

In general, abnormal human stress responses, such as fear underlying PTSD, are caused by dysfunctional neurobiological mechanisms that under normal circumstances have evolved to promote aversion to danger, or predator avoidance in the case of zebrafish (Gerlai, 2010, Stewart *et al.*, 2014b). In this regard, zebrafish display overt anti-predatory responses in reaction to direct or simulated stimuli, as well as exposure to predator or CAS (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart *et al.*, 2014b). Fear/anxiety-like behaviours displayed by zebrafish include increased geotaxis, freezing, and erratic movements (Caramillo *et al.*, 2015, Egan *et al.*, 2009). The results of the present study support these findings, since acute CAS exposure evoked fear-related behaviour in zebrafish, including fewer entries and less time in top of the test tanks (**Fig. 2-3**), as well as higher immobility and meandering (**Fig. 4-5**). Preference for the bottom of the tank is often a response to threat, and is generally considered a sensitive measure of anxiety (Kalueff *et al.*, 2013). Furthermore, immobility is often synonymous with freezing, representing a common response to a stressor or increased anxiety (Kalueff *et al.*, 2013). Likewise, meandering reflects erratic movements, and is often increased during high levels of anxiety (Kalueff *et al.*, 2013).

As noted earlier, an essential component of PTSD is fear conditioning, while zebrafish do contextualize fear (Ogawa *et al.*, 2014). Avoidance conditioning in zebrafish has also been shown (Blank *et al.*, 2009), as they refrain from swimming from the white to the dark compartment in order to avoid an electric shock. Furthermore, fear conditioning has previously been demonstrated in zebrafish by pairing visual cues with CAS (Blaser and Vira, 2014, Hall and Suboski, 1995), as in the present study (**Fig. 2-5**). Thus, our results showed that a contextual reminder elicited fear-like geotaxis, freezing, immobility and meandering in zebrafish 24-h after CAS exposure in the absence of the aversive stimulus (CAS), but that time (i.e., behaviour assessed either on day 1 or day 2) didn’t play a role in the observed behavioural response meaning the level of anxiety in zebrafish was the same on day 1 and on day 2. This indicates that a sensory reminder (e.g., a visual cue), when associated with the initial stressor, maintains the fear response in the absence of the original fearful event, similar to that observed in rodent models of PTSD and clinical PTSD. For example, using a conditioned taste aversion paradigm in rats, single prolonged stress (a rodent model of PTSD) with or without re-stress enhances associative sensory aversion learning 7 days after stressor-taste pairing (Brand *et al.*, 2008).

Alternatively, the preservation of the stress-related behaviour displayed by the zebrafish might be attributable to the original trauma itself, rather than re-experience by contextual fear memory. Although it is commonly accepted that subsequent sensory and contextual reminders of the original trauma perpetuate full-blown PTSD, our results substantiate the understanding that the intensity and duration of the initial traumatic event is integral to the development of PTSD (Flor and Nees, 2014, Kang *et al.*, 2003). To this end, repetitive memory intrusions following a traumatic experience reflect the original traumatic event without direct interplay between the two (Pace-Schott *et al.*, 2015). As such, it has been proposed that PTSD patients develop abnormal threat perception making it difficult for them to accept the traumatic event as distinct and completed (Blain *et al.*, 2013). Instead, PTSD patients experience heightened perceptions of current threat, as well as involuntary flashbacks to the traumatic event, even in the absence of contextual reminders (Blain *et al.*, 2013, Desmedt *et al.*, 2015). These flashbacks induce behavioural re-enactments during which the trauma is relived as if it was being repeated at present (Desmedt *et al.*, 2015, Van der Kolk *et al.*, 2001). Moreover, considering our findings, earlier studies have noted that residual anxiety induced by the initial stressor may recur in zebrafish in the absence of an external reminder (Piato et al., 2011). We propose that this is indicative of *de novo* fear conditioning in which trauma exposure dysregulates fear extinction and extinction retention (Lisieski *et al.*, 2018). That is to say that the contextual processing of aversive stimuli is disrupted, which diminishes the ability to use contextual cues to differentiate between aversive and safe conditions (Garfinkel *et al.*, 2014, Lisieski *et al.*, 2018). Our preliminary findings support this conclusion. However, subsequent studies should track behavioural fear responses post-trauma, assessing the time to extinction, as well as perpetuation with intermittent reminders, to fully establish translational validity of this model for PTSD research.

The overall increase in fear/anxiety-like behaviour displayed by zebrafish in the present study contributes to the face validity of CAS/contextual reminder as an effective stressor/re-experience PTSD model in zebrafish. However, future work is required to establish this model with respect to construct and predictive validity. Forthcoming biological analyses, including whole-body cortisol and whole-brain monoamine analyses, will confer construct validity for the model, while treatment response to pharmacological agents used in the treatment of PTSD, such as serotonin reuptake inhibitors (SRI), should be considered to establish its predictive validity.

A possible confounder to these findings is that time of assessment did not alter the behavioural outcome, meaning the level of anxiety in zebrafish is the same on day 1 or day 2. This implies that residual effects of the day 1 stressor (CAS) may persist in zebrafish for a limited period (Piato *et al.*, 2011), thus offering a plausible explanation for the maintenance of fear response in the absence of the original stressor on day 2. Therefore, the original trauma itself rather than its re-experience by contextual fear memory, may perpetuate aberrant behaviour, which is arguably different from the neuro-progression of PTSD over time (Yang *et al.*, 2020). Subsequent studies to track behavioural responses at several time points distal to the traumatic event will provide significant insight. Therefore, studies where zebrafish are re-tested at behavioural and biological levels (e.g., whole-body cortisol) 7 and/or 14 days after conditioning are recommended in order to confirm true validity of the model for PTSD (see Yang *et al.* (2020). Furthermore, further validation to confirm the presence of other PTSD-like symptoms, such as scototaxis and thigmotaxis, that progress over time would also be valuable, as would assessment of trauma-induced deficits in explicit memory.

Another possible limitation is whether CAS is effective to elicit a sustained anxiety response. unless zebrafish are co-presented with more profound stress procedures such as crowding, food deprivation, shaking, predator exposure, or shallow water (Song *et al.*, 2018). Future studies may therefore more rigorously evaluate the dose-dependent effects of CAS on fear conditioning responses, and whether it is indeed an adequate fear-inducing factor relevant and/or specific to PTSD. Additionally, an avenue worth pursuing is to assess the efficacy of subjecting zebrafish to a triple stressor, similar in construct to rodent trauma procedures, in eliciting sustained fear/anxiety-like behaviour as in clinical PTSD (Uys *et al.*, 2006). Although we are not aware of studies that have addressed the issue of CAS stability over time, it is feasible that the integrity of CAS deteriorates if it is stored overnight at -20ºC, as was done in this study. We therefore recommend that fresh CAS is prepared each morning before behavioural testing to increase the intensity of anxiogenic responses. On this note, zebrafish present with prominent intra- and interindividual variability in odour driven behaviours, thus confirming that CAS does *not* elicit reproducible responses (Kermen *et al.*, 2020). This adds complexity to interpreting CAS-mediated responses. Further, exclusively using females as CAS donors may also be advantageous as males of some fish species produce CAS only intermittently while females produce CAS more reliably than males (Stephenson, 2016). That said, this phenomenon has not been studied in zebrafish. Finally, conspecific blood has also been reported to evoke defensive behaviours (freezing and geotaxis) in zebrafish and provides an additional approach to inducing fear conditioning (Kermen *et al.*, 2020).

In conclusion, we hypothesised that CAS (with/without a visual cue) would evoke an anxiogenic response on day 1 that would extinguish by day 2 post-exposure but be sustained in fish co-exposed to CAS and a visual cue. Our results demonstrate that CAS exposure does indeed induce fear/anxiety-like behaviour in zebrafish. Moreover, such conditioning can be replicated by pairing CAS with a contextual reminder (spatial location), thus eliciting fear-like behaviour on day 2 but in the absence of CAS. However, anxiety responses were independent of the conditioned cue (i.e. independent of time and cue). Because fear conditioning is an essential component of PTSD, this supports using a 2-day fear conditioning protocol in zebrafish to model PTSD, although more exploratory research is needed to robustly validate the perpetuation of behavioural responses over time using this protocol. Such a protocol, once established, may provide a useful high throughput research tool to better understand the underlying pathobiology of PTSD and to investigate novel anti-PTSD drugs.

Authors’ contributions

Heslie Loots: Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Visualisation. **Marli Vlok:** Conceptualisation, Methodology, Resources, Writing - Review and Editing, Supervision, Funding Acquisition. **Stephan Steyn:** Formal Analysis, Writing - Review and Editing. **Allan Kalueff***:* Writing - Review and Editing. **Brian Harvey:** Conceptualisation, Resources, Data Curation, Writing - Review and Editing, Visualisation, Supervision, Project Administration, Funding Acquisition.

Conflict of interest

The authors declare no conflict of interest.

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4. CONCLUSION

The aim of this project was to expand the platform of post-traumatic stress disorder (PTSD) research at North-West University (NWU) by establishing a novel translational model of PTSD in zebrafish. Therefore, we assessed the fear- and anxiety-like responses elicited in zebrafish upon exposure to conspecific alarm substance (CAS) and the impact of re-experience using a contextual reminder that has previously been paired with CAS.

PTSD patients typically present with sensory hypermnesia, which is characterised as contextual reminders of the original trauma sustaining fear responses, even to the point of sensitising patients and worsening the disorder over time (Desmedt *et al.*, 2015, Parsons and Ressler, 2013). Building on this, it is commonly accepted that an animal model of PTSD must replicate the following key characteristics of clinical PTSD:

1. Typical fear- and anxiety-like behaviour induced by stress.
2. Sustained fear responses to trauma-related cues in the *absence* of the original stressor.

Keeping this in mind, the present study confirmed that CAS exposure elicits a significant anxiogenic response in zebrafish. Further, CAS increased fear- and anxiety-like behaviour in zebrafish not only during exposure, but also when zebrafish are presented with a contextual reminder. This 're-experiencing' phenomenon is indicative of PTSD-like fear contextualisation, where a conditioned stimulus is paired with an aversive unconditioned stimulus. In the current investigation spatial location acted as the conditioned stimulus and CAS as the unconditioned stimulus.

The neurobiological mechanisms that have evolved to promote avoidance of danger are typically dysregulated in PTSD patients, leading to abnormal stress responses, such as exaggerated fear (Gerlai, 2010, Stewart *et al.*, 2014). Similarly, zebrafish display overt anti-predatory responses in reaction to exposure to predators or CAS (Gerlai, 2010, Speedie and Gerlai, 2008, Stewart *et al.*, 2014). Fear- and anxiety-like behaviours displayed by zebrafish include increased geotaxis (bottom dwelling), freezing (immobility), and erratic movements (Caramillo *et al.*, 2015, Egan *et al.*, 2009), which are supported by the findings of the present study.

Because fear conditioning is an essential component of PTSD and zebrafish possess the ability to contextualize fear (Ogawa *et al.*, 2014), this species is ideal for translational PTSD studies. Accordingly, fear conditioning has previously been established in zebrafish by pairing visual cues with CAS (Blaser and Vira, 2014, Hall and Suboski, 1995), as reiterated in the current project. Thus, our results showed that a contextual reminder elicited sustained fear-like geotaxis, freezing, immobility and meandering in zebrafish 24-h after CAS exposure in the absence of the original aversive stimulus (CAS). This is similar to that observed in rodent models of PTSD and clinical

PTSD. For example, a PTSD study from our laboratory found that single prolonged stress in rats, with or without re-stress, enhances associative sensory aversion learning 7 days after stressor-taste pairing (Brand *et al.*, 2008).

We hypothesised that re-experience through the introduction of a contextual reminder of the original trauma would evoke sustained fear- and anxiety-like behaviour 24-h after conditioning (CAS exposure). Moreover, we proposed that a spatial location alone would not be robust enough as a reminder and that this would necessitate pairing CAS with a visual cue to serve as a more robust contextual reminder. However, the experimental results disproved this hypothesis, since there was no significant difference in the behaviour displayed between the CAS-exposed groups that received the visual cue and those that did not. Conversely, our research showed that CAS exposure evoked significant fear- and anxiety-like behaviour, with CAS *vs*. vehicle being the common difference between the groups rather than cue *vs*. no cue. Thus, CAS exposure, and not the presence of the visual cue, was the traumatic stressor in these experiments, both during initial exposure and re-experience. This indicates that an anxiogenic response is evoked by returning zebrafish to the context in which it was originally exposed to CAS in the absence of the aversive stimulus, whether with or without a visual cue. Therefore, the spatial location where CAS was introduced is an adequate reminder, and no pairing of a spatial reminder with a visual cue is required to facilitate fear conditioning in CAS-exposed zebrafish. Likewise, the vehicle groups that received the visual cue and those that did not, also did not display significant differences in behaviour, substantiating that the visual cue when presented alone does not induce a stress response in zebrafish.

A noteworthy finding is that time of assessment did not alter the behavioural outcome, meaning the level of anxiety in zebrafish was the same on day 1 and on day 2. Therefore, the original trauma itself, rather than re-experience by contextual fear memory, seems to perpetuate the evinced stress-related behaviour. Although subsequent sensory and contextual reminders of the original trauma are recognised to perpetuate full-blown PTSD (Flor and Nees, 2014), our findings are congruent with the understanding that the intensity and duration of the initial traumatic event is critical to the development of PTSD (Kang *et al.*, 2003). As such, repetitive memory intrusions following a traumatic experience reflect the original traumatic event without direct interaction between the two (Pace-Schott *et al.*, 2015). Accordingly, it has been suggested that PTSD patients develop abnormal threat perception making it difficult for them to accept the traumatic event as distinct and completed (Blain *et al.*, 2013). Instead, these individuals experience heightened perceptions of current danger, as well as involuntary flashbacks to the traumatic event, even in the absence of external reminders (Blain *et al.*, 2013, Desmedt *et al.*, 2015). These flashbacks elicit behavioural re-enactments during which the PTSD patient relives the trauma as if it was being repeated at present (Desmedt *et al.*, 2015, Van der Kolk *et al.*, 2001). Considering our findings, earlier studies have noted that residual anxiety induced by the initial stressor may persist in zebrafish, thus in the absence of a contextual reminder (Piato et al., 2011). We suggest that this is indicative of *de novo* fear conditioning in which trauma exposure disrupts fear extinction and extinction retention (Lisieski *et al.*, 2018). In other words, the contextual processing of aversive stimuli is impaired, leading to a reduced ability to use contextual cues to differentiate between aversive and safe conditions (Garfinkel *et al.*, 2014, Lisieski *et al.*, 2018). Our preliminary data lend credence to this conclusion. Importantly, in order to verify the translational validity of this model for PTSD, subsequent studies should track behavioural fear responses post-trauma, assessing the time to extinction, as well as perpetuation with intermittent reminders.

The overall increase in fear- and anxiety-like behaviour displayed by zebrafish in the present study contributes to the face validity of CAS ± a contextual reminder as an effective stressor ± re-experience model of PTSD in zebrafish. However, future work is required to establish this model with respect to construct and predictive validity. Building on the foundation of this project, forthcoming projects in our laboratory should further validate fear conditioning as a model of PTSD in zebrafish by performing additional behavioural assays and biological analyses (e.g. whole-body cortisol and whole-brain monoamine analyses) to establish the face and construct validity of the model. Moreover, investigating the response to pharmacological agents used in the treatment of PTSD are needed to ascertain predictive validity. A well-validated PTSD model is zebrafish will allow cost-efficient high-throughput screening (HTS) of novel PTSD treatments at the NWU.

To conclude, this project recapitulates the suitability of using zebrafish to model PTSD, although further development is necessary. Not only do zebrafish present with a good balance between system complexity and practical simplicity but they are also a time- and cost-effective addition to rodent models in neuropsychiatric research. Considering the need for a rapid, reliable and cost-effective HTS platform for novel drug discovery in PTSD, this project has added substantially to PTSD research in South Africa. Although several universities nationally are currently using zebrafish as a research tool, our laboratory is the first to focus on neuropsychiatric research, making this a very innovative project. This work has also bolstered research at the NWU by complimenting and extending its current research capacity.

4.1. Summary of observations

In summary, the primary observations from the present study are as follows:

- CAS exposure resulted in anxiety-like behaviour in zebrafish, including fewer entries and less time spent in the top zone of the test tanks, as well as higher levels of immobility and meandering.

- Contextual re-experience seemed to sustain the above-mentioned behavioural responses to CAS.
- A translational zebrafish model of PTSD based on a 2-day fear conditioning protocol, with CAS + a contextual reminder as an effective stressor + re-experience to evoke PTSD-like symptoms in zebrafish, was developed.
- The face validity of this model, specifically focussing on evidence of anxiety, has been established.

4.2. Limitations and recommendations

Further validation of this model is required, including additional behavioural assays to augment face validity, as well as biological analyses and the assessment of pharmacological PTSD treatment response to establish construct and predictive validity, respectively. Although contextual re-experience seemed to sustain anxiety following the initial exposure to CAS, our data did not fully support this conclusion and further work is needed to confirm this.

These and other potential confounders and limitations to the findings of the current project are described in table 4-1, as well as recommendations to address these uncertainties.

Table 4-1: *Limitations and recommendations.*

Limitations	***Recommendations***
The current project employed a 2-day protocol to induce PTSD-like symptoms. Anxiety seemed to be perpetuated by contextual re-experience following the initial exposure to CAS, however, our data did not fully support this conjecture. Alternatively, the original trauma itself, rather than re-experience, may have sustained fear behaviour. As such, earlier studies have noted that residual effects of the original stressor (CAS) were maintained in zebrafish (Piato *et al.*, 2011). Therefore, to determine whether PTSD is perpetuated by sensory reminder or by the original trauma, subsequent studies should track behavioural fear responses post-trauma over a longer period.	• Subsequent studies that track behavioural responses at several time points distal to initial exposure to the traumatic stressor will provide valuable insight into the progression of fear- and anxiety-like behaviours. A possible way to address this is to replicate or improve on the zebrafish model of PTSD used in a study by Yang *et al.* (2020). In their study, testing for stress-induced behavioural alterations in zebrafish started 7 days after initial exposure to the traumatic stressor with the animals euthanised on day 10 for the analysis of PTSD biomarkers (Yang *et al.*, 2020). However, to determine whether PTSD-like symptoms are perpetuated by sensory reminders or by the original trauma itself, we recommend the addition of a re-experience session prior to the behavioural assays to provide a reminder of the original stress.

Table 4-1: *Limitations and recommendations (Continued).*

Limitations	***Recommendations***
Zebrafish were subjected to a limited number of behavioural tests; thus, opportunity exists to bolster the face validity of this zebrafish model of PTSD.	• Expanding the anxiety protocol by adding the light/dark test to measure scototaxis, will reinforce the presence of PTSD-like symptoms and strengthen the face validity of the fear conditioning model of PTSD in zebrafish. • Zebrafish are highly social animals and typically swim in shoals. For this reason, we recommend the measurement of shoal cohesion in zebrafish triplets to examine disrupted social behaviour elicited by trauma exposure. • The assessment of trauma-induced deficits in explicit memory will be beneficial. To achieve this zebrafish should be subjected to a learning and memory test, such as the T-maze or Y-maze tests.
The present study only assessed the face validity of a fear conditioning-based model of PTSD in zebrafish. However, this model needs to be assessed with respect to construct and predictive validity.	• Biological analyses, such as whole-body cortisol assays, are needed to confer construct validity. This calls for application of tissue cortisol extraction methods, as well as assays to subsequently measure cortisol levels, such as enzyme-linked immunosorbent assay (ELISA) kits or, alternatively, high performance liquid chromatography (HPLC). • Serotonin and noradrenaline, along with other monoamines, play an important role in the neurobiology of PTSD (Oosthuizen *et al.*, 2005). Therefore, whole-brain analyses will add to the construct validity of a PTSD model in zebrafish. To this end, the zebrafish brain must be dissected from the skull, followed by analyses of monoamines using HPLC or ELISA. Alternatively, quantitative real-time polymerase chain reaction analyses can be employed to assess the expression of genes that code for monoamine receptors and other PTSD biomarkers (Airhart *et al.*, 2007, Stockhammer *et al.*, 2009, Tudorache *et al.*, 2013, Yang *et al.*, 2020).

Table 4-1: *Limitations and recommendations (Continued).*

Limitations	***Recommendations***
	- Forthcoming studies should examine treatment response to pharmacological agents commonly used in the treatment of PTSD, such as serotonin reuptake inhibitors (SRI), in zebrafish to establish predictive validity. Furthermore, we propose dose-ranging studies to determine the most appropriate dose of the drug to serve as positive control in the screening of novel PTSD treatments.
Only approximately 30% of individuals who survive a traumatic or life-threatening event will develop clinical PTSD (Nemeroff *et al.*, 2006). Similar to humans, animals also respond to stress in a heterogeneous manner, with zebrafish displaying divergent behavioural phenotypes (Caramillo *et al.*, 2015, Cohen *et al.*, 2003, Cohen *et al.*, 2004, Moretz *et al.*, 2007, Toms *et al.*, 2010). This raises the question whether dividing the zebrafish into well-adapted and maladapted groups following CAS exposure based on their performance in behavioural paradigms will better represent clinical PTSD.	- We suggest the application of column statistics to determine the upper and lower percentiles of distribution for behavioural tests to identify well-adapted and maladapted animals. This is of value for zebrafish research to establish how risk and resilience contribute towards the development of PTSD-like symptoms.
Of note is that zebrafish display prominent intra- and inter individual variability in fear responses (Kermen *et al.*, 2020). Indeed, a recent study shows that CAS does not induce consistent fearful responses in zebrafish (Kermen *et al.*, 2020). This complicates the interpretation of CAS mediated responses.	- Studies evaluating the dose-dependent effects of CAS on fear conditioning responses are encouraged. - The males of some fish species produce CAS only intermittently while females produce CAS more reliably than males (Stephenson, 2016). This phenomenon has not been studied in zebrafish, and therefore the exclusive use of female zebrafish as CAS donors might be advantageous. Such explorative studies are recommended.

***Table 4-1:** Limitations and recommendations (Continued).*

Limitations	***Recommendations***
An added concern is whether CAS is robust enough to evoke sustained anxiety responses unless zebrafish are co-presented with more profound stress procedures such as crowding, food deprivation, shaking, predator exposure, or shallow water (Song et al., 2018).	• Alternative alarm cues, such as conspecific blood, hypoxanthine 3-N-oxide or chondroitin sulphate, should be considered (Barkhymer *et al.*, 2019, Gallus *et al.*, 2016, Kermen *et al.*, 2020). Comparing the fear- and anxiety-like behaviours elicited by these alarm cues to that of CAS will assist in identifying the most potent alarm cue. An added benefit of using hypoxanthine 3-N-oxide or chondroitin sulphate is that, contrary to CAS, the doses of these alarm cues are ascertainable to facilitate standardisation. • A worthwhile approach is to assess the efficacy a triple stressor strategy, similar in construct to rodent trauma procedures, in eliciting sustained fear/anxiety-like behaviour in zebrafish (Uys *et al.*, 2006). For example, Yang *et al.* (2020) used an acute severe stress protocol where zebrafish were stressed in three consecutive sessions: 1) including vortex in cold water paired with light exposure, 2) shallow water exposure, and 3) restraint stress.
In the present study zebrafish were snap frozen in liquid nitrogen. This form of euthanasia is standard in zebrafish for studies evaluating the stress response (Barcellos *et al.*, 2007, De Abreu *et al.*, 2014, Tudorache *et al.*, 2013). However, this method is subsidiary to the preferred form of euthanasia in the vast majority of ordinary zebrafish studies, *viz.* chemical methods.	• Snap freezing is the accepted form of euthanasia in stress research in zebrafish because it has minimal effects on cortisol levels. It has been reported, however, that eugenol also does not interfere with the detection of increased cortisol levels (Davis *et al.*, 2015). We suggest a study comparing whole-body cortisol concentrations between zebrafish that were snap frozen and zebrafish immersed in eugenol baths. The results of such a study would affirm the most ethical yet suitable form of euthanasia for reliable whole-body cortisol determinations in zebrafish. This considers the refinement principle in the 4R’s of ethical considerations.

***Table 4-1:** Limitations and recommendations (Continued).*

Limitations	***Recommendations***
Zebrafish were allowed to habituate for 1-h after being transferred to the test tank prior to conditioning. This was done to eliminate the effect of novelty on the anxiety response. However, we deemed a habituation period unnecessary when transferring the zebrafish to the same tank for re-experience since the test tank was no longer a novel environment by this stage of the protocol. What we failed to consider was that the anxiety-like behaviour may have been exacerbated in response to the handling stress of transferring the fish from the housing tank to the test tank for re-experience.	• We advocate the development of automated techniques where manual intervention by the experimenter is reduced. This would entail the design and construction of a custom testing unit which consists of an external tank equipped with a gate mechanism which opens to a test tank (Parker *et al.*, 2013). In practice, the external tank will function as a housing tank for the duration of the study and zebrafish will need to be housed and, therefore, tested as triplets in order to prevent social isolation. For exposures and behavioural assays, the gate will be raised to allow the zebrafish to enter the test tank and lowered once all the zebrafish entered the test tank. This will be repeated at the end of the assays to allow the fish to return to the housing tank. Such a unit will therefore eliminate the need to transfer the zebrafish between the housing and test tanks. Moreover, CAS will be administered to the zebrafish with minimal outside intrusion by syringing the solution through long, flexible and transparent PVC tubing which opens into the test tank (Faustino *et al.*, 2017). Finally, drainage valves fitted at the base of the unit would allow flow for changing contaminated water (CAS, animal waste and surplus food) with fresh tank water.

4.3. Ethical statement

The Water Research Group (WRG) from the Unit for Environmental Sciences and Management at the NWU recently installed the National Aquatic Bioassay Facility (NABF) in 2017. The NABF is the largest zebrafish bioassay facility in Africa. Currently, the WRG focuses on ecotoxicology, ecology and environmental parasitology using locomotor activity as an effect endpoint (Hadfield, 2020). However, zebrafish models are rapidly gaining popularity as a research tool to study brain disorders at several laboratories globally. As such, the Animal Models Project of the South African Medical Research Council (SAMRC) Unit on Risk and Resilience in Mental Disorders under the direction of Prof Brian Harvey set out to establish anxiety and stress models in zebrafish at the Department of Pharmacology, School of Pharmacy, NWU. We must, therefore, take responsibility for the welfare of the zebrafish used in scientific studies in our department. Hence, future zebrafish research performed in our laboratory should build on the following ethical strategies realised in the present study:

- I received training and was certified in Aquatic Ectothermic Vertebrates Handling and Ethics prior to beginning the study (Addendum C).
- I studied the biological characteristics, behaviour, genetic constitution and nutritional needs of zebrafish and used this knowledge to inform the project proposal for the present study.
- A power analysis (Addendum D) based on previously published zebrafish studies (Demin *et al.*, 2017, Meshalkina *et al.*, 2018, Volgin *et al.*, 2018, Wang *et al.*, 2020) was done in collaboration with the Statistical Consultation Service (Prof Suria Ellis) of the NWU to determine the ideal sample size for the experiments in this study.
- All experiments and procedures were approved by the NWU AnimCare committee (Addendum E, NHREC registration AREC-130913-015; approval number NWU-00169-18-A5).
- The animals employed in the present study were bred in captivity and thus no animals were taken out of their natural habitat.
- The animals were transported from the NABF to a behavioural testing facility within the same building 24-h prior to testing to allow them to habituate to the housing tanks.
- Zebrafish were transported to the zebrafish behavioural laboratory, Department of Pharmacology, in large opaque buckets made of plastic (1-2 fish per 1-L tank water) to allow for large water volumes and to prevent overcrowding. The large volume also contained more oxygen and lessened temperature fluctuations during transport. The buckets were filled with tank water from the Zebtec Housing System immediately prior to transferring the zebrafish to ensure the optimal temperature, aeration, salinity and pH. The transport buckets were set up in close proximity to the Zebtec Housing System and fish were transferred to these buckets with a dip to prevent rough handling. The opaque transport buckets were covered with opaque lids to reduce visual stress from the outside. The buckets were then carefully placed on a trolley and transported from the NABF in the basement to our testing facility on the third floor via the elevator. This method of transport was chosen to reduce slopping water and to avoid injuring the fish.
- Overcrowding was prevented by housing zebrafish in housing tanks to a maximum density of 5 fish per litre tank water.
- The lateral sides of the housing tank, excluding the side facing the wall, were covered with cardboard to prevent visual stress from the outside. Light was still allowed into the tank

from the uncovered side of the tank and the hole in the canopy to maintain the light-dark cycle.

- An 8-h period passed after transportation before the fish were fed, this to allow them to first acclimatise to their new environment. The animals were then fed twice daily at 08:30 and 16:00. They received approximately half a teaspoon ZM-400 fish food per 5 fish, fed bit by bit to avoid overfeeding and resulting poor water quality.
- The animals were monitored twice daily using approved monitoring sheets (Addendum F) to verify environmental quality and evaluate fish health.
- The housing tanks, as well as buckets with extra tank water for the test tanks and water changes, were each equipped with an oxygen pump, heater and thermostat. Additionally, the room temp was also regulated to prevent temperature shock when netting the fish for relocation from the housing to the test tanks or other way round.
- Animal waste and excess food were siphoned out daily to avoid over feeding and poor water quality. Furthermore, housing tank water was changed daily to preserve water quality.
- The behavioural testing facility where the zebrafish were housed throughout the study was subject to controlled access. A sign was posted on the door to instruct individuals to keep quiet in and around the facility. The doors were kept closed and entering or exiting the facility during behavioural assays was prohibited so as not to unnecessarily disturb the fish.
- Perspex sheets were placed on the test tanks immediately after the fish were transferred to prevent them from jumping out of the tanks.
- All behavioural video recordings were recorded directly onto the computer in the behavioural testing facility and backed-up on a password protected computer in the computer room, as well as on an external hard drive. Raw data extracted from EthoVision are stored on the computer in the behavioural testing facility and backed-up on the computer in the computer room. Further, the supervisor, Prof Brian Harvey, retains hard and electronic copies of the raw data. For the purpose of credibility and transparency, and for future research purposes, the recordings and raw data will be kept for a minimum of 5 years and will be available on request for scrutiny and verification.

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ADDENDUM A

Biological Psychiatry Congress 2019 participation certificate.

BIOLOGICAL PSYCHIATRY CONGRESS

20 19

This is to certify that

Ms Heslie Loots

Has participated in / attended the following CPD Activity

Biological Psychiatry Congress 2019

at

Century City Conference Centre, Cape town

Held from

20 - 23 September

Health Professions Council of South Africa Accreditation offices approved CPD reference is as follows

Category	CPD Points	Accreditation #
Clinical	7	MDB005/047/09/2019 - Main
Total	**7**	

Endorsed by Faculty of Health Sciences - University of Pretoria

Please Keep this certificate on record for HPCSA audit purposes

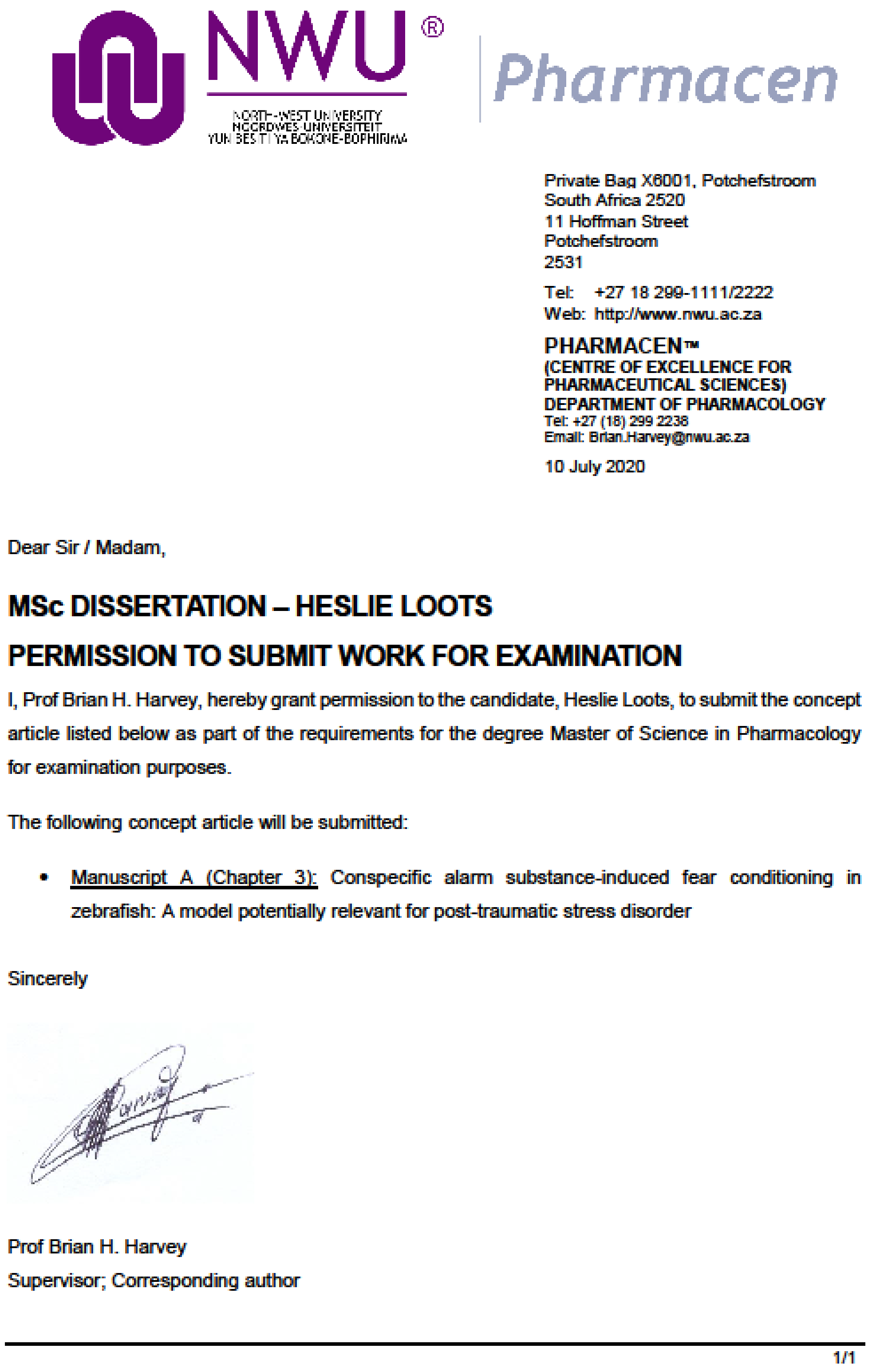
ADDENDUM B

'Author Information Pack' of Journal of Neuroscience Methods, can be found at:

https://www.elsevier.com/journals/journal-of-neuroscience-methods/0165-0270/guide-for-authors

ADDENDUM C

Letter of consent: Prof Brian H. Harvey.



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Pharmacen

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Potchefstroom
2531

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PHARMACEUTICAL SCIENCES)
DEPARTMENT OF PHARMACOLOGY
Tel: +27 (18) 299 2238
Email: Brian.Harvey@nwu.ac.za

10 July 2020

Dear Sir / Madam,

MSc DISSERTATION – HESLIE LOOTS

PERMISSION TO SUBMIT WORK FOR EXAMINATION

I, Prof Brian H. Harvey, hereby grant permission to the candidate, Heslie Loots, to submit the concept article listed below as part of the requirements for the degree Master of Science in Pharmacology for examination purposes.

The following concept article will be submitted:

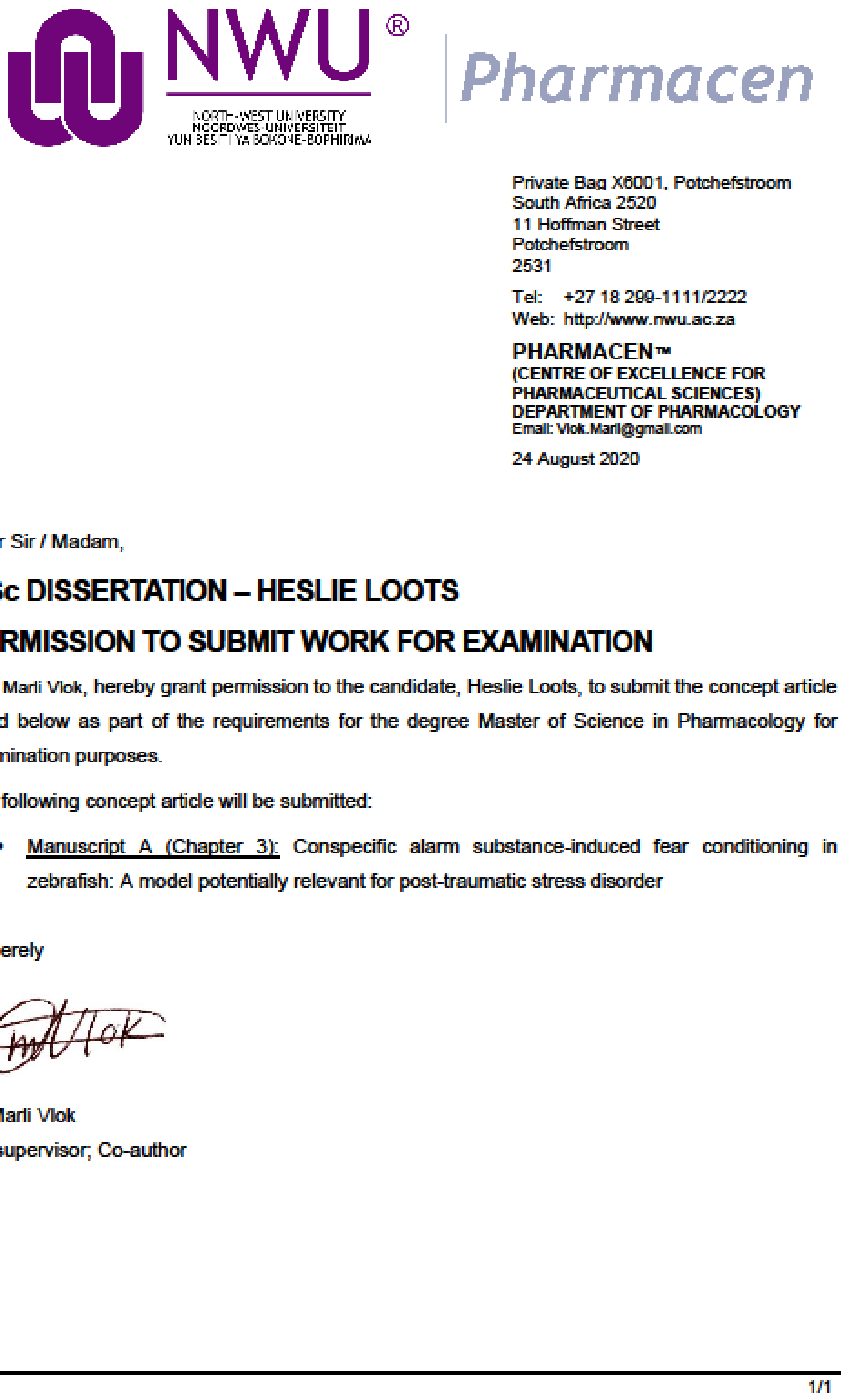
- Manuscript A (Chapter 3): Conspecific alarm substance-induced fear conditioning in zebrafish: A model potentially relevant for post-traumatic stress disorder

Sincerely

Prof Brian H. Harvey
Supervisor; Corresponding author

1/1

Letter of consent: Dr Marli Vlok.



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DEPARTMENT OF PHARMACOLOGY
Email: Vlok.Marli@gmail.com

24 August 2020

Dear Sir / Madam,

MSc DISSERTATION – HESLIE LOOTS

PERMISSION TO SUBMIT WORK FOR EXAMINATION

I, Dr Marli Vlok, hereby grant permission to the candidate, Heslie Loots, to submit the concept article listed below as part of the requirements for the degree Master of Science in Pharmacology for examination purposes.

The following concept article will be submitted:

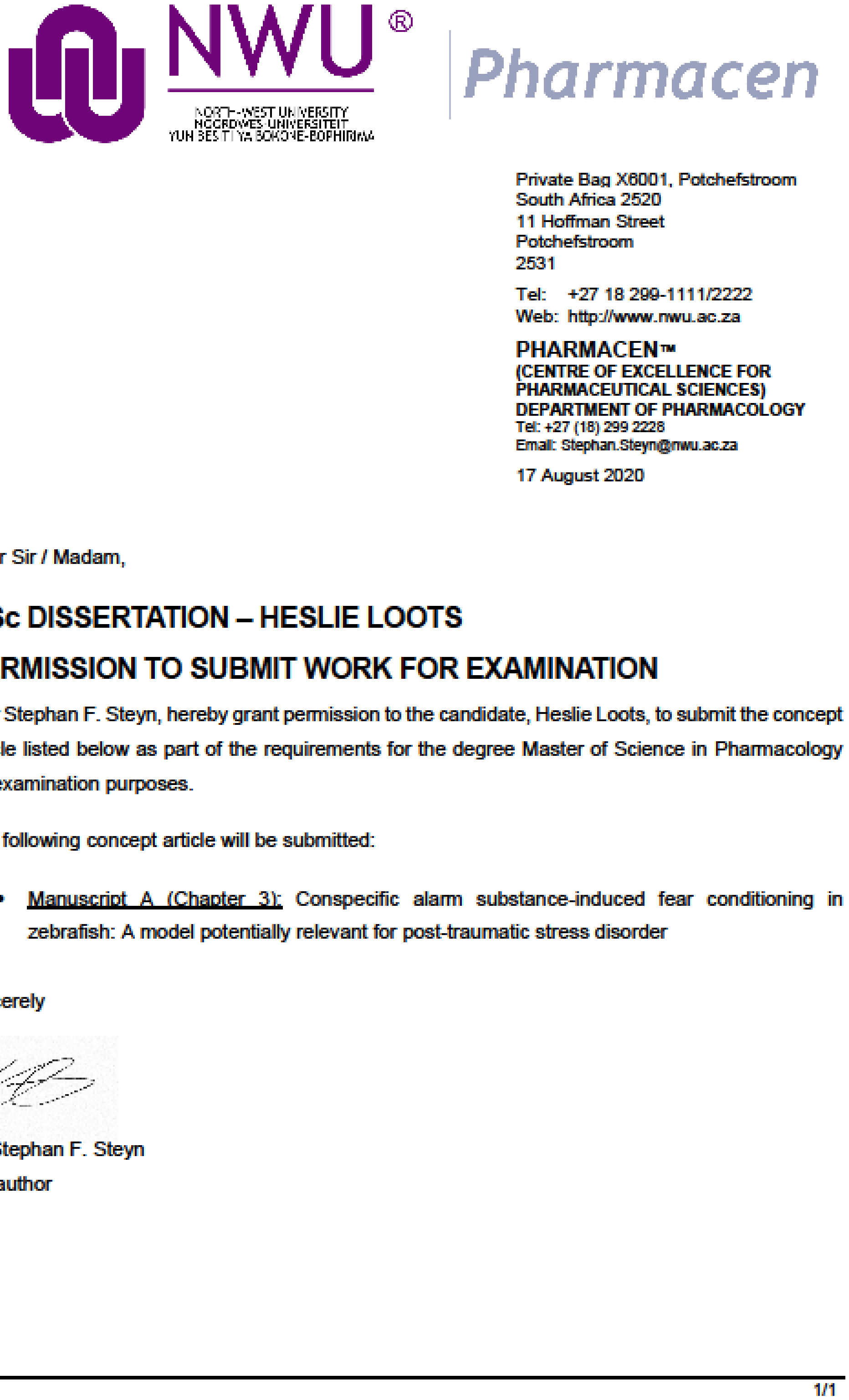
- Manuscript A (Chapter 3): Conspecific alarm substance-induced fear conditioning in zebrafish: A model potentially relevant for post-traumatic stress disorder

Sincerely

Dr Marli Vlok
Co-supervisor; Co-author

ADDENDUM C

Letter of consent: Dr Stephan F. Steyn.



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DEPARTMENT OF PHARMACOLOGY
Tel: +27 (18) 299 2228
Email: Stephan.Steyn@nwu.ac.za

17 August 2020

Dear Sir / Madam,

MSc DISSERTATION – HESLIE LOOTS

PERMISSION TO SUBMIT WORK FOR EXAMINATION

I, Dr Stephan F. Steyn, hereby grant permission to the candidate, Heslie Loots, to submit the concept article listed below as part of the requirements for the degree Master of Science in Pharmacology for examination purposes.

The following concept article will be submitted:

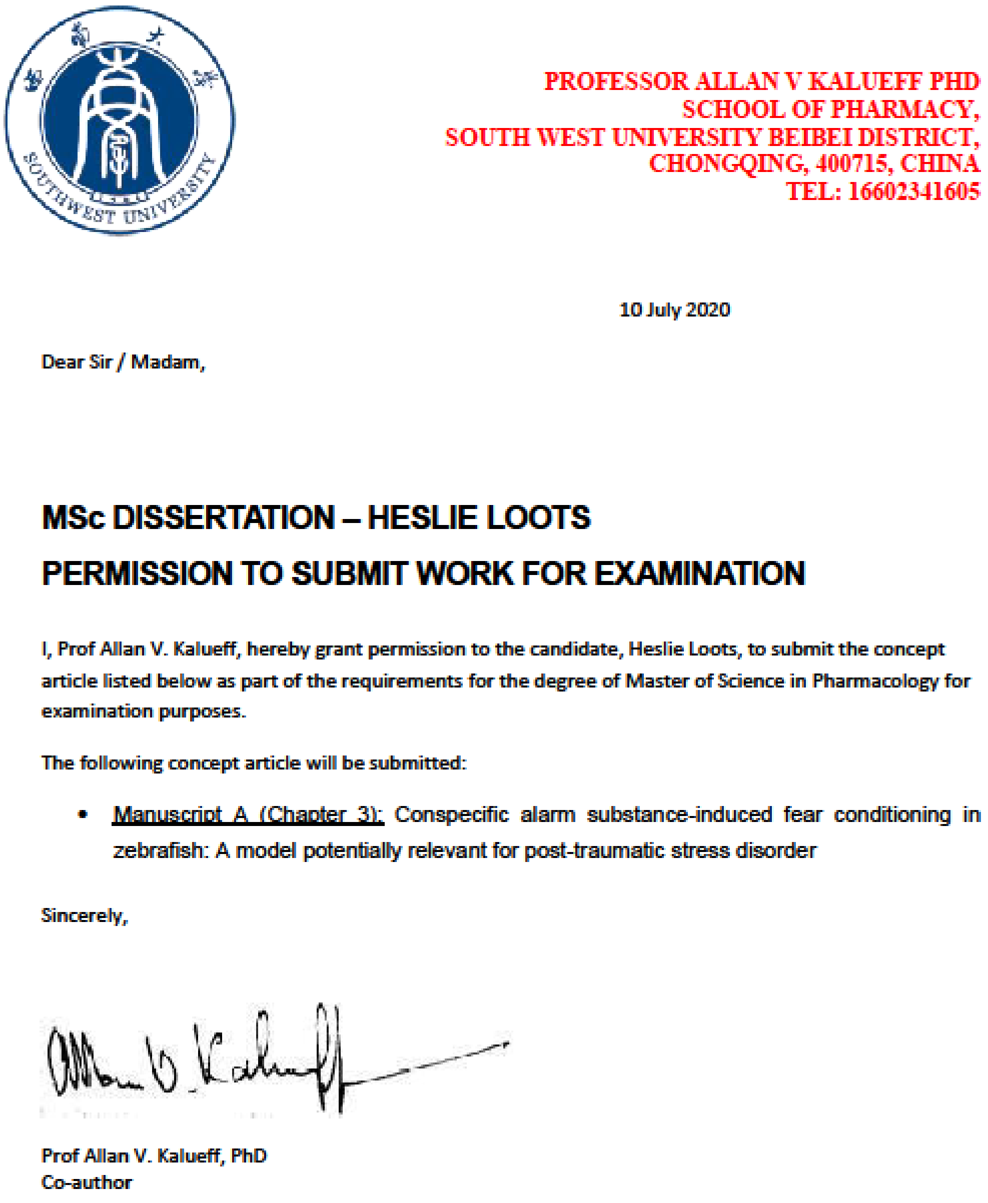
- Manuscript A (Chapter 3): Conspecific alarm substance-induced fear conditioning in zebrafish: A model potentially relevant for post-traumatic stress disorder

Sincerely

Dr Stephan F. Steyn
Co-author

ADDENDUM C

Letter of consent: Prof Allan V. Kalueff.



PROFESSOR ALLAN V KALUEFF PHD
SCHOOL OF PHARMACY,
SOUTH WEST UNIVERSITY BEIBEI DISTRICT,
CHONGQING, 400715, CHINA
TEL: 16602341605

10 July 2020

Dear Sir / Madam,

MSc DISSERTATION – HESLIE LOOTS

PERMISSION TO SUBMIT WORK FOR EXAMINATION

I, Prof Allan V. Kalueff, hereby grant permission to the candidate, Heslie Loots, to submit the concept article listed below as part of the requirements for the degree of Master of Science in Pharmacology for examination purposes.

The following concept article will be submitted:

- Manuscript A (Chapter 3): Conspecific alarm substance-induced fear conditioning in zebrafish: A model potentially relevant for post-traumatic stress disorder

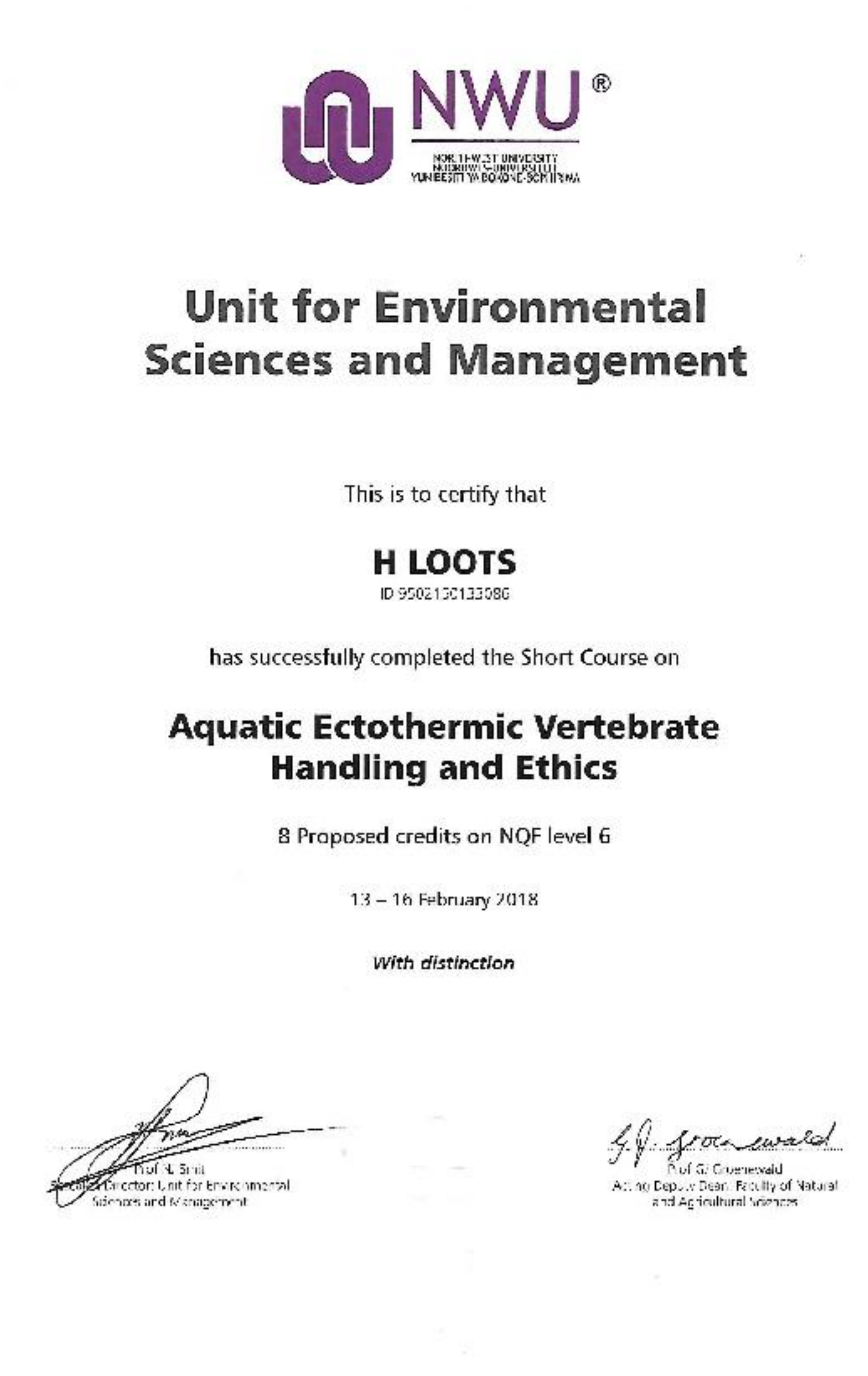
Sincerely,

Allan V. Kalueff

Prof Allan V. Kalueff, PhD
Co-author

ADDENDUM D

Zebrafish handling and ethics certification.



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Unit for Environmental Sciences and Management

This is to certify that

H LOOTS
ID 9502130133086

has successfully completed the Short Course on

Aquatic Ectothermic Vertebrate Handling and Ethics

8 Proposed credits on NQF level 6

13 – 16 February 2018

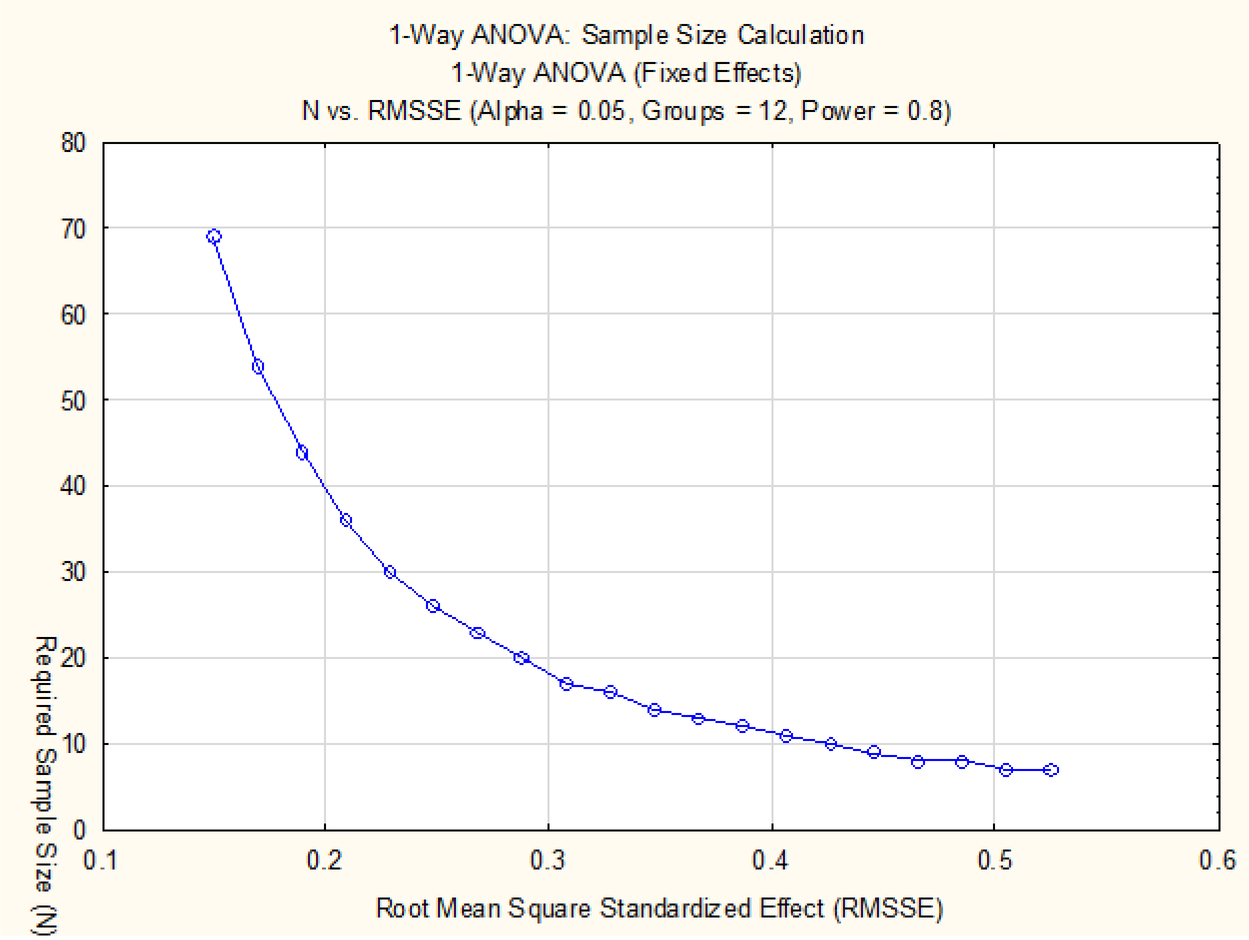
With distinction

Prof N Smit
Specialist Director: Unit for Environmental Sciences and Management

G.J. Groenewald
Prof GJ Groenewald
Acting Deputy Dean: Faculty of Natural and Agricultural Sciences

ADDENDUM E

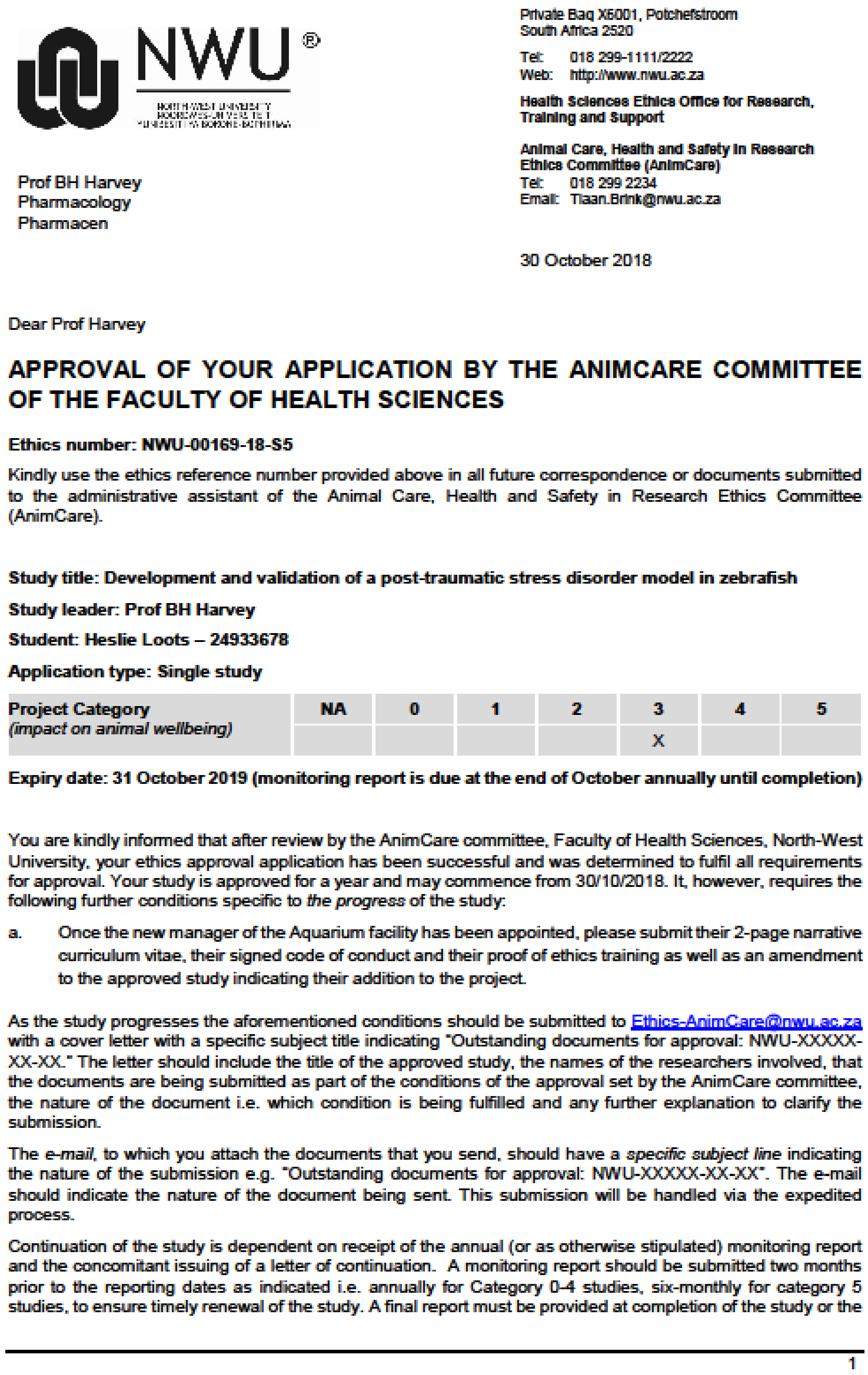
Power analysis report.



```
For a large effect 8-10 per group would be sufficient to achieve 80%
power at a 5% level of significance
For a medium effect 25 per group would be sufficient to achieve 80%
power at a 5% level of significance
```

ADDENDUM F

AnimCare committee approval letter.



Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Health Sciences Ethics Office for Research, Training and Support

Animal Care, Health and Safety in Research Ethics Committee (AnimCare)
Tel: 018 299 2234
Email: Tiaan.Brink@nwu.ac.za

Prof BH Harvey
Pharmacology
Pharmacen

30 October 2018

Dear Prof Harvey

APPROVAL OF YOUR APPLICATION BY THE ANIMCARE COMMITTEE OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00169-18-S5

Kindly use the ethics reference number provided above in all future correspondence or documents submitted to the administrative assistant of the Animal Care, Health and Safety in Research Ethics Committee (AnimCare).

Study title: Development and validation of a post-traumatic stress disorder model in zebrafish

Study leader: Prof BH Harvey

Student: Heslie Loots – 24933678

Application type: Single study

Project Category *(impact on animal wellbeing)*	**NA**	**0**	**1**	**2**	**3**	**4**	**5**
					X		

Expiry date: 31 October 2019 (monitoring report is due at the end of October annually until completion)

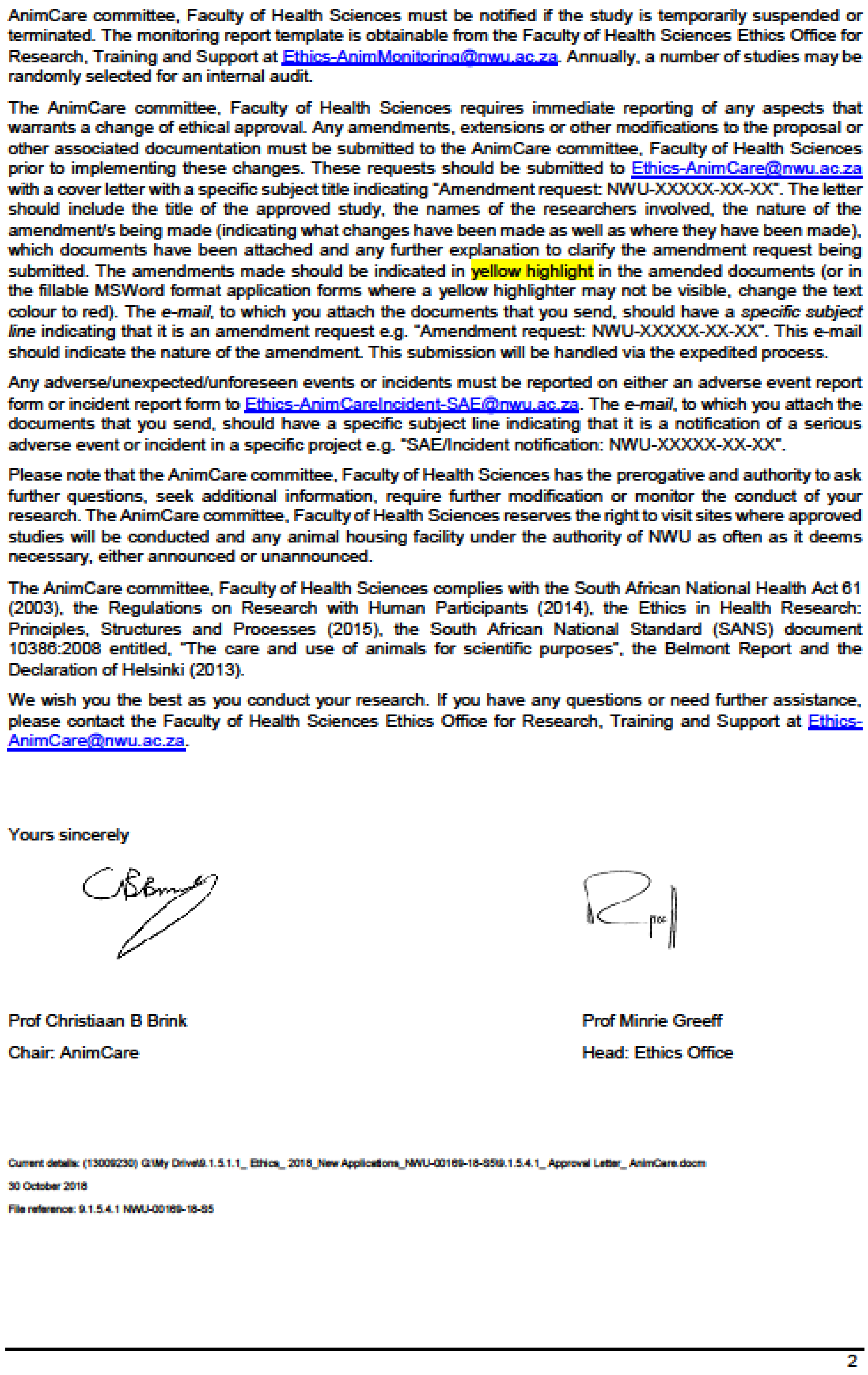
You are kindly informed that after review by the AnimCare committee, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 30/10/2018. It, however, requires the following further conditions specific to *the progress* of the study:

a. Once the new manager of the Aquarium facility has been appointed, please submit their 2-page narrative curriculum vitae, their signed code of conduct and their proof of ethics training as well as an amendment to the approved study indicating their addition to the project.

As the study progresses the aforementioned conditions should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Outstanding documents for approval: NWU-XXXXX-XX-XX." The letter should include the title of the approved study, the names of the researchers involved, that the documents are being submitted as part of the conditions of the approval set by the AnimCare committee, the nature of the document i.e. which condition is being fulfilled and any further explanation to clarify the submission.

The *e-mail*, to which you attach the documents that you send, should have a *specific subject line* indicating the nature of the submission e.g. "Outstanding documents for approval: NWU-XXXXX-XX-XX". The e-mail should indicate the nature of the document being sent. This submission will be handled via the expedited process.

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for Category 0-4 studies, six-monthly for category 5 studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the



AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The AnimCare committee, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare committee, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Amendment request: NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in yellow highlight in the amended documents (or in the fillable MSWord format application forms where a yellow highlighter may not be visible, change the text colour to red). The *e-mail*, to which you attach the documents that you send, should have a *specific subject line* indicating that it is an amendment request e.g. "Amendment request: NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment. This submission will be handled via the expedited process.

Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form to Ethics-AnimCareIncident-SAE@nwu.ac.za. The *e-mail*, to which you attach the documents that you send, should have a specific subject line indicating that it is a notification of a serious adverse event or incident in a specific project e.g. "SAE/Incident notification: NWU-XXXXX-XX-XX".

Please note that the AnimCare committee, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research. The AnimCare committee, Faculty of Health Sciences reserves the right to visit sites where approved studies will be conducted and any animal housing facility under the authority of NWU as often as it deems necessary, either announced or unannounced.

The AnimCare committee, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of animals for scientific purposes", the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely

Prof Christiaan B Brink	Prof Minrie Greeff
Chair: AnimCare	Head: Ethics Office

Current details: (13009230) G:\My Drive\9.1.5.1.1_ Ethics_ 2018_New Applications_NWU-00169-18-S5\9.1.5.4.1_ Approval Letter_ AnimCare.docm

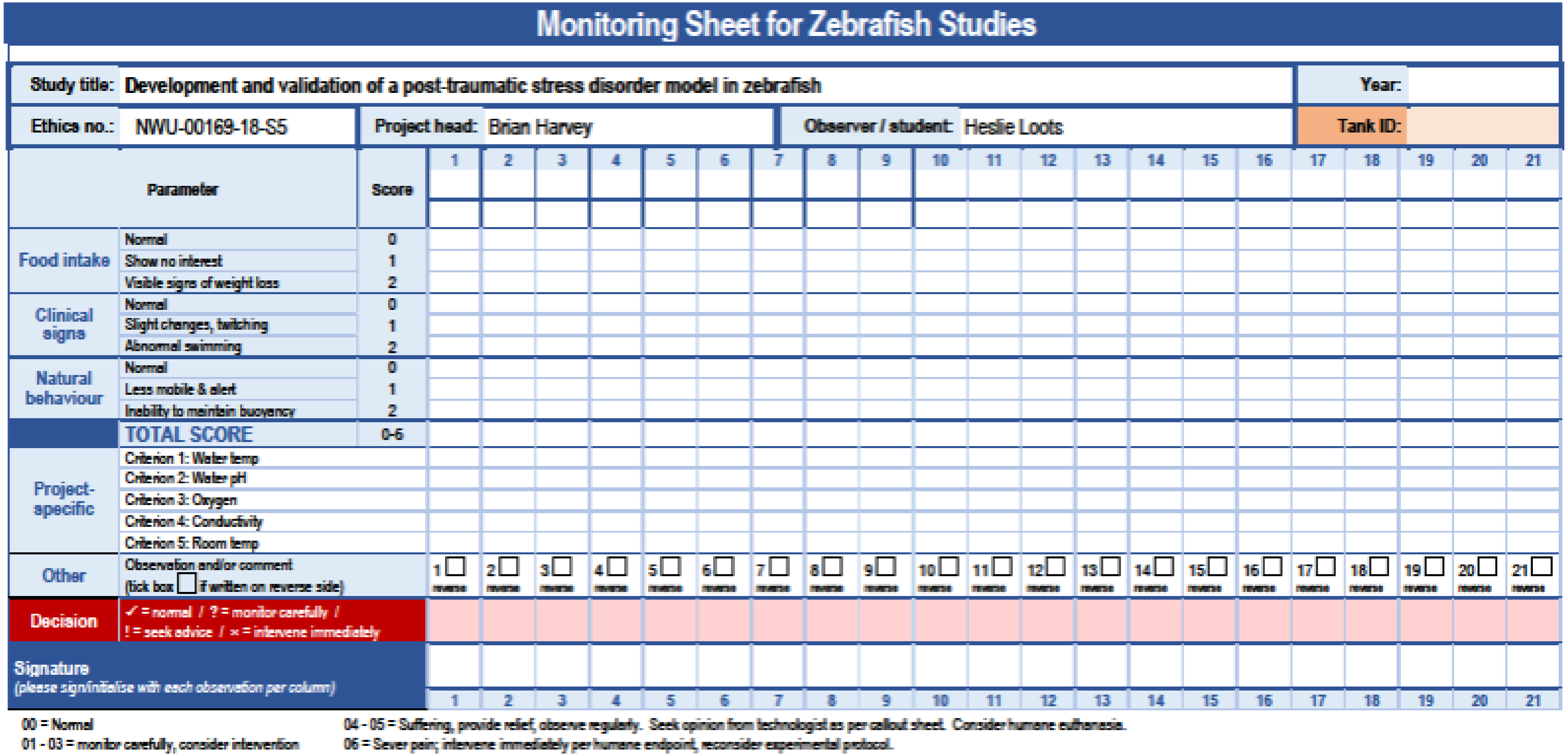
30 October 2018

File reference: 9.1.5.4.1 NWU-00169-18-S5

2

ADDENDUM G

Sample of monitoring sheet



Monitoring Sheet for Zebrafish Studies

Study title: Development and validation of a post-traumatic stress disorder model in zebrafish
Year:
Ethics no.: NWU-00169-18-S5
Project head: Brian Harvey
Observer / student: Heslie Loots
Tank ID:

Parameter		Score	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Food intake	Normal	0																					
	Show no interest	1																					
	Visible signs of weight loss	2																					
Clinical signs	Normal	0																					
	Slight changes, twitching	1																					
	Abnormal swimming	2																					
Natural behaviour	Normal	0																					
	Less mobile & alert	1																					
	Inability to maintain buoyancy	2																					
	TOTAL SCORE	0-6																					
Project-specific	Criterion 1: Water temp																						
	Criterion 2: Water pH																						
	Criterion 3: Oxygen																						
	Criterion 4: Conductivity																						
	Criterion 5: Room temp																						
Other	Observation and/or comment (tick box ☐ if written on reverse side)		1 ☐ reverse	2 ☐ reverse	3 ☐ reverse	4 ☐ reverse	5 ☐ reverse	6 ☐ reverse	7 ☐ reverse	8 ☐ reverse	9 ☐ reverse	10 ☐ reverse	11 ☐ reverse	12 ☐ reverse	13 ☐ reverse	14 ☐ reverse	15 ☐ reverse	16 ☐ reverse	17 ☐ reverse	18 ☐ reverse	19 ☐ reverse	20 ☐ reverse	21 ☐ reverse
Decision	✓ = normal / ? = monitor carefully / ! = seek advice / × = intervene immediately																						
Signature *(please sign/initialise with each observation per column)*																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

00 = Normal
01 - 03 = monitor carefully, consider intervention
04 - 05 = Suffering, provide relief, observe regularly. Seek opinion from technologist as per callout sheet. Consider humane euthanasia.
06 = Sever pain; intervene immediately per humane endpoint, reconsider experimental protocol.

Observations and/or comments, corresponding to the column on the front page of the monitoring sheet *(see reverse side)*

1.	
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" Today is gone. Today was fun.

Tomorrow is another one.

Every day,

From here to there,

Funny things are everywhere. "

- Dr. Seuss

(1960, Penguin Random House LLC. United States)

Fin