# The role of zebrafish larvae for studying anxiety-like behaviour

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Chapter 1

# General Introduction and thesis outline

## Anxiety disorders and their socioeconomic impact

Anxiety, in general, is a sustained sense of fear, worry, and distress over some future perceived threat to a person's well being. The stimulus in anxiety often poses no real threat. When anxiety leads to impairment in a person's functioning, it can be a called anxiety disorders. These disorders are often accompanied by various physiological symptoms such as chronic fatigue, dizziness, chest pain, and sleeping disorders [1]. In the EU (European Union), approximately 165 million people (total population) are affected by anxiety and other mental disorders each year [2]. Moreover, findings from the Global Burden of Disease (GBD) study performed in 2010 revealed that anxiety-related disorders were the sixth leading cause of disability in both high-income (HI) and low- and middle-income countries (LMI) [3]. The GBD study used disability-adjusted life year (DALY) as a standardized measure to capture periods of health loss (years of life lived with disability, YLDs) in addition to mortality rate (years of life lost, YLLs) [4]. The YLD rates for both male and females show a similar pattern, whereby the majority of disabilities due to anxiety disorders occur between adolescence and young adulthood [3]. Females were accounted for 65% of DALYs caused by anxiety disorder [3].

Disabilities due to anxiety disorders influence different areas of life, including education, health, income, and interpersonal relationships. The consequences of anxiety disorders are not only limited to patients and their immediate social environment but also cover an entire social fabric through economic costs [5]. The economic costs due to anxiety disorders consist of (i) direct or visible costs related to diagnosis and treatment (including counselling sessions, hospitalization, physician visits, medication, etc.); and (ii) indirect costs or invisible costs such as lost income due to mortality, disability, early retirement or absenteeism [6].

## Causes of anxiety disorders in humans

According to the diagnostic and statistical manual of mental disorders (DSM-V), anxiety disorders consist of a heterogeneous group of disorders that share anxiety as a common emotional symptom. Nevertheless, each anxiety disorder is characterized by a different aetiology, physiological features, and pathological outcome [7]. **Table 1**

summarizes the different types of anxiety disorder and their physiological characteristics according to DSM-V.

**Table 1. Summary of anxiety disorders and their physiological characteristics defined by DSM-V diagnostic criteria.** The explanations for each anxiety disorders are based on DSM-V [1] and Wiedemann [7].

| Disorder | Explanation |
|---|---|
| Generalised anxiety disorder (GAD) | Excessive worry and anxiety due to unrealistic thoughts on uncontrollable future events; physiological symptoms include restlessness, autonomic hyperactivity, sleep disturbances, and muscle tension |
| Panic disorder (PD)/ attacks | Recurrent paroxysmal anxiety that lasts between some minutes and a few hours; accompanied by different physiological sensations such as tachycardia, suffocation, trembling, sweating, abdominal distress, and dizziness. Agoraphobia (see below) develops in patients who associate specific situations or events with panic attacks |
| Social anxiety disorder (SAD) | Persistent fear of being scrutinized and negatively evaluated in social interactions or performance situations (e.g public speaking); accompanied by physiological and cognitive disturbances |
| Agoraphobia | Fear of being trapped or confined in situations where escape is not possible (e.g public transportation); symptoms include depersonalization, derealisation, dizziness, and cardiac symptoms |
| Specific phobias | Excessive and persistent fear of defined objects or situations such as fear of spiders (arachnophobia) or fear of height (acrophobia) |

Anxiety disorders are driven by a complex interplay of different factors such as genetics, biochemical, social, and psychological events [7]. Among these, environmental impacts during the developmental period are considered as one of the major factors that contribute to a variety of adaptive changes. These changes may lead to anxiety disorders at later in life. The median age for onset of anxiety disorder is 11[8], and several factors have been implicated in causing such early onset. For example, conflict in the family, lack of parental support, child-rearing style, and personality traits have been linked with the incidence of anxiety disorders during adolescence [7].

# Animal models of anxiety

Animal models are useful for investigating anxiety disorders at the functional level. Moreover, model organisms are also helpful for studying the pharmacological effects of potential anxiolytics (anti-anxiety drugs). In general there are three different types of animal behavioural models to study anxiety: (i) conditioned behaviour (conflict-based and cognitive-based tests), (ii) unconditioned behaviour (based on innate behaviour and often referred to as 'ethological-based model'), and (iii) separations models (investigates the behaviour of individuals during separation from mother or conspecifics). **Table 2** summarizes important features of two of the three types of animal behavioural models to study anxiety.

## The need for alternative animal models in anxiety research

Although rodent models have been very successful in deciphering behavioural and neurobiological aspects of anxiety and anxiety disorders, research involving

**Table 2. Two types of animal behavioural model to study anxiety.** Conditioned and unconditioned behavioural models to study anxiety, together with their important features.

| **Conditioned behaviour model** | |
|---|---|
| Conflict model | Behaviour is suppressed by an aversive stimulus. Anxiolytic-like effects of drugs are confirmed if supressed behaviour is released |
| Fear-potentiated startle response | The model was designed based on Pavlovian classical conditioning; fear-conditioning augments startle response of an organism. During the conditioning phase, a neutral stimulus is coupled with an aversive stimulus. After several pairings, animal learns that neutral stimulus is associated with a negative stimulus |
| **Unconditioned (Ethological-based model)** | |
| Elevated plus maze | Uses the conflict between exploration and aversion in elevated open spaces. Height and openness of an elevated open arm cause anxiety-like response in the animal. Increased number of entries to the open arm and time spent in the open arm indicates anxiolytic-like effects |
| Open field test | This model investigates an organism's exploratory behaviour in an open arena. Usually, the central zone of an open arena is avoided and animals prefer to stay in the edge of an arena. Increased time spent and distance moved in the central zone indicates anxiolytic-like effects |
| Light dark preference test | The number of transitions between light and dark zone is used as measures of anxiety. Rodents naturally prefer dark zone and treatments with anxiolytics increases the relative amount of time spent and distance moved in the dark zone. |

psychiatric disorders and neuropsychopharmacology is a very dynamic field that rapidly advancing. For example, in the past, scientists considered the amygdala to be the main region of the brain that governs anxiety. However, emerging studies suggest that a tiny region of the brain – the bed nucleus stria terminalis (BNST) – plays an important role in anxiety (i.e the sustained response), while the amygdala is more involved in fear (i.e the phasic response) [9].

The need for new animal models is also apparent in drug development. Thus the drug development pipeline represents a major investment of capital, human resources, and technological expertise [10]. The costs involved in discovering and developing new drugs is on an increasing trend. For example, a study done by Tufts Center for the Study of Drug Development in 2014, found that the costs of bringing a new drug to the market approach nearly $US 3 billion, with a 145% increase in cost compared to the year 2003 [11]. Some groups are seeking alternatives for preclinical trials since mammalian models such as rodents could be very expensive and time-consuming.[12, 13]

The Kalueff laboratory made a comparative analysis of cost efficiency of mouse and adult zebrafish studies [14]. In that analysis, both model organisms were given chronic fluoxetine treatment for 2 weeks ($n$ = 15/group). The use of adult zebrafish resulted in an approximately 5-fold saving (total expenditure, $562) compared to the mouse model (total expenditure, $2790). Since larval zebrafish can be adapted for high-throughput screenings (HTS) [15-17], larvae could be even more cost efficient compared to adults. This is because HTS platforms with larvae could screen hundreds of compounds per day to identify target compounds (hits) or candidate genes (biomarkers) [14]. The reasons for the cost efficiencies of zebrafish larvae are not only that the adults are cheaper to maintain than rodents, but also one female adult zebrafish can produce up to 35,000 eggs in her lifespan [18]. In principle, one egg is one test individual.

Another factor to be considered in the selection of animal models is the throughput or efficiency, measured in terms of the number of assays per unit time that can be realistically be performed. In general, rodent models are of lower throughput compared to zebrafish larvae, mainly because they are more labour intensive [19]. For example, zebrafish larvae can be simply and easily transferred into the experimental

plate (384, 96, or 24 well plates) by pipetting. This makes them highly suitable for high-throughput drug discovery and screening.

The use of animals, especially mammalian models in biological research have raised ethical concerns in the field of biology [20]. To improve animal welfare, therefore, Russell and Burch introduced the 3R concept (replacement, reduction, and refinement) [21]. Hence, efforts were initiated to replace animal models, especially mammals with alternatives. These could include, in principle, 'lower' or cold-blooded vertebrates (amphibians or teleost fish including the zebrafish), invertebrate species such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, and cell or tissue culture assays.

Despite these advantages, zebrafish larvae cannot completely replace the rodent models in the drug development pipeline, rather they exist to serve as a complementary model. Zebrafish models, especially the larval models, can in principle function as an invaluable screening tool during the pre-clinical phase, prior to rodent models to pre-filter compounds and reduce the unnecessary clinical trials using rodents [13] (**Figure 1**). Overall, zebrafish larvae permit the faster and cost-effective screening of large libraries of compounds for therapeutic effects on complex phenotypes of central nervous system (CNS) functions (for example reducing anxiety-like behaviours) [13].

## Behavioural assays using developing zebrafish larvae

Zebrafish larvae during their early stages of development already show a wide range of behavioural repertoires. Previous studies have reported a chronological sequence in the locomotor development of larval zebrafish between 1–5-day post fertilization (dpf) [22-25] (**Figure 2).** Each locomotor pattern of developing larvae has been shown to be sensitive in screening for pharmaceuticals and environmental toxicants that influence behaviour (**Table 3**).

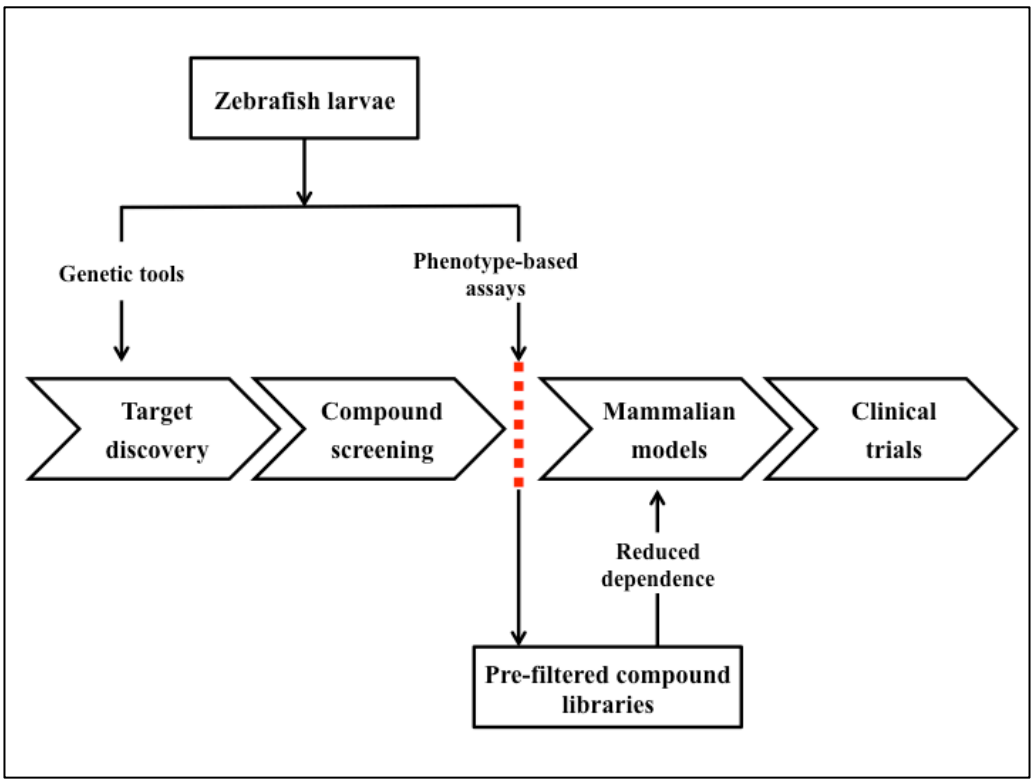


**Figure 1. Drug discovery pipeline involving larval zebrafish model.** Adapted from Figure 9 in Steenbergen *et al.*[13]

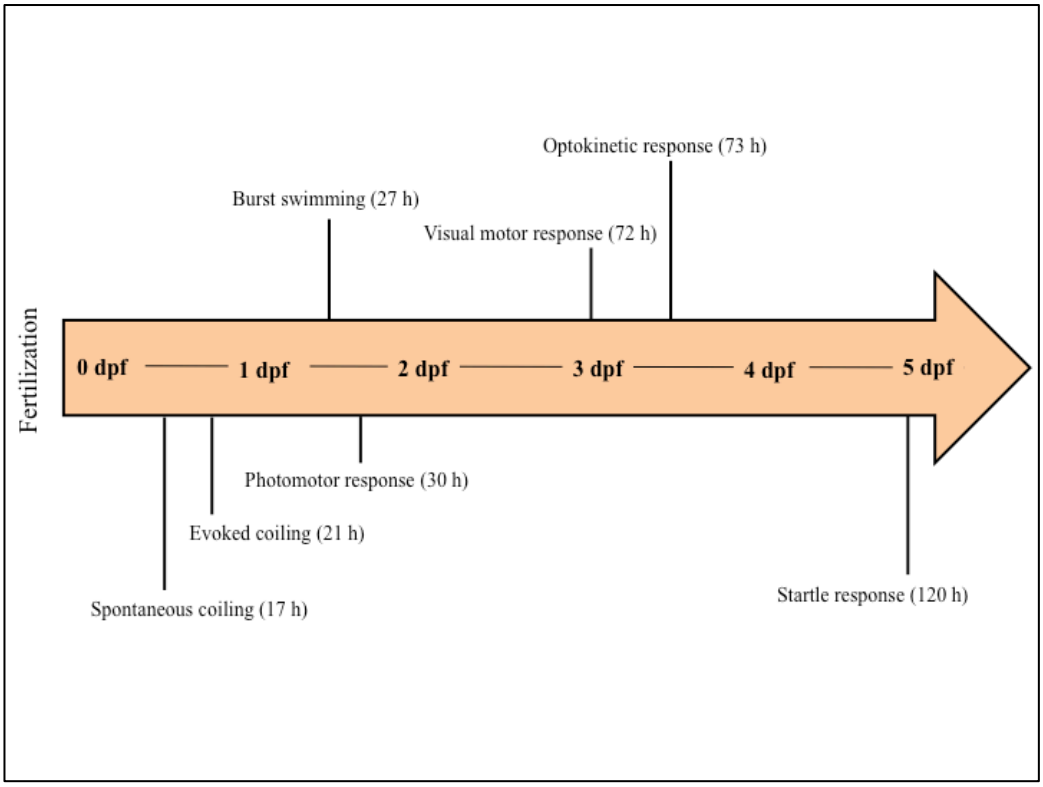


**Figure 2. The Chronological sequence of locomotor developmental stages that appear during the first five days of development in zebrafish larvae.** For references see Table 3.

**Table 3. Different types of behavioural repertoires shown by early developing zebrafish larvae (≤ 5 dpf).** The information is summarized graphically in Figure 2.

| Behaviour | Descriptions | Stimulus | Reference |
|---|---|---|---|
| Spontaneous coiling | Repeated alternating tail coils independent of sensory stimulation | None | [26] |
| Evoked coiling | Manual stimulation of embryo results in tail coiling | Touch | [23] |
| Burst swimming | Larvae lie on the side at the bottom of the petri dish. When touched, larvae swim rapidly to the other side of dish and lie again on their side | Touch | [27] |
| Photomotor response | Between 30 and 42 hours post fertilization, embryos show low levels of basal motor activity. The Intense photic stimulus causes robust and reproducible behaviours (excitation and refractory) | Light intensity | [16] |
| Visual motor response | Sudden exposure to darkness increases larval locomotion | Alternating light-dark | [28] |
| Optokinetic response | Stereotyped eye movements in response to moving objects. Larvae with fewer saccades (fast movement of the eyes) have defect visual functions | Moving objects | [29] |
| Acoustic startle response | Becomes evident after the larval auditory system has become functional | Acoustic | [30] |

Our justification for using the zebrafish as a functional and pharmacological model to study human anxiety disorders is based on the fact that anxiety-like behaviours in adult and larval (≥ 5 dpf) zebrafish can be attenuated by the gold-standard anxiolytic drug diazepam [31-37]. In addition, other psychotropic drugs used in the treatment of human anxiety disorders, such as fluoxetine and buspirone also showed anxiolytic-like effects in adult zebrafish [31-34, 38]. Conversely, anxiogenic drugs such as caffeine enhanced anxiety-like behaviours in both adult and larval zebrafish.

## Aim and outline of the thesis

The main aim of the thesis is to further explore the value of using early developing zebrafish larvae (up to 5 dpf) as a model to study anxiety-like behaviour and their pharmacological modulation with drugs. Several behavioural parameters of larval zebrafish were used in this thesis to evaluate anxiety-like behaviours, which are locomotion, startle response, and thigmotaxis. In addition to this, behavioural assays are also used to screen synthetic anxiolytics commonly used in the treatment of anxiety disorders. Finally, the toxic effects of the synthetic and herbal-based anxiolytics were also assessed on the developing zebrafish larvae.

In **Chapter 2**, I review the advantages and challenges of using zebrafish larvae as an alternative model to identify new plant-derived anxiolytics. I highlight factors that may influence the outcomes of larval zebrafish-based behavioural assays. In addition, I discuss current challenges herbal psychopharmacology research and how the hyphenation of larval behavioural assays with herbal psychopharmacology is still in its infancy.

In **Chapter 3**, I show that the larval visual-motor response assay is highly sensitive to four types of psychotropic drugs (amitriptyline, buspirone, diazepam, and fluoxetine) that have different mechanisms of action. All four drugs suppress larval locomotion both after acute and chronic pre-exposure. Chronic treatment with amitriptyline and buspirone also were toxic at the highest concentrations tested. I suggest that these effects might be explained by serotonin toxicity (syndrome).

The aim of **Chapter 4** is to further explore the implications of Chapter 3. A previous study showed that amitriptyline induces serotonin toxicity, characterized by hypolocomotion and vertical swimming in adult zebrafish. We used several larval zebrafish behavioural repertoires such as locomotion, startle response, and thigmotaxis to identify behavioural phenotypes that resemble serotonin toxicity in zebrafish larvae after treatment with serotonergic psychotropic drugs.

In **chapter 5**, I assess the developmental toxicity of the four psychotropic drugs used previously in **Chapters 3** and **4**. Together with the four synthetic drugs, I also included four herbal extracts commonly used in the treatment of various mood and psychological disorders. They are *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Withania somnifera*. The rationale behind this chapter is that the drugs and herbal extracts used here are common mainstays in the treatment of anxiety disorders among pregnant mothers.

Finally, in **Chapter 6**, I summarize and discuss the preceding chapters, and highlight the limitations of using larval zebrafish (≤ 5 dpf) to study complex affective disorders such as anxiety disorders. Furthermore, I propose a model that potentially could be useful to study anxiety disorders as a developmental disorder, whereby the models incorporate zebrafish of different ages. In addition, this model also considers individual variations that may affect an individual's propensity to be more susceptible to developing anxiety disorders. I suggest that larval zebrafish (< 5 dpf) could be more useful for studying anxiety and related disorders in a developmental model. In addition, larvae from this age group might also be useful early on the drug discovery pipeline to assess the developmental toxicity of candidate psychotropic drugs.

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**Chapter 2**

# The use of larval zebrafish (*Danio rerio*) model for identifying new anxiolytic drugs from herbal medicine

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Published in *Zebrafish. 2018. 15(4): 321 – 339*

# Abstract

Anxiety is a widespread psychiatric disorder. The search for a cure is still continuing since many of the synthetic drugs were inefficient in completely treating anxiety, yet caused some dangerous side effects until many of the drugs were withdrawn from the market. One promising source of new anxiolytics could be herbal medicines. The challenge is to screen plant extracts. Rodent models can be used for this purpose but are expensive. Moreover, rodent tests are costly and consume relatively large quantities of sample. For this reason, alternative animal models may be useful. Zebrafish larvae have many advantages for screening natural products. The main advantage is that they can be produced cheaply and in large numbers. Several studies have shown that zebrafish is a good model for studying drugs that affect anxiety. This review focuses on the use of animal models including zebrafish larvae, for studying anxiety and screening for herbal medicines that modulate anxiety. Finally, future prospects of the zebrafish larva as an alternative model in this field are also discussed.

# Introduction

Anxiety-related disorders are the most widespread psychiatric disorders affecting humans [1]. In severe cases, anxiety disorders can lead to significant impairment of daily functioning [2]. At present, anxiety disorders are diagnosed and classified based on systems outlined in the Diagnostic and Statistical Manual of Mental Disorders, 5$^{th}$ edition (DSM-V) by American Psychiatric Association or by the International Classification of Diseases, 10$^{th}$ revision (ICD-10) by World Health Organization [3]. DSM-V classifies anxiety disorders into (i) agoraphobia (ii) generalized anxiety disorder (GAD), (iii) social anxiety disorder (SAD), (iv) panic disorders (PD), and (v) specific phobias [4]. The etiology of anxiety is often complicated and co-morbid with other disorders such as depression[5], whereby both may occur together with anxiety predisposing depression or vice versa [6]. It is also possible that anxiety disorders may represent an external manifestation of a disrupted homeostatic balance (for example disruption of sleep and circadian rhythm) [7].

One factor that can contribute to the development of anxiety disorder and other psychiatric disorders is stress [8-10]. Stress is a hard concept to define, although the mechanisms underlying stress are highly conserved among vertebrates [11]. Almost every discussion on stress includes three prominent figures: (i) Claude Bernard, (ii) Walter Bradford Cannon, and (iii) Hans Selye. Claude Bernard was a French physiologist who introduced the idea of *milieu intérieur* – maintenance of the internal environment surrounding cells is essential for the living organism [12]. Later, in 1929, Cannon extrapolated the works done by Bernard and coined the term *'homeostasis'*, which refers to a range of values for internal variables [12, 13]. He further postulated that any threat to homeostasis might arise due to external or internal stimuli and could be physical or psychological. Hans Selye was an endocrinologist who pioneered research in stress syndrome. He used the word 'stress' in a physiological context to describe the body's non-specific response to any demand placed upon it [14].

Selye showed that acute exposure of rats to non-specific nocuous agents such as low temperatures, spinal shock, and intoxication with various drugs (atropine, morphine, adrenaline, etc.) produced characteristic and harmful syndromes [15]. Initially, Selye named this syndrome as "general adaptation syndrome", and later renamed it as "stress response" [16]. The word "stress" was used for the first time by

Selye to describe this syndrome in his first comprehensive monograph published in 1950. Though Selye's discovery was groundbreaking, he faced heavy criticism in the late 1940s and 1950s for naming both the cause and effect as stress [16]. Hence, the word "stressors" was used to reflect agents that trigger stress response [16]. Though there were many complaints from physicians and scientist regarding Selye's discovery and the confusion in the definition of stress, one physician[17] quoted the following *"Stress in addition to being itself, was also the cause of itself and the result of itself"*. The role of stress in anxiety will be further discussed in the next section.

Even after decades of intensive research using model organisms, *in vitro* studies, and clinical trials, the ability to treat anxiety effectively is still inadequate [18]. Although many drugs are available for anxiety (referred as 'anxiolytics'), individuals who suffer from anxiety and anxiety-related disorders are on the increase. Furthermore, the highly sedentary lifestyle that we are living nowadays can be an important contributing factor to the increased incidence of anxiety [19]. The reasons behind ineffective treatment for any type of neurobehavioral disorders are: (i) low bioavailability of drugs, (ii) ineffective drug-delivery method, (iii) lack of knowledge on genetic factors, (iv) lack of suitable model organism(s) for drug discovery and (v) failure to tailor the treatment program to the individual (i.e. failure to adopt the principles of personalized medicine) [20]. Therefore, these are important considerations for researchers from different fields of neuroscience in order to find new therapeutic drugs.

The main scope of this paper is not to review each plant species in detail in terms of its phytochemistry and pharmacotherapy as these were reviewed extensively elsewhere [21-26]. The literature is superfluous with many reviews on specific plant species (for example *Hypericum perforatum*[27, 28], *Passiflora incarnata*[29], *Valeriana officinalis[30, 31], Withania somnifera*[32]) or specific mechanistic action of herbal preparations (for example modulation of different receptors such as the gamma-aminobutyric acid (GABA receptors)[33] and serotonin receptors [34]). The main scope of this review is to discuss the perspective of using herbal anxiolytics on zebrafish larvae as a model system. Moreover, current challenges in phytochemistry and zebrafish neurobehavior were also discussed in this review.

## Etiology of Anxiety

In normal situations, our body responds to threatening stimuli via different mechanisms such as defensive behaviors, autonomic reflexes, increased alertness, and catecholamine and corticosteroid secretion [35]. It is normal to feel fearful at some points in our lives when there is a perceived imminent threat to our sense of well-being [4]. Such a perceived threat could be the taking of an exam, the giving of a public talk, or the making of an important life decision. However, if there is an anticipation of a future threat, and it is either irrational or is never resolved, then a pathological state of anxiety may develop [4]. Fear and anxiety are usually emotion-based adaptive responses to stressful stimuli or threats [36]. These responses may arise due to external inputs (auditory, olfactory, visual or somatosensory stimuli); or internal inputs from the endocrine and nervous systems [36].

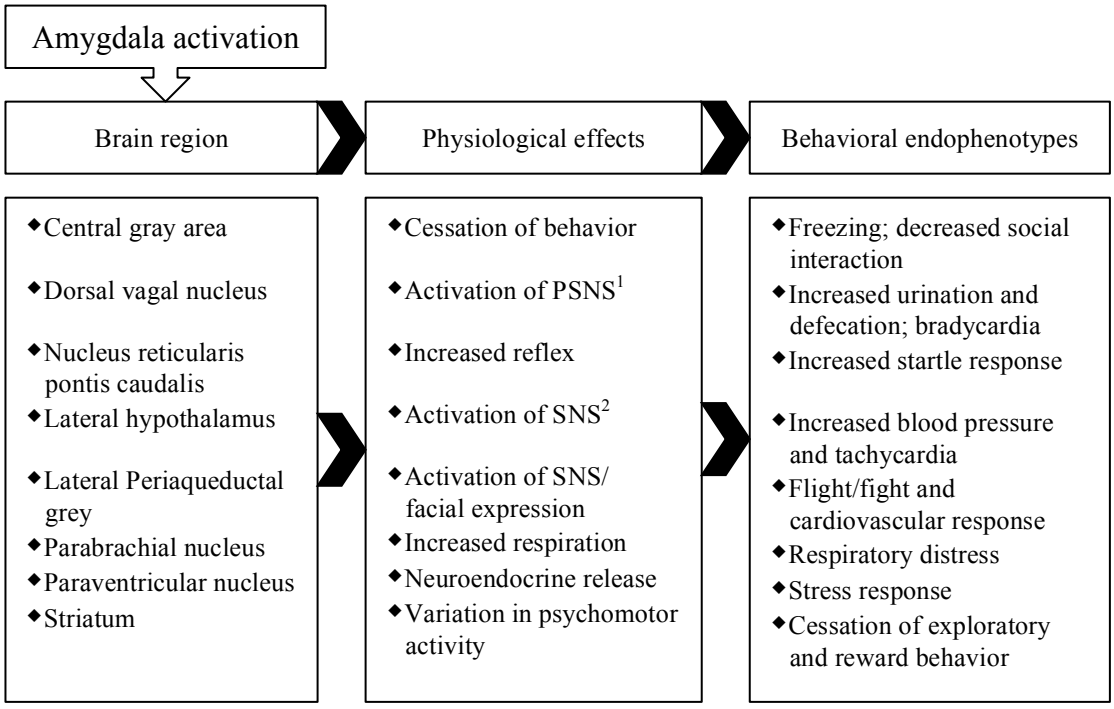
Apart from these stimuli, anxiety may also be triggered by unpleasant memories or the anticipation of stressors or threats [36]. Though anxiety and fear may represent two similar emotional conditions, they can be easily distinguished on the basis of the controllability of the threat[37] (that is, the extent to which the threat can be controlled by the individual concerned). According to Epstein's view[37], in a fear response, there is a hope of controlling the threatening situation. By contrast, anxiety appears when the attempt to control the threat (i.e. to cope) has failed, and the threat is therefore perceived to be uncontrollable or uncertain (a helpless state) [37]. Defining anxiety and fear in terms of different controllability scales has an added advantage, whereby they can relate to another psychological disorder – depression. When feelings of uncontrollability increase and last for long period, the organism enters the state of being hopeless and anxiety is replaced with depression [38, 39]. The etiology of anxiety covers different anatomical structures involving mainly the nervous and the endocrine system.

## Functional anatomy of anxiety

Disruption in the limbic system, an important emotional center of the brain, is linked to anxiety disorders [40]. The limbic system includes the hippocampus, hypothalamus, medial prefrontal cortex, and amygdala [40-42]. Resilience towards anxiety disorders is correlated with hippocampal volume and neurogenesis [40]. By contrast, the amygdala is responsible for the formation and retrieval of fearful memories. The amygdala has many interconnections with various parts of the brain

including the hippocampus, thalamus, hypothalamus [40]. The amygdala becomes activated during the fear response and this causes various behavioral responses [36, 40, 43] (**Box 1**). Neuroimaging studies in humans verify that distinct but related brain anatomical structures motivate fear and anxiety. Fear is also known as 'phasic fear', and anxiety as 'sustained fear'. Based on experimental paradigms using rodent models, nonhuman primates, and humans, Davis and colleagues have concluded that the amygdala mediates the fear response while anxiety is mostly governed by the bed nucleus of the stria terminalis (BNST) [44].

**Box 1.** Schematic diagram depicting the role of the amygdala in the fear response. Upon external stimulation, the amygdala induces various physiological effects, which in turn producing some behavioral endophenotypes that can be exploited as an index for anxiety. Adapted from Fig.2 in Davis (published in *Annu. Rev. Neurosci*. with permission of the publisher, Annual Reviews©, California, USA) and with additional information from Fig. 63.1 in Charney and Drevets (published in Neuropsychopharmacology – 5th Generation of Progress with permission of the publisher, Lippincott Williams and Wilkins©, Philadelphia, USA).




[1]Parasympathetic nervous system
[2]Sympathetic nervous system

# Theories of anxiety pathophysiology

There are many theories suggesting the pathophysiology of anxiety. Examples include the GABAergic theory, the stress response theory, and the monoamine theory. Different classes of biomolecules are involved in these theories such as neurotransmitters, hormones (adrenaline, noradrenaline, and cortisol), neurotrophins,

and neuropeptides. These biomolecules are involved in signaling in the brain, and between the central nervous system and peripheral tissues.

## GABA-ergic theory

Gamma-aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in the central nervous system. Receptors for this neurotransmitter are localized in the brain and peripheral nervous system [45]. The role of GABA in mood disorders was first identified based on the clinical efficacy of valproic acid (a GABA agonist) in the treatment of bipolar disorder [46]. Furthermore, the gold-standard anxiolytic, diazepam (Valium), a benzodiazepine, acts via the GABA pathway. Benzodiazepines do not bind to the receptor site where the endogenous ligand GABA binds, but to a different site located between α- and γ-subunits of $GABA_A$ receptors[47, 48], which is also known as the benzodiazepine site [49].

Many preclinical and clinical studies support the role of GABA in mood disorders [50-56]. It is assumed that decreased inhibitory signaling in the GABA-ergic system could be the main reason for the pathophysiology of anxiety [57, 58]. Another important role of the GABAergic system is in the regulation of inhibition of HPA axis activity. However, the GABAergic control of the HPA axis is highly susceptible to both acute and chronic stress [59]. Although the GABAergic system is well studied, it is still not known the exact role of this system in hyperactivity of HPA axis. Scientists are still speculating whether a deficit in the GABAergic system independently causes HPA axis hyperactivity that leads to mood disorders or if the dysfunction of the GABAergic system is secondary to stress-induced HPA hyperactivity in mood disorders [60].

## Stress Response Theory

The stress response is widely conserved across the vertebrate species, in order to maintain survival [61]. Nevertheless, due to our sedentary lives, this mechanism may lead to health problems [14]. In his later research, Hans Selye found that not all stress responses are bad for our health [16]. Lenard Levi's clinical and social investigations in Sweden played a prominent role in shifting Selye's mindset. According to Levi, our cerebral cortex has the ability to differentiate between adrenocorticotropic hormone (ACTH) and corticoids released under unpleasant (arguments with a spouse) and pleasant situations (pleasure of kissing a girlfriend or boyfriend) [62]. Hence, Selye

introduced the terms “eustress” and “distress” to differentiate positive and negative stress respectively [63]. Distress can be either acute (intense but of short duration) or chronic (of long duration and possibly low intensity) [64]. The concept of eustress is incomplete[65], due to the lack of clear criteria to differentiate this type of stress from distress and insufficient knowledge on the basis of eustress [66].

Perhaps the easiest way to explain the relationship between eustress, distress, and health is with the help of Yerkes-Dodson principle (depicted in **Figure 1**) [67]. According to this principle, there is a non-linear relationship between the intensity of stress levels and health. The concept of ‘*hormesis*’ could give a clearer interpretation quantitatively on the Yerkes-Dodson principle. Hormesis is a process that causes cells or organisms to exhibit a biphasic response to an increasing amount of substances or conditions [68]. In other words, lower dose exposure results in a beneficial response, while the higher dose is detrimental and toxic [68]. According to Le Fevre et al.[69], an individual’s perception and interpretation of a condition determine whether a stressor becomes eustress or distress. It could be speculated that the hump/ maximum performance (as shown in Fig. 1) is variable individually. Previous studies revealed large inter-individual variations in the stress response to psychological challenges [70-74].

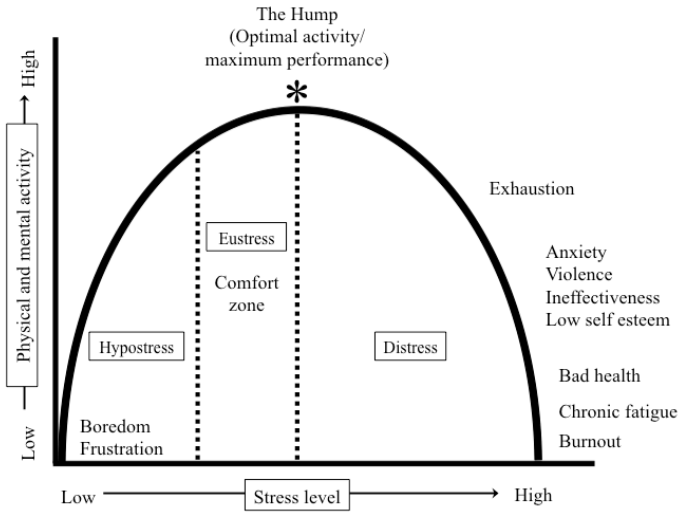



**Figure 1. Yerkes-Dodson curve showing different types of stress.** Stress left to the midpoint represents positive stress (eustress) while stress beyond this point is considered as negative stress (distress) that can affect our health. An extreme abundance of stress or hypostress can cause boredom and poor performance. Redrawn with modifications from Fig. 1 in Rapolienė *et al.* (published in *Adv. Prev. Med.* with permission of the publisher, Hindawi©, Cairo, Egypt) and Fig. 1.5 in Seaward (published in Managing Stress: Principles and Strategies for Health and Well-Being with permission of the publisher, Jones and Bartlett Learning©, Massachusetts, USA).

Physical and psychological stressors are capable of causing different biological response, including release of catecholamines, sympathetic arousal [also known as sympathetic-adrenal-medullary (SAM) axis], and hypothalamic-pituitary-adrenal (HPA) axis activation [75]. Acute and chronic stress are different[76], whereby the former is governed by the SAM axis, while the latter mainly involves HPA axis [75]. Acute stress-response is the immediate action of the sympathetic nervous system (SNS) that readies an organism for flight/fight response [75]. Upon activation, the SNS causes the adrenal medulla to release adrenaline and noradrenaline into the bloodstream [75]. These two hormones prepare our body for the threat by increasing the heart rate and blood pressure, dilating pupils and inhibiting gastrointestinal activity [75]. The main objective is to prepare the body for the threat by maximizing muscular output and reaction speed [14].

Although acute and chronic stress can both activate the HPA axis[77], chronic stress is thought to be the main cause of many stress-related diseases, since our body is constantly aroused for danger [64]. Different endocrine pathways govern the functioning of HPA-axis (as summarized here in **Figure 2**). Moreover, HPA axis activity is modulated by different parts of the limbic system, such as the amygdala and hippocampus. The amygdala elevates HPA axis activity while the hippocampus causes suppression of HPA axis activation [40]. Stressors trigger the short-term adaptive responses that involve short-term activation of HPA axis, whereby a negative feedback system via glucocorticoid receptors establish a homeostatic balance. Unfortunately, under excessive stress conditions, the HPA axis system becomes maladaptive. This causes a negative impact on the limbic system and increases the risk for many psychiatric disorders[78], including anxiety. Chronic stress is often characterized by hyperactivity of the HPA axis with elevated cortisol levels. A hyperactive HPA axis can be explained by two mechanisms. One mechanism suggests that impaired feedback inhibition is responsible for decreased glucocorticoid receptors activity. In the other, there is excess glucocorticoid signaling [79]. Furthermore, higher cortisol concentrations also cause toxic effects on the hippocampus through reduced brain-derived neurotrophic factor (BDNF) expression [80].

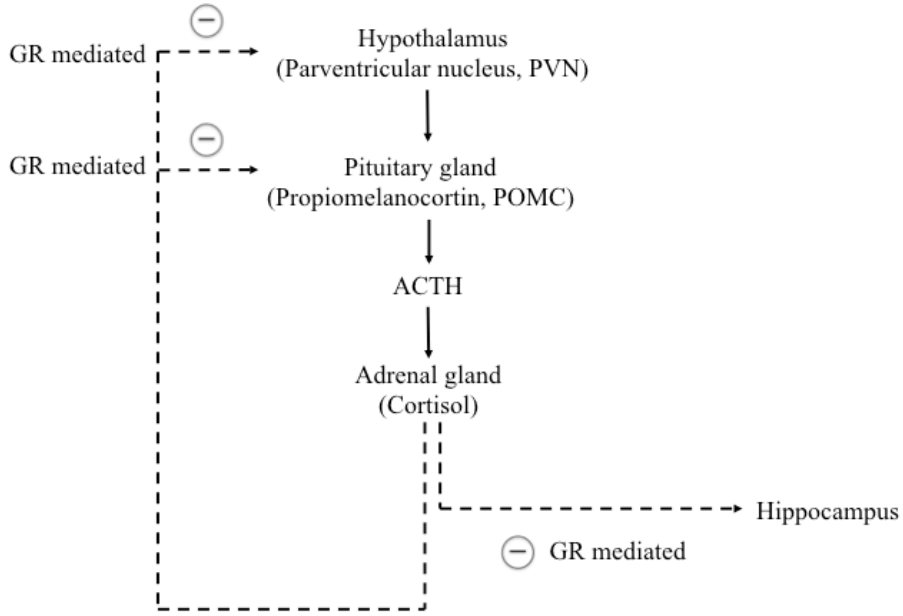



**Figure 2. General organization and functioning of the HPA-axis in humans.** The paraventricular nucleus (PVN) of hypothalamus induces propiomelanocortin (POMC) secreting cells in the pituitary gland to produce adrenocorticotropic hormone (ACTH). This hormone will activate adrenal glands of the kidney to release cortisol (the main stress hormone). A negative feedback system via glucocorticoid receptors (GR) establishes homeostasis of the HPA axis. Adapted from Fig. 1 in *Steenbergen et al.* (published in *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* with permission of the publisher, Elseiver©, Amsterdam, Netherlands).

In 1993, McEwen and Stellar proposed a new model to give a clearer explanation of the difference between acute and chronic stress. According to them allostasis and allostatic load are the important factors that distinguish acute from chronic stress [81]. To have a better understanding of this concept, it is important to incorporate the concept of homeostasis as well. Comparatively, homeostasis is defined as physiological systems that are essential for the stability of life, while allostasis is the process that maintains these systems [82]. From a practical viewpoint, homeostasis preserves set points and various boundaries of physiological states (such as pH, temperature, etc), whereas allostasis allows for a modification of these set points in order to counter challenges [82]. Therefore, by default allostasis is positive and necessary to sustain life and it actually supports homeostasis [83]. On the contrary, the allostatic load is the body's wear and tear due to the repeated activation of the adaptive response to stress [81]. The concept of allostasis and allostatic load is reviewed in detail elsewhere[66, 81-83] and beyond the scope of this review.

One of the important features of stress response theory, especially regarding anxiety, is the coping mechanism. Coping involves physiological, psychological and

behavioral responses in order to avoid a threat or distress, and applies to both animals and humans [84, 85]. It is more apparent that susceptibility to stress-induced diseases varies between individuals and may involve a coping mechanism [86]. In the human context, this mechanism is comparable to "temperament" or "personality" traits, which are essential in maintaining an adaptive capacity under changing environments [87]. In general, coping styles among individuals can be classified into two groups: active (proactive) and passive (reactive). However, there is a possibility for large inter-individual variability within these two groups [86].

In addition to the two coping styles mentioned above, there are also two ways in which an organism may respond towards a threat or negative stimulus. One is an active strategy (involving flight-fight response)[88], whereby the main goal is to eliminate the source of threat. The other one is a passive strategy (involving conservation/ withdrawal)[89] and the main aim of this strategy is protection from the consequences of threat. The active coping strategy involves the SAM axis whereas the passive coping strategy involves mainly the HPA axis (see above). Individuals turn to a passive coping strategy whenever the flight-fight response has failed (for example arrested flight[90], entrapment[91], and defeat[90]). It is assumed that anxiety is remarkably increased when the passive coping strategy are used more frequently (**Figure 3**) [86].

## Monoamine Theory

Well documented anxiolytic activity of some antidepressants such as fluoxetine (Prozac®) and amitriptyline (Elavil) suggests the involvement of the monoaminergic system in the pathophysiology of anxiety [40]. According to this theory, disruption of monoaminergic system in the synaptic cleft, specifically involving catecholamines [dopamine (DA) and noradrenaline (NA)] and the indoleamines (serotonin, 5-HT) is thought to be the main reason for anxiety [92]. One candidate gene thought to be causing a malfunction in the signal transduction of monoamines is BDNF [93]. In healthy individuals, BDNF promotes the survival of neurons in the brain. However, under a stressed condition, this gene is down-regulated. This leads to degeneration and apoptosis of neurons in the hippocampus of depleted BDNF [93].

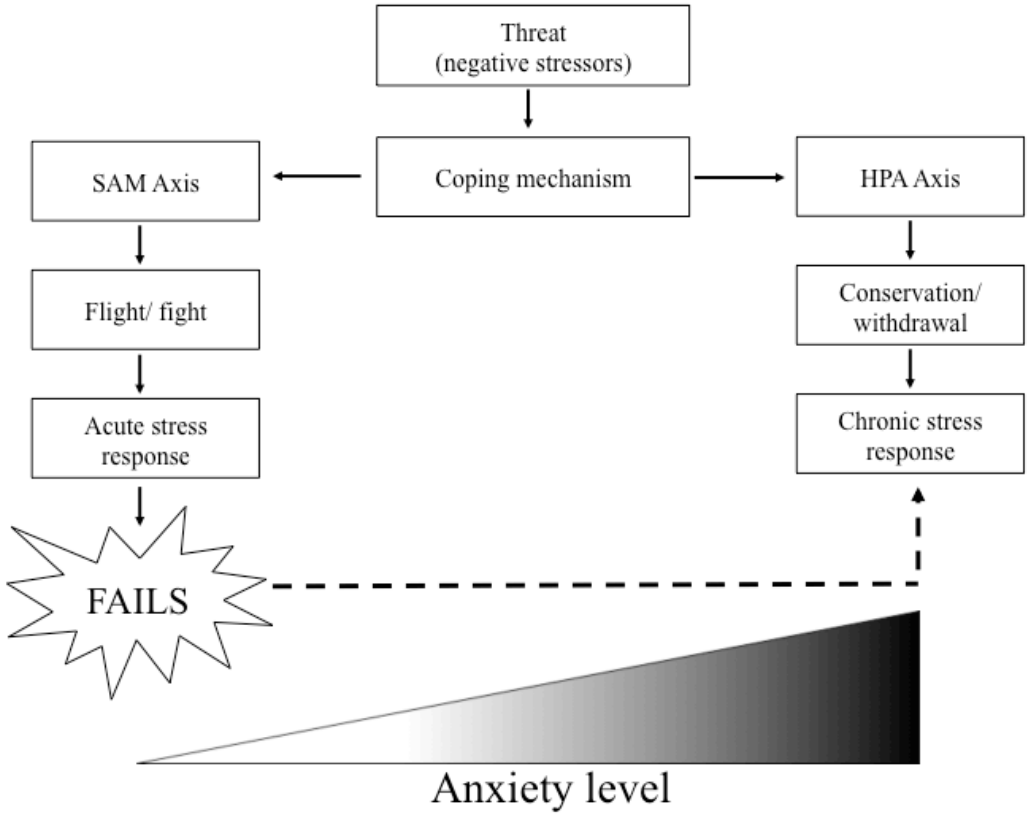



**Figure 3. Coping mechanism is a response to threat (negative stressors).** See text for details. Adapted from Figure 1 in Steimer (published in *Dialogues Clin. Neurosci.* with permission of the publisher, Les Laboratoires Servier©, Suresnes, France).

# Model Organisms for Studying Human Psychological Disorders

The purposes of using animal models to study anxiety are to (i) understand the basic mechanisms (pathophysiology) of anxiety and (ii) develop new therapies [94, 95]. McKinney and Bunney have suggested that a model organism should have the following minimum requirements in comparison with humans: (i) similar pathophysiology (face validity), (ii) comparable etiology (construct validity), (iii) common treatment (predictive validity), (iv) causes behavioral changes that can be monitored accurately, and (v) most importantly reproducible between investigators [96].

Theoretically, a model organism should reproduce all features of a human disease or disorder under investigation. However, this is rarely achieved since psychiatric disorders (including anxiety) are characterised by several clusters of symptoms [97].

Therefore, no model organism can reflect the whole range of symptoms associated with anxiety [98]. Despite this limiting factor, animal models are still useful in research concerning anxiety, since psychiatric disorders are characterized by endophenotypes [99, 100]. The term endophenotype indicates a series of behavioral characteristics that are associated with altered processes involved in particular illnesses [101]. Therefore, instead of replicating the whole syndrome of a specific psychological disorder, an animal model is more suited to replicate particular cluster of symptoms involved in anxiety [98]. Using endophenotypes to design animal models for psychological disorders offers many advantages. For instance, Bakshi and Kalin have highlighted that endophenotypes offer higher chances for construct and predictive validity in the model [98].

## Behavioral Models for Studying Anxiety

So far, the rodent model has been the most extensively and successfully-used laboratory animal in anxiety research. The rodent behavioral models used to study endophenotypes of anxiety can be broadly classified (see **Table 1**) into two categories: (i) unconditioned responses (measuring the organism's innate exploratory behaviour[98]) and (ii) conditioned responses (which often involve training, and interfere with memory and motivational processes [102]). The startle response is a common endophenotype in both conditioned and unconditioned responses. The startle response involves reflex movements upon a stimulus [103]. There are three types of startle responses observed in rodent models: (i) a general startle response (measured while the animal is not subjected to any stimuli), (ii) fear-potentiated startle (observed after the animal is exposed to a stimulus or stimuli), and (iii) context-potentiated startle (promoted by the uncertainty of whether the threat is present or not [103]).

According to Montgomery, animals exposed to a novel environment may respond either by showing an exploratory tendency (driven by curiosity) or withdrawal cues (driven by fear) [104]. There are many factors that affect these behaviors, such as the degree of novelty, the complexity of the situation, and the internal state of the animal [104-108]. Moreover, in a novel environment, animals with low anxiety levels will tend to explore the new environment, while the anxious ones will hesitate to take risks. Therefore, evaluating locomotion and exploratory tendency as the main parameters gives information on the degree of anxiety. The above-mentioned two parameters are useful for evaluating potential new anxiolytic drugs, whereby a

decreased value for these two parameters could indicate anxiolytic activity [109]. However, it is important to consider that a decline in these two parameters might not be purely anxiolytic if they cause additional effects such as locomotion inhibition, toxicity or sedation [109].

**Table 1. Rodent models of anxiety and the various tests that they include.** Adapted from Table 1 in Steimer (published in *Dialogues Clin. Neurosci.* with permission of the publisher, Les Laboratoires Servier©, Suresnes, France).

| Unconditioned response models | Conditioned response models |
|---|---|
| *1. Exploratory behavior* | *1. Conflict test* |
| • Elevated plus maze (EPM) | • Geller-Seitfer test |
| • Elevated T maze | • Vogel test |
| • Open field test (OFT) | |
| • Hole-board test | |
| *2. Light/dark preference test* (LDPT) | *2. Avoidance test* |
| • Light/dark box | • Active avoidance |
| • Light/dark open field | • Passive avoidance |
| *3. Social behavior* | • Fear-potentiated startle |
| • Social interaction test (SIT) | |
| • Stress-induced vocalization | |
| *4. Others* | |
| • Baseline startle response | |
| • Stress-induced hyperthermia (SIH) | |
| • Predator based model | |

In conditioned response anxiety models, an animal's ability to predict aversive events (fear conditioning) is exploited. In this model, an aversive stimulus (unconditioned stimulus, US), such as mild electric shock, is often paired with a neutral stimulus (conditioned stimulus, CS), such as smell, light or sound [110]. Usually, after several pairings of neutral and aversive stimuli, the animal learns that the neutral stimulus is associated with a negative experience [110]. This will eventually elicit fear responses whenever the animal is presented with a neutral stimulus only. One of the important fear responses shown by animals in this model is freezing behavior (characterized by complete cessation of movement, except respiration) [111].

An important behavioral endophenotype assessed in the open field test (OFT) is thigmotaxis. Thigmotaxis is characterized by a preference for an environment adjacent to the periphery, rather than the centre of an arena. This endophenotype has been used as a key index to measure anxiety in mammals was reported in rat[112] and mice [113]. Light dark preference test (LDPT) is another experimental model largely

based on rodents' innate aversion of brightly lit environments [114]. Behavior in LDPT reflects a conflict in animals between the preference for protected areas (for e.g., dark compartment) and innate motivation to explore a novel environment [115]. In both OFT and LDPT, the key parameters are 'percentage time' and 'percentage distance' spent in both 'safe' and 'unsafe' zones of an arena [109]. Moreover, total distance moved in two zones also included since this can give valuable information on the side effects of the drugs. The starting point for all three tests are very important as the animals may show freezing behavior when placed in an 'unsafe' zone [109].

Besides the above-mentioned experimental models, there are other models to study anxiety: one example would be by inducing chronic stress. In the field of stress research concerning anxiety, the main objective is to have a long-lasting stressor that can impair homeostatic state in order to resemble a state of being anxious [109]. There are many ways to accomplish this in the laboratory. Some examples include the following: (i) prenatal stress, (ii) olfactory bulbectomy stress, (iii) repeated restraint stress, (iv) repeated unpredictable stress, (v) repeated social defeat stress [109]. Chronic stress in mice was reported to be inducing anxious-like behavior[116] with elevated levels of DA, NA, and 5-HT levels in the cerebral cortex [117].

## Obstacle in Using Animal Models for Anxiety

Though anxiety can be modeled in the laboratory as explained above, there are several problems that need to be addressed by behavioral scientists when developing animal models of anxiety. Perhaps the first question that arises is whether anxiety is exchangeable with fear, stress, panic, or sensitivity towards an aversive situation?[118] In the animal kingdom, fear has evolved as an adaptive response to provide protection from possible dangerous environments[118], whereas anxiety is fear produced in an anticipated manner towards an imprecise threat. Despite, both fear and anxiety cannot be interchanged, but they can be modulated by the same factors such as environmental and genetic factors [118]. Therefore, it is a normal practice to use fear-related behaviors in a rodent model to investigate anxiety disorders [119]. However, in the field of neuroscience, fear and anxiety appear as two strictly different yet related paradigms [118].

Another important criterion in neurobehavioral research that is often overlooked is the ability to make implicit assumptions when designing animal models of anxiety. In

most animal models of anxiety, a random population of animals is used to study anti-anxiety drugs [120]. However, in the human population, only a small group severely affected by anxiety seek medical attention [121]. Furthermore, it is also necessary to distinguish between 'trait-anxiety' and 'state-anxiety'. According to Lister "State-anxiety is an anticipated fear one experiences at a particular moment and often increased by the presence of an anxiogenic stimulus. Conversely, trait-anxiety is a continuing attribute in an individual with no variation from time to time"[121] The difference between trait-anxiety and state-anxiety can be explained by the following example. Individuals with ophidiophobia may have trait-anxiety at a normal level and low baseline level of state-anxiety under most circumstances. However, the introduction of snake in their environment may increase the state-anxiety. Often, most behavioral studies focus on therapeutics for 'state-anxiety', whereby an animal is exposed to an anxiogenic stimulus before the effect of candidate drug is assessed. Although this approach is easy, fast, and logical, it oversees important factors that contribute to high trait-anxiety, which might not be beneficial to a chronically anxious individual [121].

As mentioned earlier, a good experimental model must have good predictive validity. This is hampered by the ambiguous psychological and pharmacological theories of anxiety. For instance, pharmacologically it is validated that there are standard anxiolytic drugs. Despite this fact, it is undeniable that there is a dispute on which drugs should be used as standards [121]. According to Lister pharmacological validity alone cannot make a good model for anxiety. This is because of many drugs used for anxiety cause various side effects including ataxia, anterograde amnesia, and sedation [121]. Another important remark by Lister is that if an anxiolytic drug is functional in a particular experimental paradigm, it is not necessary for that particular paradigm to be assessing anxiolysis [121]. This is even harder with drugs that have multiple behavioral effects. For example, anxiolytic drugs often function as anticonvulsants as well [122]. Some studies have reported the ability of drugs to antagonize convulsive actions of PTZ as anxiolytic agents. Lister argued that such experimental models must be classified as a correlation model[123] and not as an anxiety model.

## Pharmaceuticals for Anxiety

Anxiety disorders are very heterogeneous in nature; therefore not all anxiety patients are the same, clinically [98]. As mentioned earlier, the pathophysiology of anxiety overlaps with other psychological disorders, such as depression. Initially in the 1960's the treatment for anxiety and depression were distinctly different, whereby the diagnostic notions were clearly dichotomized into major depressive disorder (MAD) and generalized anxiety disorder (GAD), while other anxiety disorder subtypes were clustered together [93]. However, starting from the 70s and 80s, antidepressants overlapped with anxiolytics used in treating anxiety disorder subtypes [93]. At present, the pharmacological treatment for anxiety includes benzodiazepines, 'non-benzodiazepine anxiolytics', tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRIs) [124].

Benzodiazepines affect the GABA system[23] via different mechanisms such as induction of ionic channel transmission, alteration of membrane structures [125], inhibition of GABA transaminase or glutamic acid decarboxylase [33], or just simply by binding to the benzodiazepine site of GABA receptor. Increased GABA neurotransmission produces a damping effect on stimulatory pathways, which eventually provides a relaxing effect and thus alleviating anxiety [126].

TCAs include amitriptyline, clomipramine, doxepin, and trimipramine. This class of drugs targets the norepinephrine and serotonin reuptake mechanism located on the presynaptic membrane of the noradrenergic and serotonergic neurons. By doing so, TCAs increase the accessibility of noradrenaline and serotonin to their corresponding postsynaptic receptors and thus allow enhanced neurotransmission [127].

Additionally, monoamine oxidases (bound to the outer membrane of mitochondria[128]) regulate monoamine levels by breaking down endogenous monoamines (NA, 5-HT, and DA) released in the neuronal cytoplasm in order to avoid excessive build-up and lethal interactions [129]. MAOIs prevent the catabolism of monoamines by blocking the actions of this enzyme. This results in an escalated concentration of monoamines at the synaptic cleft and at postsynaptic receptors [130].

## Motivation to use Natural Products for the Treatment of Anxiety

Many medicines of plant origin have been used for centuries to calm the mind. In most Asian countries traditional herbal medicines have a long history of usage in disease prevention and treatment. Herbal medicines also appear to be popular in the

West. For example, European countries spent $4.96 billion on over-the-counter herbal medicines in 2003 [131]. In the same year, Ginkgo and St. John's Worst were the most commonly reimbursed herbal medicines among German health insurance providers [131]. More information on the prevalence and the type of herbal medicines used by adults who experience anxiety and anxiety disorders is reviewed elsewhere [132]. In Asia, the two main traditional medicinal systems that exploit plant-based treatment are Ayurvedic medicine and traditional Chinese medicine (TCM). Some examples of the plant species used as anxiolytics in those systems include ashwagandha (*Withania somnifera*), passionflower (*Passiflora incarnata*), St. John's Wort (*Hypericum perforatum*), and valerian root (*Valeriana officinalis*). It is also important to note that in Western Europe there was a centuries-long tradition of using plants as anxiolytics and these plants were listed in the official Pharmacopoeias. For example, the British Pharmacopoeia of 1885 lists valerian root among numerous other plant remedies [133].

Most synthetic drugs act according to a 'single-disease/single-target/single-drug' strategy [134]. However, herbal medicines exert their therapeutic actions via interactions of multiple active compounds (known as a synergistic effect). This synergistic effect can be defined as a collective effect produced by a combination of compounds rather than from an individual contribution alone [135]. The concept of synergism is common in traditional medicinal systems [136]. Moreover, there is a possibility for conventional modern (allopathic) medicine to overlook complex mechanisms underlying anxiety. Therefore, the reductionist approach seen in treatments using synthetic compounds could be one of the reasons for the ineffective treatment of anxiety and other psychological disorders. Herbal extracts are speculated not to be directly involved in pathophysiological processes, but instead alter the absorption, distribution, metabolism, and excretion (ADME) of bioactive compounds, or even reduce their side effects [137].

Often, herbal medicines are reported to have 'adaptogenic' effects and are referred to as 'adaptogens' [21]. In 1947, Dr. Nikolai Lazarev introduced the term 'adaptogen' [22]. Adaptogens are substances that are suggested to produce a state of raised resistance, enabling an organism to manage different kinds of stressors [138]. According to Breckhman and Dardymov [139], an adaptogen must have the following: (i) produce a nonspecific response, (ii) have a normalizing effect on the

body, and (iii) do not influence normal body functions. However, The European Medicine Agencies has expressed doubts about the adaptogen concept [140].

## The Challenges in the Research of Herbal Medicine

Plant-based anxiolytics have for many years been assayed on rodent-based behavioral models (reviewed by Sarris *et al.*[26]), Moreover, many preclinical[141-147] and clinical studies[148-153] have identified the anxiolytic activity of herbal medicines. Nevertheless, there are several challenges hampering the progress of herbal psychopharmacology (summarized in **Table 2**).

**Table 2. Current challenges present in research involving herbal psychopharmacology.** Adapted from information in Sarris *et al.* (published in *Eur. Neuropsychopharmacol*. with permission of the publisher, Elseiver©, Amsterdam, Netherlands).

| Challenges | Explanations |
|---|---|
| Predictability of model | • Evidence from *in vitro* model cannot be extrapolated to human clinical applications [26]<br>• *In vivo*, herbal constituents undergo biotransformation [26] |
| Experimental design | • Difficult to standardize experimental design [26]<br>• Improper standardization causes poor translation between different studies [26] |
| Incomplete studies | • Therapeutic effects of main active ingredients do not guarantee same effect for crude extracts [154]<br>• Traditional medicinal systems assume synergy to be the main reason for therapeutic effects [137] |
| Low bioequivalence | • Different commercial herbal preparations have different bioequivalence that needs to be evaluated [26]<br>• Difficult to assess safety and efficacy due to low bioequivalence [26] |
| Poor replication | • Therapeutic effect is not reproducible in different laboratories [26]<br>• For example, *Piper methysticum* in Europe yielded positive results, however, similar results are not replicable in the United States. |
| Practical flaws | • Herbal extracts have flaws that hamper translation into therapeutic application [26], some examples are:<br>▪ Inability to cross the blood-brain barrier [155]<br>▪ Poor aqueous solubility [155]<br>▪ Propensity to degrade easily [155] |
| Safety and efficacy | • These are highly dependent on the chemical composition of the extract, which is influenced by numerous factors [26] including:<br>▪ Phytochemical variability<br>▪ Environmental conditions (temperature, rainfall and etc.)<br>▪ Exposure to pests and microbial infections<br>▪ Parts of the plant used for extraction<br>▪ Preparation method (harvest time, storage, and extraction)<br>▪ Quality of soil |

Despite all these bottlenecks, scientists assume that the integration of omics-technology (systems biology) into phytomedicine will advance this field since this will pave the way to explore different areas of this field. This approach often includes

various biochemical inter-disciplines, such as pharmacogenomics, proteomics, and metabolomics [26]. An example of an application of omics-technology is studying the epigenetic effects of herbal extracts using proteomic analysis [26]. *Hypericum perforatum* was used in two epigenetic studies [156, 157], which revealed the regulation of different genes and proteins involved in synaptic and energy metabolism function. Therefore, systems biology may provide answers to many questions in phytomedicine, such as clinical efficacy, pharmacodynamics, synergy effects, and toxicity.

## The zebrafish in Neurobehavioral Research

The zebrafish (*Danio rerio*) is now an important model organism in neuropharmacology. Both adults and larvae are extensively studied to increase our understanding of the brain function, dysfunction and their genetic and pharmacological modulation [158]. Neurobehavioral tests to assess anxiety in zebrafish are adopted from rodent models [159]. Such tests include open field tank, light-dark tank, and novel tank diving test [158].

Zebrafish has high genetic and physiological homology to humans [158]. Other features of zebrafish are a central nervous system (CNS) similar to that of mammals including mouse and humans[160-162], high fecundity (a single female can produce up to 300 eggs at a time[163]), rapid embryonic development (major organs form within 1 day post fertilization (dpf)[158], easy maintenance at high densities in the laboratory [164], sexual maturation within four months [164], and external development of optically transparent early embryos [164]. This latter feature facilitates the direct observation of tissue and organs development, as well as the *in vivo* injection of drugs or genetic constructs [158]. External development of a transparent embryo is not a feature of development found in mice (*Mus musculus*) and rats (*Rattus rattus*) [164].

The close similarity between mammalian and zebrafish behavioral paradigms can be exploited to study anxiety, fear, post-traumatic disorders, and other stress-related human psychiatric conditions. Previous findings suggest that behavioral studies using zebrafish as the model organism can span multiple behavioral domains including anxiety[165-170], depression[171, 172], neurodegeneration[162], serotonin

syndrome[173], and sleep disorders [174-177]. In fact, former studies have already shown that both larval and adult zebrafish are sensitive to all major classes of neurotropic drugs, including antipsychotics[178, 179], anxiolytics[168, 170], and antidepressants [180, 181].

Last but not least, another major advantage of zebrafish is that they are cost-effective model. They are good candidates for high throughput screening (HTS)[178, 182] and the costs of *in vivo* screening of one drug in the zebrafish are approximately US$300, which is 500 times cheaper than similar rat assays [183].

## Zebrafish Larvae in Neurobehavioral Research for Anxiety

Here the use of zebrafish larvae in anxiety research is discussed. There are several behavioral phenotypes that can be considered as an anxiety-like behavioral domain in zebrafish. Kalueff *et al.* made an extensive catalog of zebrafish behavioral phenotypes for multiple behavioral domains, including for anxiety [184]. Although this catalog is primarily based on adult zebrafish, it can be a good reference to study similar behavior in larval zebrafish. Relevant behavioral phenotypes include: (i) alarm reaction, (ii) burst swimming, (iii) corkscrew swimming, (iv) erratic movement, (v) escape behavior, (vi) freezing, (vi) hyperactivity burst, (vii) immobility, (viii), meander, (ix) photokinesis, (x) startle response, and (xi) thigmotaxis.

Often there is an overlap between zebrafish anxiety-like behavior and fear-related behavior [166, 168, 169]. The fear response is cue-oriented, due to a direct reaction to a currently present stimulus [166, 169, 185-187]. By contrast, the anxiety response is more diffuse since it is produced by potential (but not present) aversive stimuli. Currently, there is no clear distinction in larval zebrafish between these two behavioral phenotypes; however, some phenotypes (e.g. the alarm reaction) are more relevant for assessing fear; others (e.g. withdrawal) more closely represent anxiety-like behavior [184].

Most experimental models of anxiety in zebrafish larvae are based on (i) the visual motor response (VMR) test, (ii) thigmotaxis (inner/outer zone preference), and (iii) scototaxis (light/dark zone preference). The VMR test is an example of a response to startle stimuli and is often measured as the distance that larvae swim following stimuli. Zebrafish larvae can be startled by different stimuli such as acoustic, tactile and visual stimuli [188-190]. All these stimuli create different

responses in the larvae; for example, a sudden transition to darkness is characterized by large-angle (‘O-bend’ shaped) turns [189].

The theory behind scototaxis test is similar to the rodent experimental model; however, zebrafish larvae showed a natural preference for a bright environment instead of for a dark environment [183]. The authors justified this observation based on the fact that zebrafish are diurnal and rodents are nocturnal. However, there is a problem in their justification since adult zebrafish, though also being a diurnal displayed preference to the dark compartment in LDPT [115, 165, 191, 192]. Contradicting to these findings, some authors have reported adult zebrafish showing a preference for the light environment [159, 193]. Reasons for these discrepancies seen in adult zebrafish are not clear but are likely due to different experimental designs used by these different laboratories [194]. Miklósi and Andrew suggested that maturation of melanophores in zebrafish could be the reason for the age-related switch in the preference for light/ dark[195], but this claim needs further clarifications. In essence, how the age-related switch changes the preference of light/ dark in zebrafish is still not thoroughly studied.

Stephenson and colleagues have shown that zebrafish preference for light/ dark is dependent on ambient light levels and olfactory stimulation [196]. Results from this study could provide a potential explanation for the contradicting observations in adult zebrafish. According to the authors, at lower light intensity levels, zebrafish devoid of food odor preferred lighter environment than darker and this preference reversed with increasing light intensity. These highly interesting observations suggest a trade-off between food foraging and the risk of predation. Moreover, this study is a good example showing approach-avoidance motivational conflict suggested by Maximino and colleagues [168]. Scototaxis behavior in zebrafish cannot be explained solely based on avoidance of the white compartment alone or approach to the black compartment alone.

Results from behavioral studies using cavefish have suggested that thigmotaxis is linked to exploration or predator avoidance (approach-avoidance motivational conflict as seen in scototaxis) [197]. Schnörr *et al*. published interesting results for thigmotaxis analysis using zebrafish larvae [198, 199] They found that zebrafish larvae (as young as 5 dpf) express anxiety by showing thigmotactic behavior. In that study, anxiolytics (diazepam) attenuated the wall-hugging (thigmotactic) behavior,

while anxiogenic drugs (e.g. caffeine) enhanced that behavior.

The HPA axis, which governs stress responses, is also conserved in teleost fish such as the zebrafish (where it is referred to as the hypothalamic-pituitary interrenal (HPI) axis). The homology between the HPA and HPI axes in terms of anatomy and molecular constituents could result in similar functional organization and physiology of the stress response [200]. Due to these remarkable qualities, a range of complementary assays can be developed to assess the correlation between behavioral and endocrine systems in relation to stress response in larval zebrafish [200]. For instance, it is possible to translate chronic mild stress (CMS) paradigm in zebrafish larvae to evaluate their behavioral profile.

There are a few important factors that need to be addressed when screening for potential anxiolytics using zebrafish larvae (explained in **Table 3**). Relevant example studies for each of these factors included. These factors can have a huge influence on the outcome of research and warrant proper attention. Anxiolytic drugs have been reported to have side effects, such as impairment of visual sensitivity [201, 202]. Benzodiazepines have been previously shown to affect visual sensitivity at high concentrations [201]. Since the light/dark preference test relies on an intact vision, larval zebrafish could struggle to distinguish white and dark zones when exposed to such drugs.

Airhart *et al.*[203] studied the effect of fluoxetine (an SSRI) on larval zebrafish locomotion and found that larvae exposed to fluoxetine on 4 or 5 dpf showed reduced spontaneous swimming activity (SSA) at 6 dpf with no recovery until 14 dpf. Fluoxetine increases postsynaptic concentrations of serotonin, due to the inhibitory effect on serotonin transporter protein (SERT) [204]. This study also showed a significant reduction of SERT and 5-$HT_{1A}$-receptor transcripts in the spinal cord but not in the brain after fluoxetine treatment. The authors also suggested that fluoxetine neurotoxicity on intraspinal ventromedial neurons could have caused cessations of movement.

**Table 3. Factors that may influence the outcome of larval zebrafish behavioral assays.**

| |
|---|
| **1. Age**<br>• Behavioral assays are performed at 5–7 dpf as most organs are already fully developed.<br>• Same compounds may yield different results if bioassays are performed using larvae of different ages.<br>Example study<br>(I) Ali *et al*.: $LC_{50}$ values of 60 water-soluble compounds declined as the embryo developed. |
| **2. Individual variation**<br>• Coping style is the individual difference in response to stress exposure.<br>Example study<br>(I) Tudorache *et al.*: Individual zebrafish larvae can be classified into early and late emerges. |
| **3. Route of delivery**<br>• Lipophilic compounds are difficult to dissolve in water (immersion exposure) and affects bioavailability and uptake mechanisms.<br>• Immersion exposure technique of hydrophilic compounds can cause unwanted side effects.<br>Example study<br>(I) Bailey *et al.*; Nilsson & Fange; Stray-Pederson; Finney *et al*.: Dissolving compounds in water may affect oxygen exchange in the gills and swim bladder of aquatic organism.<br>(II) Ordas *et al*.: Rifampin and moxifloxacin adhere to the skin of larvae. |
| **4. Strain difference**<br>• Strain type influences general locomotor activity and thigmotactic behavior.<br>• Different strains respond differently to anxiolytic compounds.<br>Example study<br>(I) Egan *et al*.: Adult leopard strain has a higher baseline anxiety level and can be useful in screening anxiolytic compounds.<br>(II) Norton: Analyzed behavior of different wild type strains [AB, Casper, Tubiengen (TU), and Wild Indian Karyotype (WIK)]. TU strain spent lesser time in the outer zone than others. |
| **5. Solvent**<br>• Solvents that used to dissolve the lipophilic compounds can alter locomotor activity at a very lower concentration.<br>Example study<br>(I) Hallare *et al*.: Sub-toxic levels of DMSO increased hsp70 levels in zebrafish embryo and larvae. |
| **6. Temporal factor**<br>• Time frame of a day highly influences behavioral profile of zebrafish larvae<br>Example study<br>(I) Burgess & Granto; MacPhail *et al*.: Zebrafish larvae are hyperactive in the beginning of a day and have stable baseline activity in the afternoon. |

The study by Airhart *et al.* shows significant side effects of fluoxetine that can be mistaken for an anxiolytic effect. Moreover, clinical studies have shown that SSRIs exposure at therapeutic levels during the third trimester of pregnancy causes children

to have lower APGAR (appearance, pulse, grimace, activity, and respiration[205, 206]) scores at birth compared to control group without exposure. The APGAR score provides a quick summary of the health of a newborn baby [206, 207]. Therefore, it is essential to know if the effects of any anxiolytic compounds represent a therapeutic effect or a toxic effect.

## Zebrafish Larvae in Natural Product Research

Unlike the rodent counterpart, zebrafish larvae have not been used extensively for plant-based anxiolytic activity research. Nevertheless, some researchers have exploited zebrafish larvae for other bioassays using plant extracts or even plant-based pure compounds. One of the earliest reports of a plant-based product bioassay using zebrafish as the animal model was on characterizing pro-angiogenic properties of *Angelica sinensis* using transgenic lines of zebrafish [208]. In another study, anti-angiogenic properties of East African medicinal plants were investigated using zebrafish bioassay-guided fractionation. Crawford *et al.* used thin-layer chromatographic (TLC) to fractionate and isolate bioactive compounds responsible for the anti-angiogenic effect [209].

Earlier, zebrafish larvae were used in behavioral assays to identify herbal medicines with antiepileptic (anticonvulsant) properties. These studies involved extracts of *Valeriana officinalis*[210], *Solanum torvum*[211], and *Salvia miltiorrhiza* [212]. Although these three studies focus on antiepileptic activity, they can yield useful information for anxiety research. For example, in those studies, they used pentylenetetrazole (PTZ) to induce epilepsy-like seizures in zebrafish larvae. This compound is known to inhibit $GABA_A$ receptors [213, 214]. Studies with *in vitro* assays revealed that crude herbal valerian extracts and their active constituents such as valerenic acid[215], alkaloids, and lignans, could interact with $GABA_A$[216], glutamates[217], adenosine[218], and serotonin receptors [34]. These receptors are important in neurochemical modulation of anxiety. This study shows that zebrafish larvae can, in principle, be effectively used for screening plant extracts for anxiolytic effects.

Torres-Hernandez and colleagues[210] also used PTZ as an agent to stimulate epileptic seizures in zebrafish larvae. Swimming speed in light and dark conditions, together with light-dependent zone preference, were used as measures of behavioral

activity. The study revealed that crude extract at all concentration tested alleviated PTZ-induced epilepsy. Moreover, the authors reported that valproic acid (VPA: a synthetic analog of valeric acid, which is naturally found in the valerian plant) also reversed the effects of PTZ. However, there are many concerns on the observations from this paper. The main lacking information is the phytochemical profile of the plant extract. Therefore, it is not practical to compare the behavioral changes induced by a crude extract with a pure compound known to be present in the plant (VPA).

Another surprising outcome of the previously mentioned study is that the crude valerian extract alone did not produce any toxic effects after 24 hours of exposure at concentrations 7 mg/ml. This concentration is higher compared to the concentrations used in an unpublished pilot study done at Leiden University (Plant Sciences and Natural Product Laboratory). This study revealed that dried methanol extracts of valerian root were extremely toxic to the larvae (exposure at 4 dpf) even at a very low dosage (~62.5 µg/ml; Muniandy and colleagues, unpublished data). Moreover, according to the authors, the pure compound valerenic acid showed extreme toxicity in their bioassays even at low concentrations. This prompts us to wonder whether valerenic acid was really extracted completely, or even present in the plant samples used in this study. The method of extraction could be the reason for the toxicity differences in both studies.

Torres-Hernandez and colleagues used 48 well plates in the zone preference analysis. This choice of well plate has potential issues. Previously, thigmotaxis behavior in wild-type zebrafish larvae was published using 24 well plates [198, 199]. According to these studies, the width of the inner and outer zone of an arena should be at least equivalent to, or higher than, the length of the larva (c. 4mm at 5 dpf). Based on this criterion, 48 well plate arenas are too small for thigmotaxis analysis. Furthermore, the larvae in that study were acclimatized in darkness for 27 minutes prior to the onset of alternating light and dark conditions. Starting with an aversive condition before recording the behavioral pattern is not optimal as it can influence the basal behavioral pattern and may induce freezing in animals with a high anxiety level [109].

Other studies have examined the anticonvulsant activity of the plants *Solanum torvum*[211] and *Salvia miltiorrhiza*[212] in zebrafish larvae using a new strategy that combines high-performance liquid chromatography (HPLC) microfractionation with

at-line anticonvulsant bioassay. Challal *et al.* reported that both *S. torvum* and its isolated active constituents (triterpene glycosides) showed anticonvulsant activity in PTZ-induced activity. Unlike the valerian study, here the phytochemical profiles of different extracts were reported. A methanol extract of *S. torvum* was chosen for further fractionation since it reduced PTZ-induced activity. Furthermore, the larvae were chronically exposed (18 hours) to crude methanol extract or to isolated compounds before subsequent treatment with PTZ and behavioral analysis. Unfortunately, the study did not include a behavioral profile for the larvae treated with plant extract alone. This information would have been useful because, for example, it is possible that the plant extract alone might cause some physiological sensation that reduces the motor response; this, in turn, could be misinterpreted as an anticonvulsant activity.

The study using *S. miltiorrhiza* reported similar anticonvulsant activity in both crude extract and with purified active constituents (tanshione II and militrone). Unlike the previous study with *S. torvum*, the authors chose an acute exposure (1 h) regime since chronic exposure (3 hours) caused bradycardia, loss of posture, and delayed touch responses to the larvae beyond maximum tolerated concentrations. The toxicity differences at different time exposure in these two studies might be explained in terms of the chemical structure of the purified compounds. Another feature of the study is that the larvae were pre-incubated in 1% DMSO before subsequent exposure to either PTZ or the plant extract. At this concentration, DMSO was not toxic to the larvae; however, care must be taken in interpreting behavioral data when DMSO used as a solvent. This is because DMSO is shown to increase heat shock protein 70 (hsp70, a marker for stress response[219]) levels even at low concentrations in larval zebrafish [220].

Though some studies have shown that zebrafish larvae can be used to explore the therapeutic potentials of plant extracts, this promising animal model needs much more evaluation and optimization. Indeed, plant-based extracts and purified compounds have been reported to be toxic to fish (ichthyotoxic). For example, ichthyotoxicity has been reported for flavonoids[221, 222] and saponins [223-225]. Furthermore, it has been reported that flavonoids exhibit developmental toxicity in developing zebrafish embryos [226]. Saponins are considered to be extremely toxic for poikilothermic (cold-blooded) animals even though they have low oral toxicity for mammals [227,

228] This phenomenon may be attributed to the poor absorption from the gut of mammals [227].

The main reason why plant extracts could be toxic to aquatic organisms, including the zebrafish, is that they disrupt the balance of water chemistry. As plant compounds decompose in the water, dissolved oxygen in the water may be depleted and the fish can become stressed. When under stress, fish exhaust the energy reserves devoted to maintaining the immune system [229]. This may eventually lead to the death of the fish. Another potential reason for plant toxicity towards fish is provided by the example of saponins. Saponins have been shown to damage the gills of fish and are traditionally used as fish toxins [223]. For example, saponins of *Camellia sinensis* (tea) seed cake resulted in the death of tilapia within 5-6 hours of exposure in the water [230]. Rio *et al.* found that saponin toxicity to mummichog fish (*Fundulus heteroclitus*) increased in the water than when injected intraperitoneally[231], which implies that the saponins were actively absorbed by gill membranes. Moreover, saponins induced toxic effects in different fish species (rainbow trout and Chinook salmon) through damages to the intestinal mucosa [232]. A very recent study done in the Philippines showed that water extracts from *Ocimum sanctum* L. (holy basil) and *Tamarindus indica* L. (Tamarind) leaves were highly embryotoxic and teratogenic for zebrafish embryo [233].

# Conclusion

Plant-based therapy for anxiety and other neurological disorders have existed for a long time. However, as with any therapeutic drugs, there are also some issues that need to be addressed when herbal medicines are considered as a means of treatment. The most important issues are efficacy and safety. The high-throughput nature of zebrafish assays can be exploited to investigate herbal medicines. Although zebrafish larvae cannot completely replace the rodent model, they can be a good complement [159].

Bioactivity-guided fractionation is an essential technique to isolate and identify both active and toxic compound in a natural product. Yet, analyzing natural products such as plant extracts is a challenging task since they are made up of a complex matrix with several closely related compounds [234]. Classical bioactivity guided

fractionation is time-consuming[235], labor-intensive[235], and requires multiple chromatographic procedures and large quantities of plant material [236]. On the other hand, zebrafish larvae based screening paradigms only need lower amounts of material at microgram scale [237]. Challal *et al.* have established a new method by combining zebrafish behavioral assay with microfractionation technique to identify bioactive compound from traditional herbal medicine [238].

Therefore, microfractionation technique hyphenated with zebrafish larvae bioassay could be useful in search of new anxiolytic compounds from natural product. However, the hyphenation of these two techniques needs to be further validated in different behavioral paradigms. Moreover, the zebrafish bioassay-guided fractionation technique requires further optimization as well to account for the complexity of herbal extract. Finally, it should be noted that there is evidence that plant extracts can be toxic to fish, even when their toxicity in mammals is low. In a nutshell, plant product screening using zebrafish-based bioassays is still at its infancy stage. However, the vastly growing different -omics technologies (metabolomics, genomics, proteomics etc.) should be hyphenated with different zebrafish-based *in vivo* assays (behavioral, physiological, and toxicology) in order to improve herbal drug discovery efforts. Furthermore, this approach could be an efficient way of identifying novel bioactive molecules in traditionally used herbal medicines.

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**Chapter 3**

# Chronic treatment with serotonergic psychotropic drugs causes locomotor suppression and toxicity in 5-day zebrafish larvae

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*Manuscript in preparation*

# Abstract

There is a demand for development of new psychotropic drugs to treat anxiety disorders. Although zebrafish larvae have a distinct behavioral repertoire by 5 days post fertilization (5 dpf), it is not fully known whether such young larvae respond to psychotropic drugs in the same way that adult humans and mammals do. Here we have examined the behavioral response of 5 dpf larvae after treatment with amitriptyline, buspirone, diazepam, and fluoxetine. We chose these drugs because diazepam is a gold-standard anxiolytic, while the remaining three drugs are commonly used for treating various anxiety disorders. Visual motor response (VMR) was chosen as a behavioral assay because larvae show startle response after sudden exposure to darkness. We measured larval locomotion (total distance moved) and burst activity (maximum velocity) after acute (1 min) and chronic (24 h) treatments with the four drugs. All drugs suppressed larval locomotion and burst activity in the challenge phase. However, amitriptyline and buspirone also suppressed larval locomotion in the basal phase both after acute and chronic exposure. Hence, reduction in locomotion in the challenge phase may not in itself represent anxiolytic effects; it may also indicate toxicity. This is supported by our observation that chronic exposure to two drugs (amitriptyline and buspirone) caused high mortality at the highest concentrations. Moreover, unlike diazepam that produced a monotonic suppression after acute and chronic exposures, the three serotonergic drugs (amitriptyline, buspirone, and fluoxetine) produced nuanced dose responses in larval locomotion. We suggest that 5 dpf larval serotonergic systems are still too immature to be fully responsive to the complex pharmacodynamics of the serotonergic drugs. Future studies could include older larvae and various biochemical analyses (neuroanatomical imaging, gene expression, and toxicity) and behavioral analyses (thigmotaxis, scototaxis, and swimming plus maze test) to yield a comprehensive understanding of how psychotropic drugs work in zebrafish larvae.

# Introduction

Rodent models have been used widely to study the pathogenesis of affective disorders such as anxiety through various behavioral, genetic, and pharmacological assays [1-5]. Another vertebrate model organism that gained much popularity for studying anxiety and other mood disorders is the zebrafish [6-12]. Traditionally, rodent behavioral assays such as the open field-test, light dark box test, social behavior test, and novelty-based tests were used to assess anxiolytic effects of drugs [13-16]. These were later adapted to the zebrafish model [17, 18]. Some examples of the resulting assays in zebrafish include the novel tank test [19-21], light-dark preference test [19, 22, 23], open field tank test [24-26], shoaling test [27-29], and novel object approaching test [30]. Zebrafish are good candidates for studying anxiety because they have physiological and functional similarities with mammals (including humans and rodents) in brain neurotransmitters and their receptors [31-35]. Moreover, there is much evidence pointing to the fact that environmental factors (exposure to novel environments and aversive stimuli) that cause anxiety are similar in zebrafish and rodents [11, 36].

Zebrafish embryos and larvae have advantages over adults for large-scale drug screening. They are small enough to be easily plated out into multiwell plates and this feature can be adapted in high-throughput screens (HTS). HTS has been used in the past to screen different types of drugs [37-41]. In one example, Kokel *et al*. used zebrafish embryos to screen thousands of small molecules to identify neuroactive compounds [41]. In another study, a behavioral profile for zebrafish embryos was established using 60 water-soluble compounds [42].

In addition to their suitability for use in HTS, larvae offer other features that can be helpful to study behavioral, genetic, and pharmacological factors related to anxiety. These features include low husbandry cost, high fecundity, and optical transparency [43-47]. The optical transparency of zebrafish larvae facilitates imaging techniques to study the internal development of organs and tissue systems [48, 49]. Moreover, larval zebrafish are robust for preclinical studies to understand the biodistribution, toxicity, and efficacy of the test compounds [50-52].

There are many behavioral phenotypes that can be used to assess anxiety in zebrafish larvae and screen drugs with anti-anxiety effects. One of the most important behavioral phenotypes identified in larval zebrafish is the startle response [53, 54].

The visual motor response (VMR) is a type of startle response seen in zebrafish larvae at around 3 dpf (days post fertilization), which becomes robust at 5 dpf [55]. The VMR in zebrafish larvae is initiated by sudden exposure darkness [42, 55-57]. A recent study examined 3-dimensional swimming patterns including a downward (diving) response in zebrafish larvae (between 6-12 dpf). Two different experimental setups were used [58]. A cubical tank in the first experiment was used to characterize 3-D swimming patterns after visual and auditory stimuli. In another experiment, tubular tanks were used to record vertical swimming with a visual stimulus only.

In a typical VMR assay, zebrafish larvae are arrayed in a multiwell plate (normally a 96 well plate) to screen either a single or multiple drugs at different concentrations at a time point. VMR is usually measured as distance swum by a larva following the stimulus (lights off). This response variable can be measured using commercially available apparatus such as the ZebraBox (ViewPoint, Lyon, France), DanioVision™ (Noldus, Wageningen, The Netherlands), and Zantiks MWP (Zantiks UK) or with in-house systems [59, 60]. In our opinion, larval zebrafish VMR assays can serve as the first line of a battery of behavioral tests to screen new candidate drugs for anxiety. Moreover, the compatibility of VMR assays with high-throughput drug screening makes zebrafish larvae an excellent choice in preclinical anxiety model research.

In the current study, we have analysed the effects of selected drugs in the VMR assay. The drugs examined were amitriptyline (Elavil), buspirone (Buspar), diazepam (Valium), and fluoxetine (Prozac®). These drugs are used to treat anxiety and anxiety disorders in humans. Moreover, all four drugs have been shown to cause anxiolytic-like effects in adult zebrafish models using different behavioral assays [20, 61-64]. Animal experiments in the drug discovery pipelines are used to determine the efficacy, pharmacokinetics, pharmacodynamics, and toxicity of candidate drugs [51, 52]. In this context, zebrafish larvae are suitable for pre-clinical studies since they can be adapted to high throughput screening assays.

According to Dutch animal laws, larvae are considered to be experimental animals when they become free feeding. This is approximately 5dpf when the yolk sac is consumed, and 5dpf is, therefore, the limit that we have respected in this study. Larvae of this age already show a wide range of behavioral repertoires such as evoked

swimming[65], photomotor response[41], optokinetic response[66], and also VMR [67].

The goal of the current study was to develop a 96-well plate based assay, potentially adaptable for HTS screening of candidate anxiolytic drugs, and based on the 5dpf zebrafish larvae in the VMR assay. We chose four psychotropic drugs commonly prescribed for anxiety and anxiety disorders to validate our assay. Two behavioral parameters were recorded: they are locomotion (measured as total distance moved in mm) and burst activity (measured as maximum velocity in mm/s).

# Materials and methods

## Ethics statement

Animal experimental procedures conducted in this study were all carried out in accordance with the Dutch Animals Act (http://wetten.overheid.nl/BWBR0003081/2014-12-18), the European guidelines for animal experiments (Directive 2010/63/EU; https://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX:32010L0063&qid=1531309204564&from=N) and institutional regulations.

## Zebrafish husbandry

Male and female adult zebrafish (*Danio rerio*) of ABTL wild type strains were maintained in our facility according to standard protocols (zfin.org). Zebrafish eggs were obtained by random pairwise mating of zebrafish. Approximately 10 adult zebrafish (equal male to female ratio) were placed together in small breeding tanks the evening before eggs were required. The breeding tanks have mesh traps to prevent the eggs from being eaten by the adult fish. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (approximately 80 eggs per dish) containing 40 mL fresh embryo medium (EM). Unfertilized, unhealthy and dead embryos were identified under a stereomicroscope and discarded using a plastic Pasteur pipette immediately after plating into Petri dishes. The procedure for the preparation of EM is based on a previously published protocol [42, 68-70].

At 1 dpf, the embryos were again screened and any dead or unhealthy embryos were removed before the healthy embryos were transferred into 96 well plates (one

embryo per well). The transfer was done on 1 dpf to minimize potential damage when transferring at later stages. Chances of damaging the larvae after post-hatching are greater relative to the pre-hatching during transfer [71]. Throughout all procedures, the embryos and the solutions were kept in an acclimatized room at 28 ± 0.5 °C, under a light-dark cycle of 14 hours light and 10 hours dark (lights switch on at 08:00).

## Exposure to psychotropic drugs

Zebrafish larvae were exposed to amitriptyline (Sigma-Aldrich, catalogue number PHR1384), buspirone (Sigma-Aldrich, catalogue number B7148), diazepam (Duchefa Farma, catalogue number 5372) and fluoxetine (Sigma-Aldrich, catalogue number F132). These drugs are referred to hereafter as AMI, BUS, DZM, and FLU respectively. **Table 1** shows the concentrations ranges used for each pharmaceutical and the spatial distribution across the 96 well plates. These concentration ranges were chosen based on previously published works [65, 72-74]. The desired final concentrations were prepared from a stock solution. Prior to the VMR behavioral assay, the larvae were pre-exposed with the pharmaceuticals either for 1 minute (acute exposure) or for 24 hours (chronic exposure). Larvae remained in the test solutions throughout the behavioral analysis. All behavior analyses were conducted with 5 dpf larvae. Hence, chronic exposure was initiated on 4 dpf larvae.

**Table 1. Concentration ranges used in this study and their locations in the 96 well plates.** N = 48 for both controls and untreated larvae.

| | | Location in 96 well plates (C=Column) | | | | | |
|---|---|---|---|---|---|---|---|
| | | **C1 & C7** | **C2 & C8** | **C3 & C9** | **C4 & C10** | **C5 & C11** | **C6 & C12** |
| **Drug/ DMSO concentration µg/ml [%]** | AMI | 0 | 0.625 | 1.25 | 2.5 | 5 | 10 |
| | BUS | 0 | 6.25 | 12.5 | 25 | 50 | 100 |
| | DZM | 0[0] | 0[0.02] | 0.71[0.02] | 1.42[0.02] | 2.84[0.02] | 5.68[0.02] |
| | FLU | 0 | 0.4 | 0.8 | 1.6 | 3.2 | 6.4 |

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine. All drugs were dissolved in embryo medium except for DZM, which was dissolved using DMSO. The final concentration of DMSO in each DZM treatment is 0.02%.)

## Experimental procedure

All behavioral experiments were done in a ZebraBox (ViewPoint, Lyon, France) recording apparatus, equipped with video camera (Point Grey FlyCap 2, Richmond, Canada) and recording software (ViewPoint, Lyon, France). Video analysis was later

done using Ethovision® XT 10 (Noldus Information Technology, Wageningen, Netherlands). Larvae were allowed to acclimatize in the test apparatus for 10 minutes in chronic exposure before recording. Plates were immediately transferred into the testing apparatus in acute exposure experiments.

## Visual motor response (VMR) assay

The experimental design for the VMR assay (shown in **Figure 1**) is adapted from a previous study published in our laboratory [42]. The VMR assay consists of 10 min basal phase (light switched ON, L1), 4 minutes of challenge phase (light switched OFF, D) and 10 minutes of recovery phase (light switched ON, L2). Forty-eight larvae were used for each treatment in this study. We were interested in analyzing larval locomotion from the basal and challenge phases. Locomotion was measured as total distance moved (mm). Data from the basal phase represent the activity of larvae at rest, while the challenge phase represents the response to stimulus and it is, therefore, interesting to see whether drugs can modulate the challenge phase. The recovery phase is present to allow zebrafish larvae to recover from the shock of light stimulus; this is mainly useful in studying habituation. Therefore we do not include it in our data because it is not relevant. In addition to general locomotion, we were also interested in assessing larval burst activity (best captured by maximum velocity) in the challenge phase. This behavioral repertoire appears in larvae at 2 dpf and often associated with escape response [75].

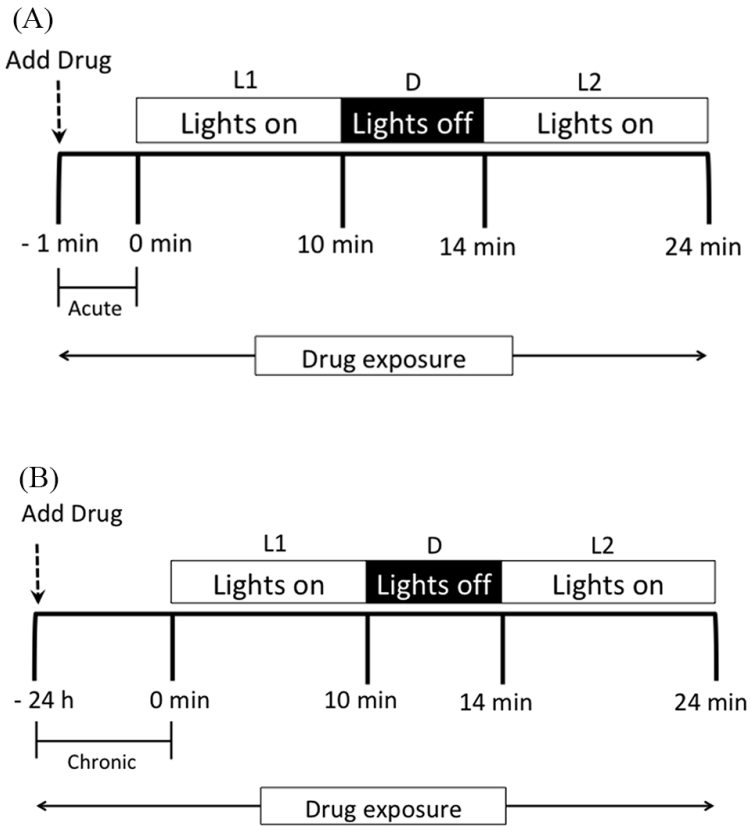


**Figure 1**. **Experimental design for visual-motor response assay.** Drugs were continuously present during the assay period as indicated by the box/arrow 'Drug Exposure' (Pre-exposure: 1 min is acute (A) while 24 h is chronic (B). Time points in the schematic diagram are not shown to scale. Larvae were allowed to acclimatize in the test apparatus for 10 min in chronic exposure before recording. Plates were immediately transferred into the testing apparatus in acute pre-exposure experiments. Key: L1, basal phase; D, challenge phase; L2, recovery phase

## Statistical analyses

Behavioral data from locomotion was analyzed using a mixed model with repeated measures. Data from larval burst activity were analysed using a linear model. Residuals from the regression models were checked for normality using a Q-Q plot. When the normality test failed, Kruskal-Wallis tests with a Pairwise Mann-Whitney U-test as post hoc analysis were chosen to compare controls with treatments. Effect sizes and degrees of freedom were always reported. Each bar in the bar chart represents mean ± SEM (standard error of the mean). All statistical analyses were done using RStudio© (version 1.1.456). N was 48 for both controls and drug-treated larvae and results from these statistical analyses were considered significant when $p < 0.05$.

# Results

## Locomotion after acute treatment with AMI, BUS, DZM, and FLU

DZM (**Figure 2A** and **Table 2**) treatment also reduced zebrafish larvae locomotion in the basal phase at all concentrations. Controls alone for the solvent (0.02% DMSO) showed no effect on larval locomotion in the basal phase. Larval locomotion in the challenge phase decreased at all concentrations of DZM, including the larvae treated with 0.02% DMSO only. Larvae treated with acute DZM also had 100% survival rate.

AMI (**Figure 2B** and **Table 2**) reduced locomotion in the basal phase at all concentrations. Locomotion was also reduced after dark challenge at all concentrations compared to the untreated larvae. The survival rate after acute exposure to AMI was 100% for all concentrations.

BUS (**Figure 2C** and **Table 2**) caused a reduction in locomotion in the basal phase from 12.5 μg/ml onwards. Larvae exposed to acute BUS treatment showed reduced distance moved only at 6.25, 50, and 100 μg/ml after the dark stimulus. The survival rate for this pharmaceutical compound was 100% at all concentrations.

FLU (**Figure 2D** and **Table 2**) produced a different response, compared to the other compounds described above, after acute exposure in the basal phase. It increased larval locomotion only at concentrations of 0.8 and 1.6 μg/ml. By contrast, this drug decreased larval movement in the challenge phase at all concentrations. Similar to the other drugs, acute FLU treatment was also not toxic to the larvae at all concentrations.

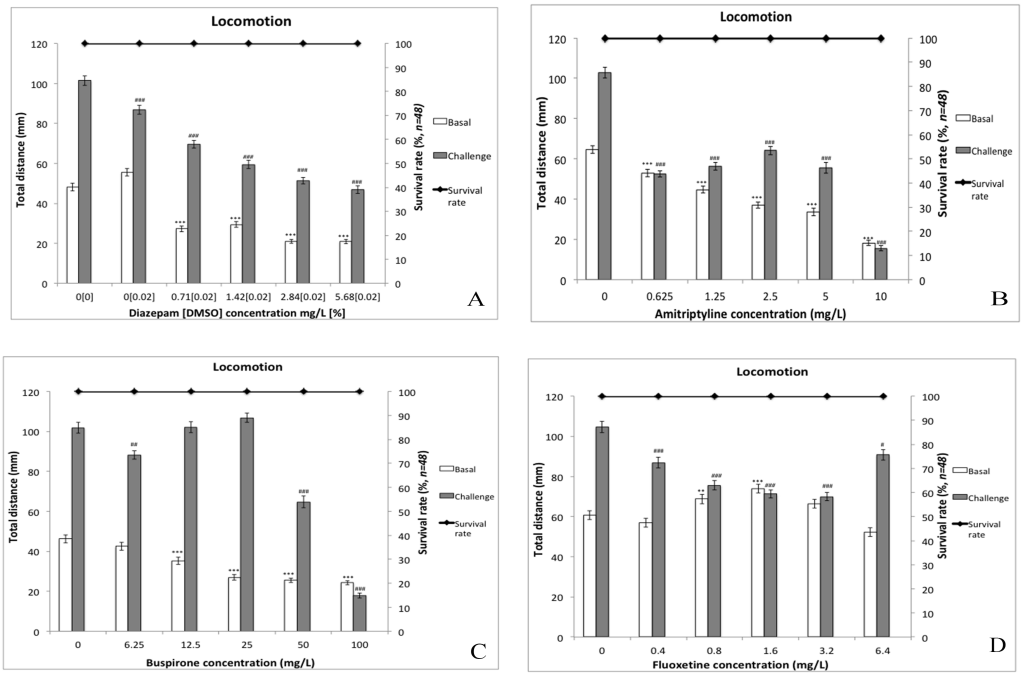


**Figure 2. Locomotion (mean total distance moved, mm) of larval zebrafish after acute exposure psychotropic drugs.** A, DZM; B, AMI; C, BUS and D, FLU. Error bars = ± standard errors of mean (SEM) values. Statistical symbols: (*) = statistical significance comparing control and treatment, basal phase; (#) = statistical significance comparing control and treatment, challenge phase. ** p-value < 0.01, *** p-value < 0.001, # p-value < 0.05, ## p -value <0.01 and ### p-value <0.001. Secondary (line) plot at top of chart = survival rate. *Key*: [ ], final concentration DMSO. Abbreviations: AMI, amitriptyline; BUS, buspirone; DZM, diazepam; DMSO, dimethylsulfoxide; FLU, fluoxetine.

**Table 2. AMI, BUS, DZM, and FLU concentrations that caused significant effects on larval locomotion in the basal (light switch on) and challenge phase (light switch off) after acute exposure.** DMSO (Dimethylsulfoxide) is used to dissolve DZM. Abbreviations: *H* = Kruskal-Wallis chi-squared values; df = degrees of freedom.

| Drugs (Phase) | Comparison (Control⇔Drug concentration) | Locomotion (distance moved in mm) | *p*-values | Test statistics |
|---|---|---|---|---|
| | | Mean ± SEM, *n* | | |
| AMI (basal) | 0⇔0.625 | 52.89 ± 1.77, *n* = 48 | <0.001 | |
| | 0⇔1.25 | 44.71 ± 1.70, *n* = 48 | <0.001 | |
| | 0⇔2.5 | 37.03 ± 1.68, *n* = 48 | <0.001 | *H* = 469.81, df = 5 |
| | 0⇔5.0 | 33.57 ± 1.81, *n* = 48 | <0.001 | |
| | 0⇔10.0 | 18.14 ± 1.32, *n* = 48 | <0.001 | |
| AMI (challenge) | 0⇔0.625 | 52.51 ± 1.55, *n* = 48 | <0.001 | |
| | 0⇔1.25 | 56.27 ± 1.96, *n* = 48 | <0.001 | |
| | 0⇔2.5 | 64.08 ± 2.02, *n* = 48 | <0.001 | *H* = 515.44, df = 5 |
| | 0⇔5.0 | 55.53 ± 2.77, *n* = 48 | <0.001 | |
| | 0⇔10.0 | 15.60 ± 1.37, *n* = 48 | <0.001 | |
| BUS (basal) | 0⇔12.5 | 35.26 ± 1.79, *n* = 48 | <0.001 | |
| | 0⇔25 | 26.97 ± 1.35, *n* = 48 | <0.001 | *H* = 56.019, df = 5 |
| | 0⇔50 | 25.55 ± 1.14, *n* = 48 | <0.001 | |
| | 0⇔100 | 24.31 ± 1.04, *n* = 48 | <0.001 | |
| BUS (challenge) | 0⇔6.25 | 88.29 ± 2.15, *n* = 48 | <0.01 | |
| | 0⇔50 | 64.76 ± 3.00, *n* = 48 | <0.001 | *H* = 540.98, df = 5 |
| | 0⇔100 | 17.86 ± 1.25, *n* = 48 | <0.001 | |
| DZM (basal) | 0[0]⇔0.71[0.02] | 27.28 ± 1.48, *n* = 48 | <0.001 | |
| | 0[0]⇔1.42[0.02] | 29.38 ± 1.47, *n* = 48 | <0.001 | *H* = 288.17, df = 5 |
| | 0[0]⇔2.84[0.02] | 20.92 ± 1.02, *n* = 48 | <0.001 | |

| | | | | |
|---|---|---|---|---|
| | 0[0]⇔5.68[0.02] | 20.94 ± 1.13, *n* = 48 | <0.001 | |
| DZM (challenge) | 0[0]⇔0[0.02] | 86.85 ± 2.35, *n* = 48 | <0.001 | |
| | 0[0]⇔0.71[0.02] | 69.64 ± 1.96, *n* = 48 | <0.001 | |
| | 0[0]⇔1.42[0.02] | 59.38 ± 2.07, *n* = 48 | <0.001 | *H* = 376.91, df = 5 |
| | 0[0]⇔2.84[0.02] | 51.38 ± 1.71, *n* = 48 | <0.001 | |
| | 0[0]⇔5.68[0.02] | 46.75 ± 1.90, *n* = 48 | <0.001 | |
| FLU (basal) | 0⇔0.8 | 68.81 ± 2.41, *n* = 48 | <0.01 | *H* = 87.861, df = 5 |
| | 0⇔1.6 | 73.95 ± 2.13, *n* = 48 | <0.001 | |
| FLU (challenge) | 0⇔0.4 | 86.81 ± 2.69, *n* = 48 | <0.001 | |
| | 0⇔0.8 | 75.61 ± 2.30, *n* = 48 | <0.001 | |
| | 0⇔1.6 | 71.27 ± 1.99, *n* = 48 | <0.001 | *H* = 147.84, df = 5 |
| | 0⇔3.2 | 69.92 ± 2.04, *n* = 48 | <0.001 | |
| | 0⇔6.4 | 90.70 ± 2.57, *n* = 48 | <0.05 | |

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine)

## Locomotion after chronic treatment with AMI, BUS, DZM, and FLU

Diazepam exposure (**Figure 3A** and **Table 3**) did not affect larval locomotion in the basal phase at any concentration used. However, in the solvent controls (0.02% DMSO), larvae showed increased locomotion in the basal phase. After the dark challenge, the larval movement was decreased at all concentrations of DZM. Furthermore, 0.02% of DMSO solvent alone also reduced locomotion.

Amitriptyline (**Figure 3B** and **Table 3**) significantly reduced locomotion at all concentrations where there was no mortality, both in the basal and challenge phase. At the two highest concentrations all larvae died. After 24 hours of exposure to buspirone (**Figure 3C** and **Table 3**), larvae showed reduced locomotion in the basal phase from 12.5 µg/ml onwards. However, at 6.25 µg/ml BUS increased locomotion in the challenge phase. After the dark stimulus, only the two highest concentrations of BUS were associated with reduced larval locomotion. At the two highest concentrations of BUS (50 and 100 µg/ml) survival rate was 85.42 and 4.17%, respectively. Larval locomotion in the basal phase was reduced after 24h treatment with fluoxetine treatment (**Figure 3D** and **Table 3**) only at 0.8 – 3.2 µg/ml. Larval locomotion after the dark stimulus was reduced at all concentrations of FLU.

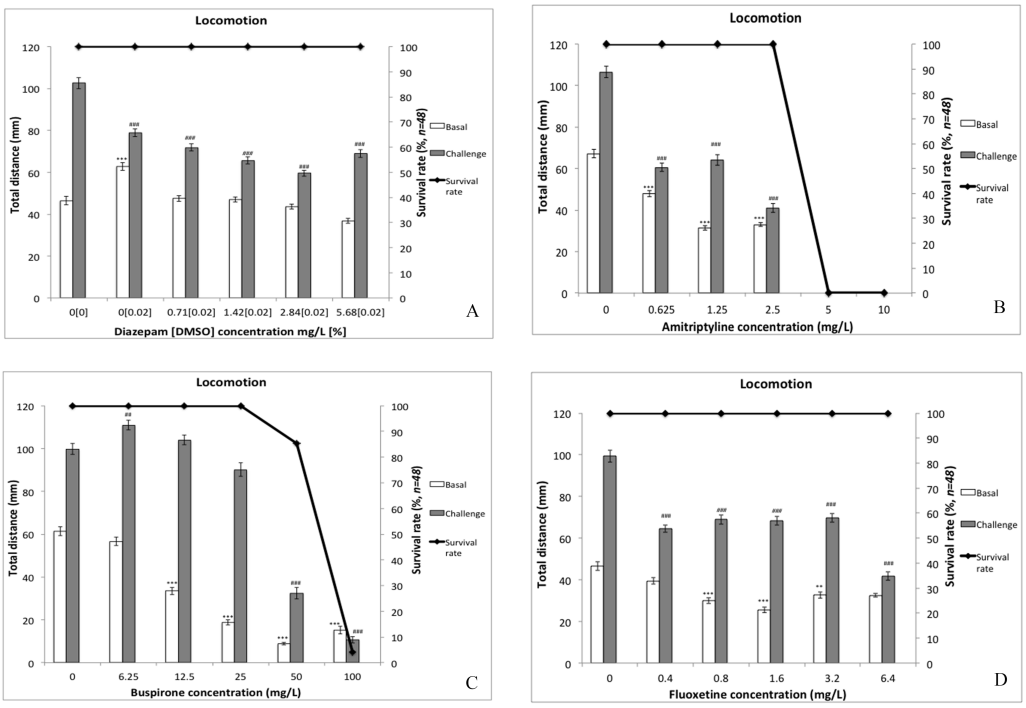


**Figure 3. Locomotion (mean total distance moved, mm) of larval zebrafish after chronic exposure to psychotropic drugs.** A, AMI; B, BUS; C, DZM and D, FLU. Error bars = ± standard errors of mean (SEM). Statistical symbols: (*) = statistical significance comparing control and treatment, basal phase; (#) = statistical significance comparing control and treatment, challenge phase. ** p-value <0.01, *** p-value <0.001, ## p-value <0.01 and ### p-value <0.001. Secondary (line) plot at top of chart = survival rate. *Key*: [ ], final concentration DMSO. Abbreviations: AMI, amitriptyline; BUS, buspirone; DZM, diazepam; DMSO, dimethylsulfoxide; FLU, fluoxetine.

**Table 3. AMI, BUS, DZM, and FLU concentrations that caused significant effects on larval locomotion in the basal (light switch on) and challenge phase (light switch off) after chronic exposure.** DMSO (Dimethylsulfoxide) is used to dissolve DZM. Abbreviations: H = Kruskal-Wallis chi-squared values; df = degrees of freedom.

| Drugs (Phase) | Comparison (Control⇔Drug concentration) | Locomotion (distance moved in mm) | *p*-values | Test statistics |
|---|---|---|---|---|
| | | Mean ± SEM, *n* | | |
| AMI (basal) | 0⇔0.625 | 47.83 ± 1.50, *n*= 48 | <0.001 | |
| | 0⇔1.25 | 31.48 ± 1.13, *n*= 48 | <0.001 | *H* = 219.06, df = 3 |
| | 0⇔2.5 | 33.03 ± 0.86, *n*= 48 | <0.001 | |
| AMI (challenge) | 0⇔0.625 | 60.61 ± 1.96, *n*= 48 | <0.001 | |
| | 0⇔1.25 | 64.20 ± 2.59, *n*= 48 | <0.001 | *H* = 282.18, df = 3 |
| | 0⇔2.5 | 40.95 ± 2.05, *n*= 48 | <0.001 | |
| BUS (basal) | 0⇔12.5 | 33.49 ± 1.72, *n*= 48 | <0.001 | |
| | 0⇔25 | 18.76 ± 1.17, *n*= 48 | <0.001 | *H* = 644.36, df = 5 |
| | 0⇔50 | 8.96 ± 0.63, *n*= 47 | <0.001 | |
| | 0⇔100 | 15.26 ± 1.82, *n*= 2 | <0.001 | |
| BUS (challenge) | 0⇔6.25 | 110.99 ± 2.39, *n*= 48 | <0.01 | |
| | 0⇔50 | 32.48 ± 2.68, *n*= 47 | <0.001 | *H* = 308.34, df = 5 |
| | 0⇔100 | 10.66 ± 1.82, *n*= 2 | <0.001 | |
| DZM (basal) | 0[0]⇔0[0.02] | 62.71 ± 1.80, *n*= 48 | <0.001 | *H* = 130.77, df = 5 |
| DZM (challenge) | 0[0]⇔0[0.02] | 78.87 ± 1.87, *n*= 48 | <0.001 | |
| | 0[0]⇔0.71[0.02] | 71.78 ± 1.65, *n*= 48 | <0.001 | |
| | 0[0]⇔1.42[0.02] | 65.67 ± 1.69, *n*= 48 | <0.001 | *H* = 205.51, df = 5 |
| | 0[0]⇔2.84[0.02] | 59.52 ± 1.50, *n*= 48 | <0.001 | |
| | 0[0]⇔5.68[0.02] | 68.95 ± 1.98, *n*= 48 | <0.001 | |
| FLU (basal) | 0⇔0.8 | 29.87 ± 1.41, *n*= 48 | <0.001 | *H* = 105.26, df = 5 |

| | | | | |
|---|---|---|---|---|
| | 0⇔1.6 | 25.49 ± 1.25, *n*= 48 | <0.001 | |
| | 0⇔3.2 | 32.61 ± 1.42, *n*= 48 | <0.01 | |
| FLU (challenge) | 0⇔0.4 | 64.54 ± 1.84, *n*= 48 | <0.001 | |
| | 0⇔0.8 | 68.89 ± 2.17, *n*= 48 | <0.001 | |
| | 0⇔1.6 | 68.32 ± 2.19, *n*= 48 | <0.001 | $H$ = 262.84, df = 5 |
| | 0⇔3.2 | 69.62 ± 2.05, *n*= 48 | <0.001 | |
| | 0⇔6.4 | 41.85 ± 2.05, *n*= 48 | <0.001 | |

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine)

### Burst activity

Acute and chronic treatments with AMI (**Figure 4A** and **B; Table 4**) resulted in reduced burst activity at all concentrations tested. With chronic exposure, the two highest concentrations tested (50 and 100 mg/L) data are not shown because both concentrations showed 100% mortality. Acute BUS resulted in a decreased burst activity only at 100 mg/L only, while chronic exposure resulted in reduced burst activity at concentrations that were toxic: 50 and 100 mg/L (**Figure 4C** and **D; Table 4**). DMSO, which was used, as a carrier solvent for DZM, had no impact on burst activity in either acute or chronic treatment (**Figure 4E and F; Table 4**). With acute exposures, all concentrations of DZM tested caused a reduction in burst activity. By contrast, chronic exposure only resulted in a reduction in burst activity at 0.71, 1.42, and 2.84 mg/L. Acute exposure to FLU significantly lowered burst activity at 0.4, 0.8, 1.6, and 6.4 mg/L. However, chronic exposure to FLU reduced burst activity in larvae only at 0.8, 1.6, and 3.2 mg/L (**Figure 4G and H; Table 4**).

## Discussion

In this study, we used the VMR assay adapted for zebrafish larvae to assess the effects on the locomotion of four important, widely prescribed psychotropic drugs. Based on recordings of larval locomotion and burst activity in the VMR assay performed in 96 well plates, we find that the assay holds promise for the evaluation of psychotropic drugs. Of the four drugs tested, diazepam gave classic linear (monotonic) dose-response on total distance moved both in acute (1 min) and chronic (24 h) exposure. The other three drugs gave a more heterogeneous response that was sometimes more difficult to interpret.

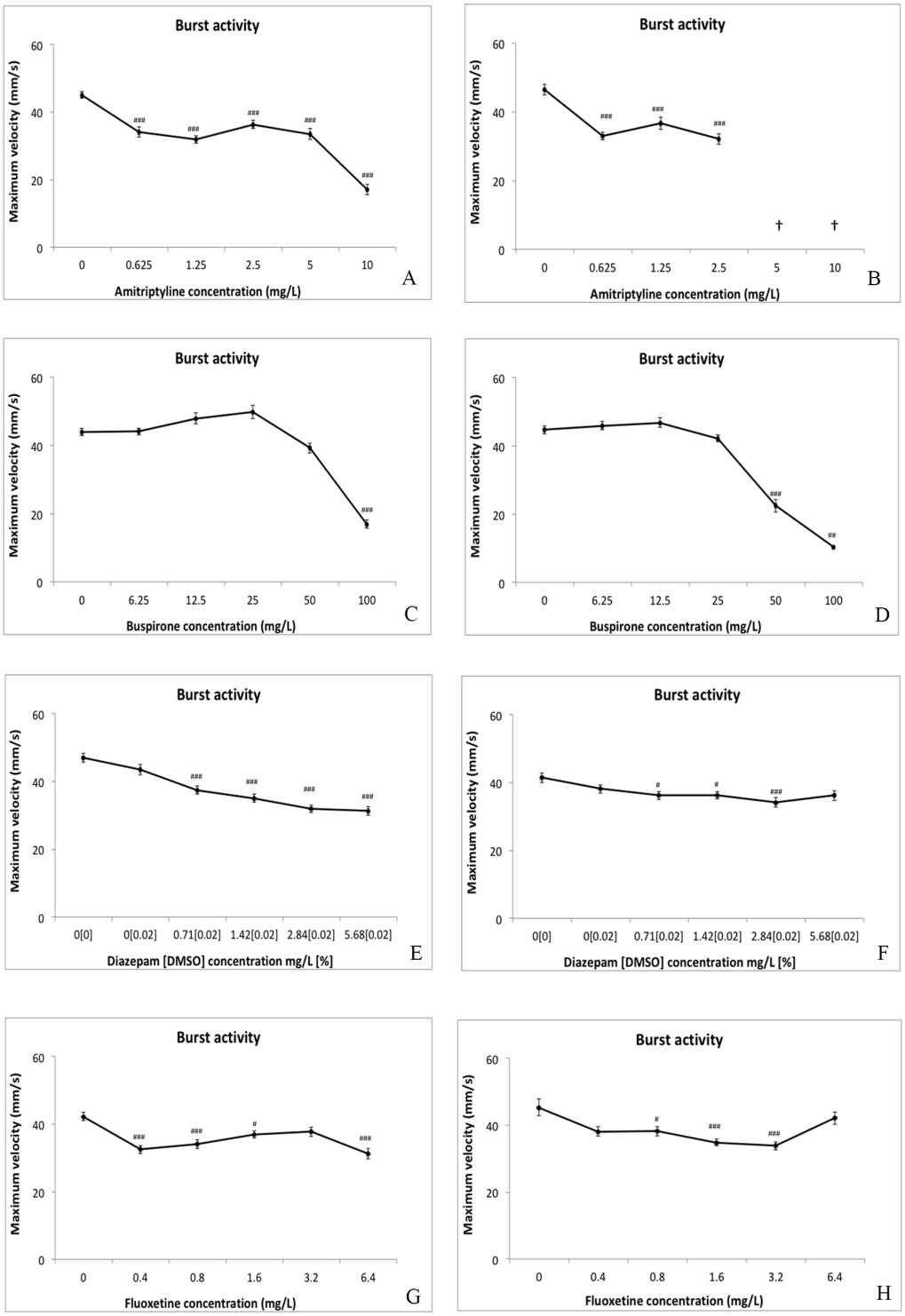


**Figure 4. Impact of AMI, BUS, DZM, and FLU on larval burst activity (mean maximum velocity, mm/s) after exposure to psychotropic drugs.** A, C, E, and G = acute exposure. B, D, F, and H = chronic exposure. Note that the survival rate of larvae is similar to that reported in FIG 2 and 3. Error bars = ± standard error of mean (SEM). Statistical symbols: #, *p*-value <0.05; ##, *p*-value <0.01; ###, *p*-value <0.001. *Key*: [ ], final concentration DMSO; †: larvae with 100% mortality. Abbreviations: AMI, amitriptyline; BUS, buspirone; DZM, diazepam; DMSO, dimethylsulfoxide; FLU, fluoxetine.

**Table 4. AMI, BUS, DZM, and FLU concentrations that caused significant effects on larval burst activity in the basal (light switch on) and challenge phase (light switch off) after chronic exposure.** DMSO (Dimethylsulfoxide) is used to dissolve DZM. Abbreviations: *H* = Kruskal-Wallis-chi squared values; df = degrees of freedom; AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine.

| Drugs (Exposure) | Comparison (Control⇔Drug concentration) | Burst activity (maximum velocity in mm/s) | *p*-values | Test statistics |
|---|---|---|---|---|
| | | Mean ± SEM, *n* | | |
| AMI (acute) | 0⇔0.625 | 34.06 ± 1.45, *n*= 48 | <0.001 | |
| | 0⇔1.25 | 31.82 ± 1.10, *n*= 48 | <0.001 | |
| | 0⇔2.5 | 36.33 ± 1.18, *n*= 48 | <0.001 | *H* = 124.59, df = 5 |
| | 0⇔5 | 33.48 ± 1.69, *n*= 48 | <0.001 | |
| | 0⇔10 | 17.01 ± 1.51, *n*= 48 | <0.001 | |
| AMI (chronic) | 0⇔0.625 | 32.95 ± 1.15, *n*= 48 | <0.001 | |
| | 0⇔1.25 | 36.63 ± 1.74, *n*= 48 | <0.001 | *H* = 49.887, df = 3 |
| | 0⇔2.5 | 32.17 ± 1.57, *n*= 48 | <0.001 | |
| BUS (acute) | 0⇔100 | 16.91 ± 1.23, *n*= 48 | <0.001 | *H* = 128.07, df = 5 |
| BUS (chronic) | 0⇔50 | 22.45 ± 1.84, *n*= 47 | <0.001 | *H* = 81.401, df = 5 |
| | 0⇔100 | 10.40 ± 0.26, *n*= 2 | <0.05 | |
| DZM (acute) | 0[0]⇔0.71[0.02] | 37.45 ± 1.19, *n*= 48 | <0.001 | |
| | 0[0]⇔1.42[0.02] | 35.00 ± 1.25, *n*= 48 | <0.001 | *H* = 92.785, df = 5 |
| | 0[0]⇔2.84[0.02] | 31.88 ± 1.14, *n*= 48 | <0.001 | |
| | 0[0]⇔5.68[0.02] | 31.31 ± 1.32, *n*= 48 | <0.001 | |
| DZM (chronic) | 0[0]⇔0.71[0.02] | 36.18 ± 1.16, *n*= 48 | <0.05 | |
| | 0[0]⇔1.42[0.02] | 36.32 ± 1.09, *n*= 48 | <0.05 | *H* = 25.862, df = 5 |
| | 0[0]⇔2.84[0.02] | 34.17 ± 1.48, *n*= 48 | <0.001 | |
| FLU (acute) | 0⇔0.4 | 32.44 ± 1.18, *n*= 48 | <0.001 | |
| | 0⇔0.8 | 34.07 ± 1.30, *n*= 48 | <0.001 | *H* = 40.135, df = 5 |
| | 0⇔1.6 | 36.93 ± 1.03, *n*= 48 | <0.05 | |
| | 0⇔3.2 | 31.22 ± 1.53, *n*= 48 | <0.001 | |
| FLU (chronic) | 0⇔0.8 | 38.12 ± 1.36, *n*= 48 | <0.05 | |
| | 0⇔1.6 | 34.82 ± 1.02, *n*= 48 | <0.001 | *H* = 37.996, df = 5 |
| | 0⇔3.2 | 33.78 ± 1.84, *n*= 48 | <0.001 | |

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine)

Previous behavioral studies used larval zebrafish to analyze anxiolytic drugs only in a single exposure regime [42, 72, 76]. For example, Richendrfer *et al*. exposed larvae to diazepam and fluoxetine for 2 h in behavioral assays that assessed escape responses [72]. In another study, zebrafish larvae were exposed to diazepam for 45 min to evaluate anxiolytic properties [76]. The effects of acute (short term) and chronic (long term) exposures to these compounds were not reported together in these studies. Little or no work before the current study has been done using a 96-well plate format to assess larval behavior in response to different types of anxiolytic drugs both after acute and chronic treatments.

Interestingly, we found that BUS and AMI are toxic in chronic but not in acute exposure at the same concentrations. For example, AMI at 5 and 10 mg/L and buspirone at 100 mg/L were not toxic to the larvae in acute exposure but were lethal in chronic exposure. Hence, a side-by-side comparison of acute and chronic

treatments is helpful for identifying toxic concentrations that can mimic the desired therapeutic effect. Thus, locomotor suppression could be an unwanted toxic effect or a valuable anxiolytic effect. In addition, the larval VMR assay (acute and chronic) might be useful as a first line of testing when performing HTS or developing new anxiolytic drugs.

A previous study from our laboratory identified four types of dose responses in larval locomotion after 96 h exposure to a panel of 60 water-soluble compounds [42]. Those responses were: (i) monotonic suppression, (ii) monotonic stimulation, (iii) biphasic response [stimulation followed by suppression], and (iv) no effect. In the current study, we also found heterogeneous dose responses.

In contrast to the classic monotonic dose response that we found for diazepam (see above) all serotonergic drugs tested here produced complex dose response effects in the VMR assay. For example, acute and chronic BUS exposure caused a complex dose-response during the challenge phase that deviated from a simple, monotonic suppression of locomotion. Acute FLU produced an optimum curve (inverted 'U') in the basal phase and pessimum curve (U-shaped) dose response in the challenge phase. We assume that the heterogeneity or complexity of effects produced by serotonergic drugs (AMI, BUS, FLU) in the study might be explained based on the pharmacodynamics of those drugs and also on the ontogeny of the serotonergic system of developing larvae. We shall now consider this assumption in more detail.

In humans, the serotonergic drugs used in the current study are presumed to cause their pharmacological activity by adapting, over an extended period of time, the serotonin neurotransmitter system in the brain. According to that presumption, those drugs do not act rapidly at the site of the receptors itself (for example diazepam) [77]. Buspirone acts as a full agonist at 5-HT$1_A$ autoreceptors and a partial agonist of postsynaptic 5-HT$1_A$ receptors [78]. The autoreceptors functions as a brake system that inhibits further release of serotonin after the initial neurotransmission event. Hence, chronic treatment is necessary for humans to desensitize the autoreceptors and increase postsynaptic activation, which is responsible for the therapeutic lag [79].

On the contrary, amitriptyline and fluoxetine are reuptake inhibitors of serotonin that actually decrease serotonin levels initially in the synapse but require chronic treatment before elevating serotonin levels to maximum concentrations where the

pharmacological effects are seen [80]. These findings from humans would explain the fact that we do not see a simple, monotonic response to serotoninergic drugs in our study. We should note at this point that our usage in this study of the terms 'acute' and 'chronic' are arbitrary, and therefore we cannot be sure whether our 'chronic' exposures equate to human 'chronic' exposure.

Another issue that might affect the outcome of our experiments is that we are using rapidly developing larvae and not an adult stage as is true of the human studies. Therefore we cannot necessarily assume that the ontogeny of the serotonergic system is complete at 5 dpf in zebrafish larvae. This immaturity might explain the complex pharmacodynamics elicited by serotonergic drugs in our system. By complex or heterogeneous we mean the non-monotonic suppression of locomotion in larvae treated with serotonergic drugs.

Further explanations for the non-monotonic responses that we observed with serotonergic drugs might come from recently published studies. For example, Tufi *et al*. proposed that changes in neurotransmitter levels during early larval zebrafish development might lead to abnormal development of the CNS (central nervous system) [81]. The authors studied different neurotransmitter profiles in early larvae (≤6 dpf) with and without pesticide treatment. Two main developmental periods or age ranges of zebrafish larvae were studied, namely: the first two days of development and 3-6 dpf. Based on hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry (MS/MS), there were significant changes in the concentrations of many neurotransmitters and their precursors within the two tested periods. However, serotonin concentrations, by contrast, were relatively stable throughout each developmental period tested. The authors suggested that there might be some essential function of serotonin in development. If this is the case, then it could explain the toxicity of AMI and BUS observed in our study. Other studies have also suggested an important role in development for neurotransmitters in the vertebrates.

During early development, neurotransmitters are important in regulating the normal development of the CNS [81]. For example, serotonin was shown to be important for early developmental neurogenesis in Sprague-Dawley rats [82]. Similar importance for this neurotransmitter was also observed in zebrafish, in which it promotes the embryonic development of motor neurons via 5-HT$1_A$ receptors [83]. In

another study, manipulation of serotonin levels in early zebrafish larvae could alter the expression of genes involved in diverse physiological functions including behavior, development, reproduction, and neuroendocrine systems [84]. Global gene expression analysis in early zebrafish larvae has shown that fluoxetine influences the expression of multiple genes involved in processes such as stress response, and DNA binding, replication, and repair [84].

Further examples of the developmental roles of serotonin in zebrafish larvae include the finding that intraspinal serotonergic neurons exhibit great developmental changes between 3 and 4 dpf but stabilize at 5 dpf [85]. Hence, it is possible that the targeting of serotonergic signaling with drugs at these ages could produce unwanted effects on locomotor activity. In support of this possibility, Airhart *et al*. showed that chronic exposure of zebrafish larvae to fluoxetine at 3 – 4 dpf caused a sustained reduction in larval locomotion that lasted until 14 dpf [65]. According to the authors, reduction in movement could be due to neurotoxicity to intraspinal ventromedial neurons. This suggestion was based on the observation of decreased levels of expression of SERT (serotonin transporter) and 5-$HT_{1A}$ receptor transcripts, in the spinal cord. Another recent study also reported that exposure to the SSRI fluoxetine during the early development of zebrafish (1000-cell stage to 3 dpf) had a profoundly negative impact on the expression of several serotonin receptors [86].

Finally, we would like to discuss our choice of organic solvent (dimethylsulfoxide, DMSO) that we used to dissolve diazepam. According to our results, acute exposure to DMSO had no significant effect on locomotion. However, our experiments with chronic exposure to diazepam experiments did, in fact, show slightly increased larval locomotion in the basal phase in 0% DMSO treated larvae compared with 0.02% DMSO treated. Hallare *et al*. reported that DMSO increased hsp70 protein (marker for stress response) levels in zebrafish embryos and larvae even at very low concentrations [87]. The DMSO concentration in the present study is within the range of concentrations used in the Hallare *et al*. study, which showed elevated hsp70 levels. It is clear, therefore, that DMSO alone can have effects on locomotion in certain exposure regimes.

# Conclusions and future perspectives

Our study shows that the behavior of 5-day zebrafish larvae is sensitive to four commonly used psychotropic drugs. All four drugs tested had an effect on general locomotion and maximum velocity, but only diazepam gave a classic, monotonic dose-response. Therefore, we argue that the VMR assay alone cannot be relied on for the assessment of candidate anxiolytic drugs. Additional assays for anxiolytic assessment might usefully include those based on thigmotaxis. Thus, previous work from our laboratory analyzed thigmotactic responses in zebrafish larvae. This study showed anxiolytic-like and anxiogenic-like response to diazepam and caffeine, respectively, in zebrafish larvae.[73] Other assays such as the light-dark preference test (scototaxis) could also be added to the battery of behavioral assays.

Most drugs used in the current study induced a monotonic suppression in locomotion even in the basal phase both after acute and chronic exposure, which warrants further investigations. Diazepam was the only drug that did not affect larval locomotion in the basal phase after chronic exposure, except at the highest concentration. The monotonic suppression observed in larvae treated acutely with diazepam could be due to sedative effects. In addition to this, the behavioral and mortality effects were seen in larvae treated with serotonergic drugs could be due to either developmental response or serotonin toxicity (serotonin syndrome). Presence of serotonin toxicity in adult zebrafish was shown earlier after treatment with amitriptyline [88]. Therefore, the high throughput nature of zebrafish larvae could be easily used to assess serotonin toxicity of drugs that target the serotonergic system.

In summary, our findings show that a behavioral analysis based on VMR assay using zebrafish larvae is not only sensitive for the identification of potential anxiolytic effects but also valuable in providing a measure of toxic effects of drugs. However, incorporating older larvae than 5 dpf and various physiological analyses (neuroanatomical imaging, gene expression profiling, and toxicity profiling, etc.) will provide a comprehensive understanding on the pharmacology of the anxiolytic drugs in zebrafish larvae.

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Chapter 4

# Serotonin toxicity-like phenotypes in zebrafish larvae – chronic treatment with serotonergic psychotropic drugs fails to attenuate thigmotaxis

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*Manuscript in preparation*

# Abstract

Serotonin toxicity is a life-threatening disorder observed in patients consuming serotonergic drugs excessively. We report the presence of phenotypes that resemble symptoms of serotonin toxicity in 5 days post fertilized (dpf) zebrafish larvae treated with serotonergic psychotropic drugs only (amitriptyline, buspirone, and fluoxetine), but not after exposure to diazepam. We used behavioural assays that evaluated larval locomotion, startle response, and thigmotaxis, which commonly used as proxies for anxiety-like behaviour to identify the serotonin toxicity. Untreated zebrafish larvae show reduced thigmotaxis levels during a dark challenge phase. Overall, larval zebrafish retained the reduced thigmotaxis levels after acute pre-exposure to all drugs. However, chronic pre-exposure to amitriptyline and fluoxetine impaired this robust behavioural activity. To confirm our hypothesis that serotonergic drugs could cause serotonin toxicity in zebrafish larvae, we evaluated larval burst activity after the vibrational stimulus. Amitriptyline and buspirone impaired the response to the stimulus. Our results suggest that zebrafish larvae show phenotypes resembling serotonin toxicity after chronic treatment with serotonergic drugs. Moreover, only acute exposure to amitriptyline (2.5 mg/L) and diazepam (0.71 and 1.42 mg/L) attenuated thigmotaxis resembling putative pharmacological effects. In conclusion, we suggest that young larvae are at a critical time point of development that may affect the outcome of the behavioural response to environmental stimuli.

# Introduction

Anxiety-related disorders are recognised as one of the great challenges of the 21st century, in terms of health, economy, and society [1]. The current major pharmaceutical for anxiety-related disorders include benzodiazepines such as diazepam and 'non-benzodiazepines' anxiolytics such as buspirone.[2] Moreover, health practitioners often prescribe antidepressants for anxiety-related disorders since depression is frequently comorbid with anxiety [3]. Some examples of antidepressants are selective serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), and monoamine oxidase inhibitors (MAOIs).

The use of antidepressants is very common and is increasingly intensifying among all age groups [4-7]. Serotonin toxicity (serotonin syndrome) is one of the main health concerns often resulting from the excessive use of antidepressants [8, 9]. Excessive activation of central and peripheral serotonin receptors results in the clinical manifestation of serotonin toxicity [10]: (i) altered mental status, (ii) central nervous system (CNS) activation, and (iii) autonomic hyperactivity [11, 12]. These symptoms can range from a mild manifestation to being lethal [11]. Progression from mild to moderate conditions causes altered mental status (agitation, confusion, etc.), insomnia, and hypertension, and severe symptoms include muscular rigidity, seizures, and coma [13].

In most cases, serotonin toxicity is reported in patients who have consumed a combination of antidepressants [14]. However, this condition was also reported after an overdose of a single serotonergic agent [15]. According to the American Association of Poison Control Centers, in 2011, there were 1,757 serious outcomes due to SSRIs prescription, with 11 being mortal [16]. The number of serotonin toxicity incidents reported are likely an underrepresentation and the actual cases could exceed the number of reported cases since this condition is frequently under-diagnosed [17].

Given the above, the increasing prevalence of serotonin toxicity has become an important biomedical concern [8, 9, 18]. So far, rodent studies have been useful in resembling clinical phenotypes of serotonin toxicity [19-24]. For example, models lacking serotonin transporter (*SERT*) gene have been developed that displayed

elevated extracellular serotonin levels [19, 21, 23]. Although rodent studies have been useful in elucidating the neurochemistry of serotonin toxicity, a limitation consists in inbred strains which do not replicate genetic variations seen of humans [25]. In contrast, zebrafish species show considerable genetic polymorphism and therefore less inbreeding than in rodents [26, 27]. Moreover, larval zebrafish offer low husbandry cost, rapid development, and are useful in high throughput screenings [28-30]. These features can be very helpful in preclinical drug screenings and modeling [29, 30].

Zebrafish are increasingly used to study human brain disorders, including neurological toxidromes (a constellation of signs and symptoms associated with a particular substance or group of substances [31]), because of their strong similarity with human and non-human vertebrates on major brain structures, neurotransmitters, receptors, hormones and functionality [32-36]. Previous studies revealed larval zebrafish to be highly sensitive to a wide range of serotonergic drugs such as amitriptyline, buspirone, and fluoxetine, leading to changes in behaviour associated with anxiety-like phenotypes [37-40]. A recent study revealed serotonin toxicity like behavioural phenotype in adult zebrafish after acute exposure with the antidepressant amitriptyline [41].

In addition, zebrafish larvae have been used extensively in studying anxiety-like responses using behavioural assays such as the visual motor response (VMR), scototaxis (dark/light preference), and thigmotaxis (preference of peripheries/avoidance of open fields). Especially high thigmotactic behaviour in an open arena indicates a low degree of exploratory behaviour, which is associated with anxiety [42, 43]. This behavioural phenotype is evolutionarily conserved across various vertebrate species [43-45]. In addition to this, Thigmotactic behaviour can be reduced by the administration of different types of anxiolytics such as diazepam [38, 46] and fluoxetine [38].

The pharmaceuticals used in this study were amitriptyline (Elavil), buspirone (Buspar), diazepam (Valium), and fluoxetine (Prozac). These drugs are presumed to be causing their therapeutic effects via the following pharmacological interventions: (i) amitriptyline elevates neurotransmitter at the synaptic cleft by blocking reuptake of serotonin and norepinephrine [47, 48], (ii) benzodiazepines interact with the $GABA_A$ receptors in the central nervous system (CNS) [49, 50], (iii) buspirone acts as a full

agonist at presynaptic and partial agonist at postsynaptic serotonin receptors [51, 52], and (iv) fluoxetine increases serotonin concentration in many areas of the brain by blocking the reuptake pumps [53].

### Objectives of study

The objective of the current study is to evaluate the incidence of serotonin toxicity in larval zebrafish, by exploring larval behaviour after exposure to serotonergic drugs. Our hypothesis was that chronic treatment with all serotonergic drugs used in this study, but not diazepam (negative control), would induce behavioural responses that resemble serotonin toxicity. Several behavioural parameters were used to assess the presence of serotonin toxicity like phenotypes in the larvae: (i) general locomotion patterns, (ii) thigmotaxis in response to a dark challenge and (iii) startle response induced by the vibrational stimulus.

## Materials and methods

### Ethics statement

Animal experimental procedures conducted in this study were all carried out in accordance with the Dutch Animals Act (http://wetten.overheid.nl/BWBR0003081/2014-12-18), the European guidelines for animal experiments (Directive 2010/63/EU; http://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX:32010L0063) and institutional regulations.

### Zebrafish husbandry

Adult zebrafish (*Danio rerio*) of ABTL wild type strains were maintained in the facility according to the local animal welfare regulations and standard protocols (zfin.org). Zebrafish eggs were obtained by natural spawning (family crossings). Fertilization was performed by at the beginning of the light period. The eggs were harvested the following morning and transferred into 92 mm Ø Petri dishes (approximately 80 eggs per dish) containing 40 mL fresh embryo medium (EM) as a vehicle (control). This medium consists of 10% Hank's balanced salt solution at a concentration of 0.98 g/L in milli-Q water (resistivity = 18.2 MΩ cm), with the addition of sodium bicarbonate at 0.035 g/L to adjust pH to 7.46. Similar buffer medium has been used previously [54, 55]. Unfertilized, unhealthy and dead embryos

were identified under a stereomicroscope and discarded using a plastic Pasteur pipette.

At 1 dpf, the embryos were again screened and any dead or unhealthy embryos were removed before being transferred into 24 well plates. 24-well consist of wells with a diameter of 15.4 mm, which is sufficiently large enough to allow free swimming behaviour in zebrafish larvae [46], necessary to measure thigmotaxis [56]. Each well of a 24 well plate contained one embryo. Throughout all procedures, the embryos and the solutions were kept at 28 ± 0.5°C, under a 14:10 hours light: dark cycle (lights switches on at 08:00).

## Pre-exposure to pharmaceuticals

Zebrafish larvae were exposed to amitriptyline (AMI, Sigma-Aldrich, PHR1384), buspirone (BUS, Sigma-Aldrich, B7148), diazepam (DZM, Duchefa Farma 5372) and fluoxetine (FLU, Sigma-Aldrich, F132) at different range of concentrations (see **Table 1**), prepared from a stock solution. The larvae were subjected to two different pre-exposure regimes prior to the initiation of behaviour analysis, i.e. acute (1 min) and chronic exposure (24 h). The larvae remained in the pharmaceutical solutions throughout the behavioural test. All behavioural tests were conducted at 5 dpf larvae. Hence, chronic exposure was initiated on 4 dpf larvae.

**Table 1. Concentration ranges used in this study and their locations in the 24 well plates.** N = 48 for both controls and untreated larvae.

| | | Location in 24 well plates (C=Column) | | | | | |
|---|---|---|---|---|---|---|---|
| | | **C1** | **C2** | **C3** | **C4** | **C5** | **C6** |
| **Drug/ DMSO concentration µg/ml [%]** | AMI | 0 | 0.625 | 1.25 | 2.5 | 5 | 10 |
| | BUS | 0 | 6.25 | 12.5 | 25 | 50 | 100 |
| | DZM | 0[0] | 0[0.02] | 0.71[0.02] | 1.42[0.02] | 2.84[0.02] | 5.68[0.02] |
| | FLU | 0 | 0.4 | 0.8 | 1.6 | 3.2 | 6.4 |

(DMSO = Dimethylsulfoxide. All drugs were dissolved in embryo medium except for DZM, which was dissolved using DZM. The final concentration of DMSO for each DZM treatment is 0.02%.)

## Behavioural tests

In this study, we used (i) general locomotion patterns and (ii) thigmotaxis, as a response to a dark challenge [46] and a vibrational stimulus [57] to identify phenotypes that could resemble serotonin toxicity. Dark challenge experiments were

conducted in a ZebraBox (ViewPoint, Lyon, France) recording apparatus, equipped with a video camera (Point Grey FlyCap 2, Richmond, Canada) and recording software (ViewPoint, Lyon, France). Video footage was later analysed using Ethovision® XT 10 (Noldus Information Technology, Wageningen, Netherlands) software. Vibrational stimulus experiments were conducted using an inbuilt tapping device in the DanioVision™ DVOC-0040 set up while video analysis was simultaneously performed using Ethovision XT 11.5 (both from Noldus Information Technology, Wageningen, Netherlands).

## *Dark challenge*

After an acclimatization period of 10 minutes during the light phase (L), the larvae were exposed to sudden darkness during the dark challenge of 4 minutes D), causing an immediate and significant increase in swimming activity (**Figure 1A**). The light intensity during L was 163.20 ± 17.25 (mean ±SD) lux, the light intensity during D was 0 lux. During L and D, (i) general locomotion and (ii) thigmotaxis were measured and compared between these two phases.

General locomotion was measured as total distance moved (in mm) over 10-minute intervals across the whole area of the well (arena). Thigmotaxis was measured as two different values. Absolute values represent the distance travelled away from the periphery of an arena (Ethovision XT 10.0 reference manual; in our study, a well of a 24 well plate is considered as an arena). Relative values represent percentage (%) of the total distance moved (TDM, mm) in the outer zone of the well ($\%TDM_{out}$), i.e. a peripheral zone alongside the walls of the well, with a width of approximately one body length (4 mm) [46]. Therefore, thigmotaxis was calculated as a ratio between TDM in the outer zone ($TDM_{out}$) and TDM over the whole test arena [58], consisting of the TDM in the outer ($TDM_{out}$) and TDM of the inner zone ($TDM_{in}$). This calculation is necessary to correct individual differences as recommended by Bouwknecht and Paylor [58]. ($\%\mathrm{TDM_{out}}$) was used to assess the pharmacological effects of the four drugs. A similar variable was used previously to show anxiolytic and anxiogenic effects in zebrafish larvae previously [46, 59].

$$\%TDM_{out} = \frac{TDM_{out}}{Total\ distance\ travelled\ in\ arena\ (TDM_{out} + TDM_{in})} \times 100$$

## *Vibrational stimulus*

To test whether observed differences in the dark challenge exposure are due to the drugs reducing anxiety-like behaviour, or disruption of motor neurons, a reflexive startle response was elicited by a vibrational stimulus (DanioVision™ version DVOC-0040 reference manual). The experimental timeline (**Figure 1B**) was identical to the dark challenge, followed by an additional recovery light phase and then a vibrational stimulus (tapping) at the highest intensity level [57]. After two more seconds, the observation period ended. We chose maximum velocity (mm/s) as a response variable for this analysis to estimate the startle response to the tapping stimulus [57].

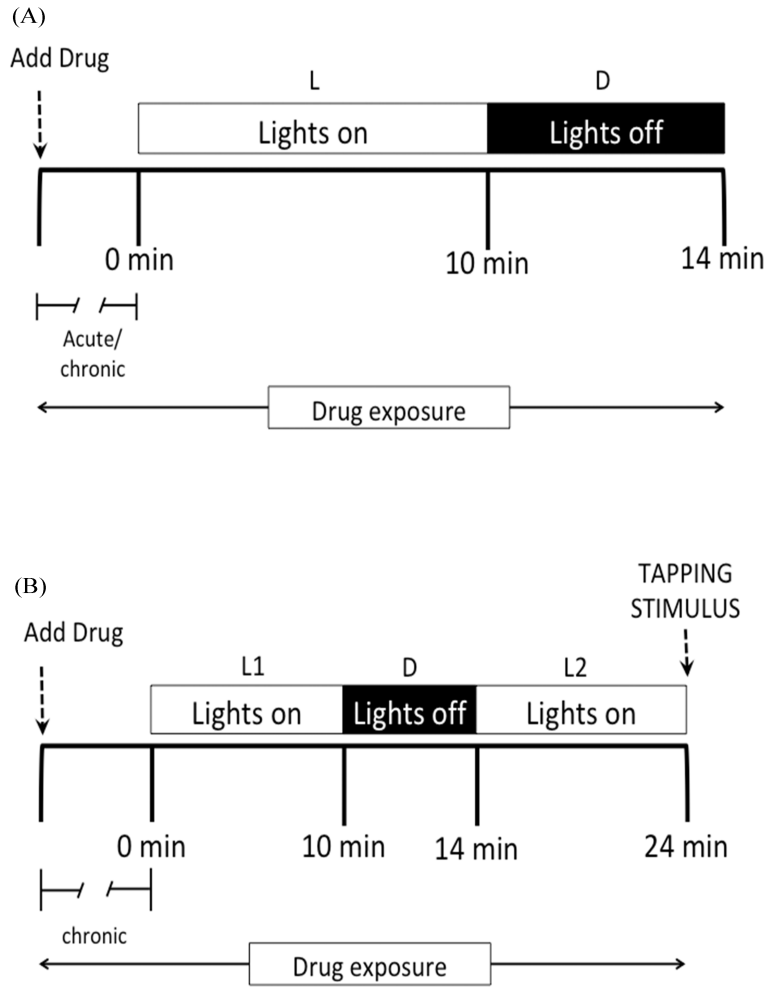


**Figure 1. The experimental scheme used in the present study.** (A) Thigmotaxis analysis with dark challenge stimulus. Data collected from both basal (L) and challenge (D) phase were used to assess absolute thigmotaxis levels (distance travelled away from the periphery of the wells in 24-well plate). Relative thigmotaxis levels were extracted from dark challenge phase. (B) Larval burst activity measured as maximum velocity after vibrational stimulus induced by tapping.

## *Statistical analyses*

Behavioural data from general locomotion, relative thigmotaxis level, and larval burst activity were analysed using linear models. Residuals from the regression model were checked for normality using a Q-Q plot. One-way ANOVA with Dunnett's post hoc analysis was used to compare treatments with control larvae if the assumption of normality is not violated. When the normality test failed, Kruskal-Wallis test with Pairwise Mann-Whitney U-test as post hoc analysis was chosen. Effect sizes and degrees of freedom were always reported. Behavioural data from absolute thigmotaxis level were analysed using mixed model with repeated measures. Distance travelled away from the periphery between basal and dark challenge was compared using the estimated marginal means (emmeans) package in R studio. All statistical analyses were done using RStudio© (version 1.1.456). N was 48 for both controls and drug treatments, and significance was accepted at $p<0.05$. In order to estimate a possible plate and positional effect, we performed a Moran's I test, with full special weighing and nearest neighbours only as parameters ($n=3$, $N=24$, $p<0.05$). In all cases, there was no significant plate or positional effect.

# Results

## Acute drug treatment

### *General locomotion*

AMI and BUS (**Figure 2A** and **C**, **Table 2**) showed a dose-response in locomotor activity resulting in a significant reduction starting at 2.5 mg/L for AMI and 25 mg/L for BUS. The reduction measured for both drugs at the maximum concentration was 75%. DZM (**Figure 2E**; **Table 2**) showed a plateau dose response, but with a significant reduction starting from 1.42 mg/L. Finally, FLU (**Figure 2G**; **Table 2**) showed an optimum dose response upon acute treatment with maximum locomotion levels at 0.8 and 1.6 mg/L. There was no significant effect of the DZM solvent DMSO on locomotion.

### *Absolute thigmotaxis level (distance travelled away from the periphery)*

Untreated zebrafish larvae from the control groups show reduced thigmotaxis level (increased swimming activity/ distance travelled away from the periphery) during dark challenge phase compared to the basal phase. Acute exposure to all

concentrations of the drugs tested did not alter this robust behavioural activity whereby larvae retained the increased swimming activity during the dark challenge compared to the basal phase (**Figure 2B**, **D**, **F**, and **H**).

**Table 2. AMI, BUS, DZM, and FLU concentrations that caused significant effects on general locomotion of zebrafish larvae in the basal phase after acute and chronic exposure.** DMSO (dimethylsulfoxide) is used to dissolve DZM. Abbreviations: *H* = Kruskal-Wallis chi-squared values; df = degrees of freedom.

| Drugs (Exposure) | Comparison (Control⇔ Drug Dose) | General locomotion (distance moved in mm) Mean ± SEM, *n* | *p*-values | Test statistics |
|---|---|---|---|---|
| AMI (acute) | 0⇔2.5 | 1179.14 ± 90.09, *n = 48* | ≤ 0.05 | |
| | 0⇔5.0 | 544.23 ± 43.74, *n = 48* | ≤ 0.001 | *H* = 125.47, df = 5 |
| | 0⇔10.0 | 424 ± 39.79, *n = 48* | ≤ 0.001 | |
| AMI (chronic) | 0⇔1.25 | 504.51 ± 64.4, *n = 47* | ≤ 0.001 | |
| | 0⇔2.5 | 271.35 ± 16.6, *n = 46* | ≤ 0.001 | *H* = 201.26, df = 5 |
| | 0⇔5.0 | 127.24 ± 10. 2, *n = 38* | ≤ 0.001 | |
| | 0⇔10.0 | 2.59 ± 2.77, *n = 37* | ≤ 0.001 | |
| BUS (acute) | 0⇔25 | 1168.67 ± 82.74, *n = 48* | ≤ 0.05 | |
| | 0⇔50 | 698.81 ± 47.88, *n = 48* | ≤ 0.001 | *H* = 134.67, df = 5 |
| | 0⇔100 | 421 ± 25.68, *n = 48* | ≤ 0.001 | |
| BUS (chronic) | 0⇔6.25 | 696.78 ± 96.87, *n = 48* | ≤ 0.05 | |
| | 0⇔12.5 | 423.50 ± 53.02, *n = 48* | ≤ 0.001 | *H* = 60.83, df = 4 |
| | 0⇔25 | 219.05 ± 31.14, *n = 41* | ≤ 0.001 | |
| | 0⇔50 | 184.44 ± 66.67, *n = 32* | ≤ 0.001 | |
| DZM (acute) | 0[0]⇔1.42[0.02] | 1111.03 ± 95.81, *n = 48* | ≤ 0.05 | |
| | 0[0]⇔2.84[0.02] | 854.13 ± 65.34, *n = 48* | ≤ 0.001 | *H* = 45.06, df = 5 |
| | 0[0]⇔5.68[0.02] | 936.20 ± 82.57, *n = 48* | ≤ 0.001 | |
| DZM (chronic) | 0[0]⇔5.68[0.02] | 728.41 ± 74.89, *n = 48* | ≤ 0.01 | *H* = 26.80, df = 5 |
| FLU (acute) | 0⇔0.8 | 1833.83 ± 97.30, *n = 48* | ≤ 0.001 | *H* = 22.61, df = 5 |
| | 0⇔1.6 | 1798.22 ± 87.69, *n = 48* | ≤ 0.001 | |
| FLU (chronic) | 0⇔0.8 | 761.82 ± 91.27, *n = 48* | ≤ 0.001 | |
| | 0⇔1.6 | 752.38 ± 88.50, *n = 48* | ≤ 0.001 | *H* = 98.09, df = 5 |
| | 0⇔3.2 | 475.34 ± 51.61, *n = 48* | ≤ 0.001 | |
| | 0⇔6.4 | 331.26 ± 38.56, *n = 48* | ≤ 0.001 | |

## Chronic drug treatment

### *General locomotion*

Chronic treatment of AMI (**Figure 3A**; **Table 2**) caused a significant drop in locomotion from concentrations of 1.25 mg/L and above. Moreover, chronic exposure to amitriptyline caused lethal effects at the hree highest concentrations. BUS (**Figure 3C**; **Table 2**) led to a reduction of locomotion at 6.25 mg/L and above, and lethal effects starting at 25 mg/L, with 100% mortality at 100 mg/L. DZM led to a reduced in locomotion only at 5.68 mg/L (**Figure 3E**; **Table 2**). FLU caused a reduction in locomotion starting at 0.8 mg/L (**Figure 3G**; **Table 2**). Chronic exposure to DMSO had no measurable effect.

### *Absolute thigmotaxis level (distance travelled away from the periphery)*

Chronic treatment with amitriptyline and fluoxetine impaired increased swimming activity in the dark challenge phase at certain concentrations. For example, 2.5 and 5 mg/L of amitriptyline reduced larval swimming activity resulting in similar levels of

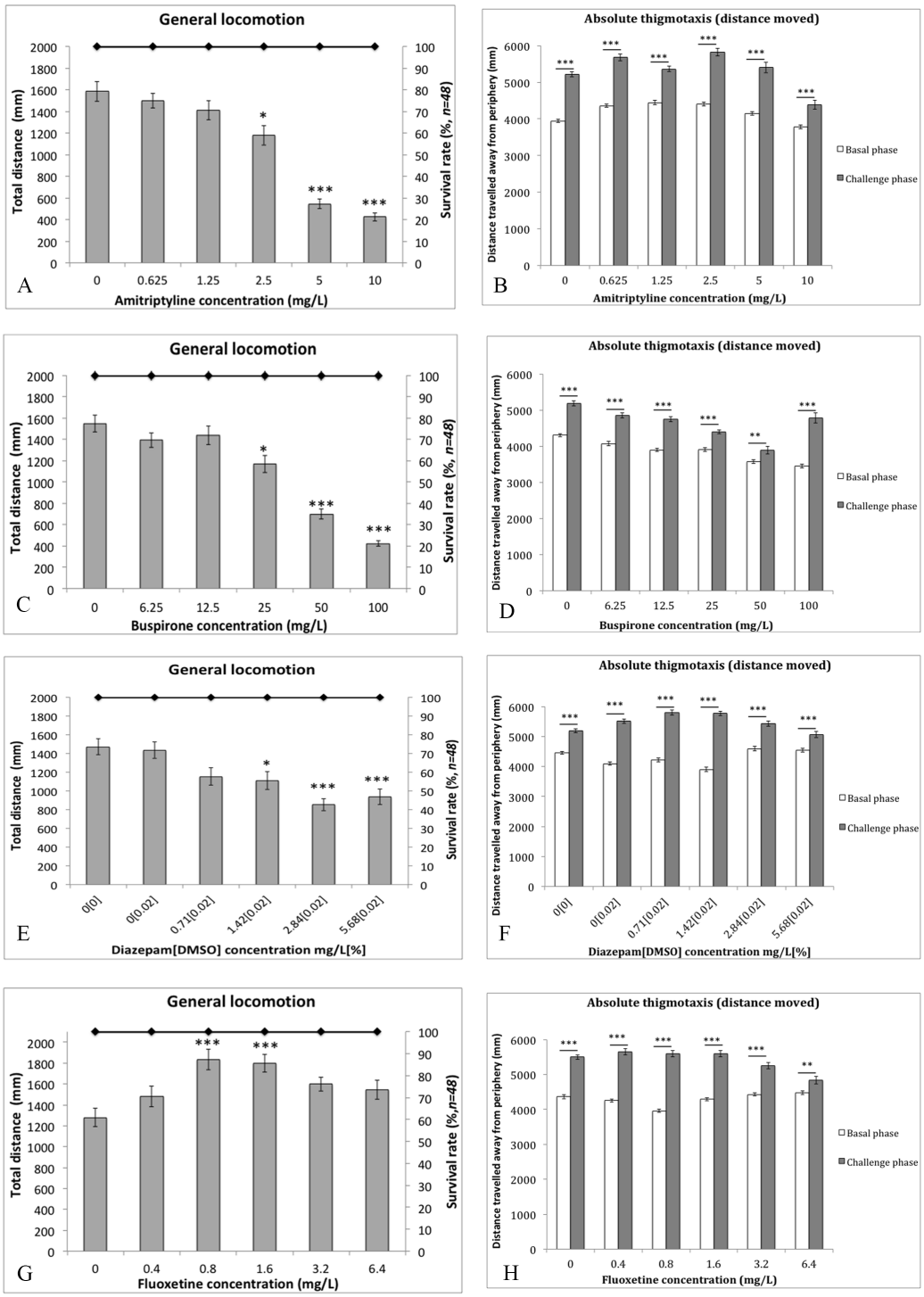


**Figure 2. Impact of drugs on general locomotion (A, C, E, and G) and absolute thigmotaxis level (B, D, F, and H) after acute exposure.** Absolute thigmotaxis is measured as distance traveled away from the periphery of the wells. Line represents survival rate. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001. Abbreviation: DMSO = dimethylsulfoxide.

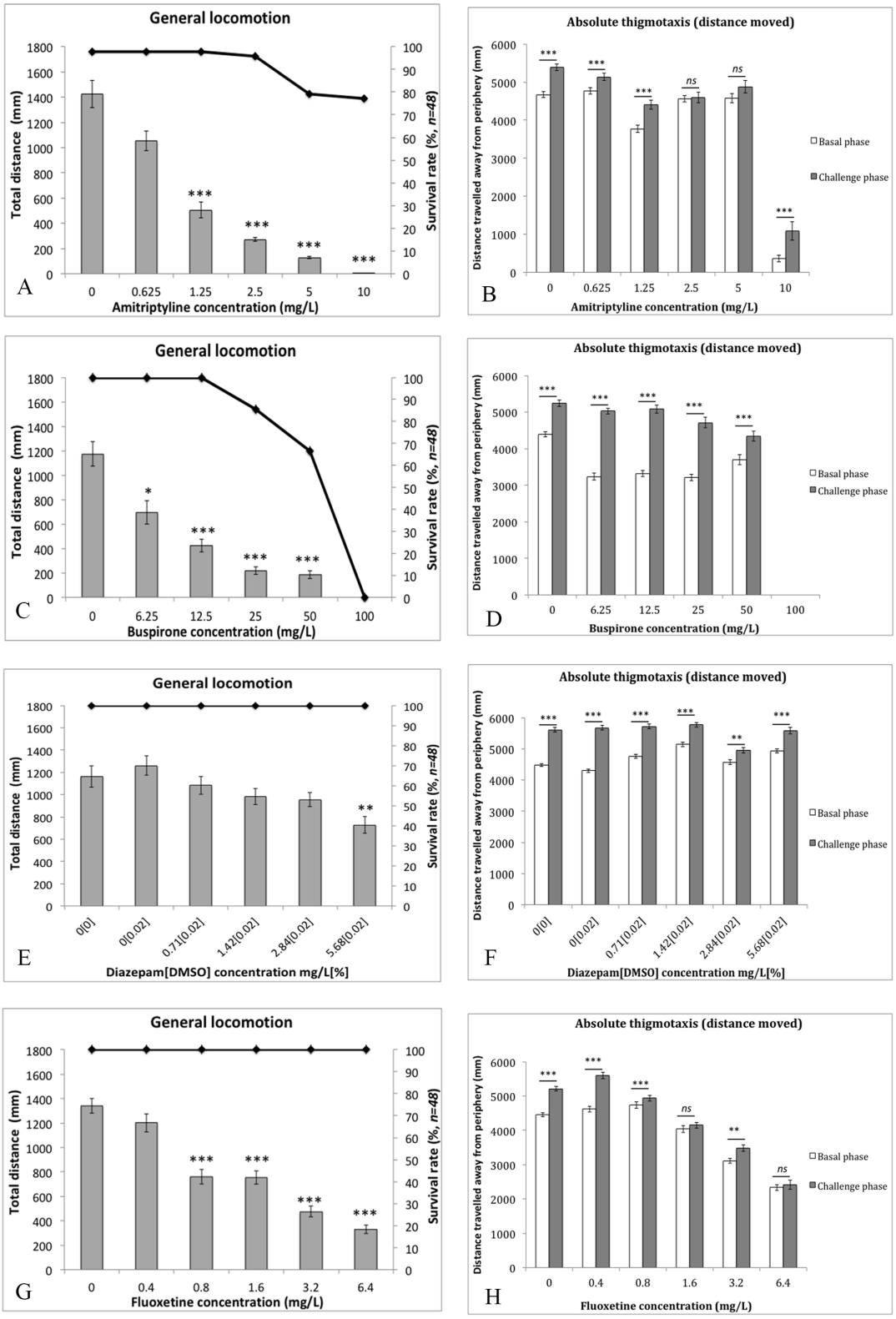


**Figure 3. Impact of drugs on general locomotion (A, C, E, and G) and absolute thigmotaxis level (B, D, F, and H) after chronic exposure.** Absolute thigmotaxis was measured as distance travelled away from the periphery of the wells. Line represents survival rate. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001. *ns*: statistically not significant. Abbreviation: DMSO = dimethylsulfoxide.

thigmotaxis, measured as distance travelled away from the periphery, in both basal and dark challenge phase (**Figure 3B**). At a concentration of 10 mg/L, larval swimming activity in both basal and dark challenge phase dropped to less than 20% compared to the untreated larvae, resulting in significant ($p \leq 0.001$) differences between the phases. FLU at 1.6 and 6.4 mg/L also induced reduction of swimming activity resulting in equal thigmotaxis level in both basal and the dark challenge phase (**Figure 3H**). Chronic treatment with BUS and DZM did not alter thigmotaxis level in larvae after dark challenge phase (**Figure 3D** and **F**), whereby larval swimming activity significantly differed between basal and dark challenge phase overall concentrations.

## Relative thigmotaxis level (% TDM in outer zone)

When measuring thigmotaxis as % TDM in outer zone, acute treatment with diazepam ($H_{(5)} = 38.538$, $p \leq 0.001$; **Figure 4A**) significantly reduced % TDM in the outer zone at 0.71 ($n = 48$, value = 60.33 ± 1.30) and 1.42 mg/L ($n = 48$, value = 62.96 ± 1.55) relative to the untreated larvae. Larval preference for the outer zone was not changed after chronic exposure to diazepam at all tested concentrations (**Figure 4B**). Acute amitriptyline (**Figure S1A**; **Table S1**) significantly reduced larval movement in the outer zone at 5 mg/L, however, at 10 mg/L % TDM in the outer zone increased compared to the untreated larvae. Larvae exposed chronically to amitriptyline showed increased % TDM in the outer zone at 1.25, 2.5 and 5 mg/L (**Figure S1B**; **Table S1**). Larval movement in the outer zone increased after both acute and chronic treatment with Buspirone (**Figure S1C** and **D**; **Table S1**) and fluoxetine (**Figure S1E** and **F**; **Table S1**).

## Vibrational stimulus

One-way ANOVA test and Dunnet's post hoc analysis showed that larvae exposed chronically to amitriptyline ($F_{(2,63)} = 7.313$, $p \leq 0.05$) and buspirone ($F_{(4,87)} = 2.961$, $p < 0.05$) did not respond to the high intense vibrational stimulus at certain concentrations. For example, 10 mg/L of amitriptyline reduced the burst activity of larvae after the vibrational stimulus (**Figure 5A**; Table 3). Buspirone at 6.25 and 12.5 mg/L also caused a reduction in maximum velocity (**Figure 5B**; Table 3). All other drugs had no effect on larval burst activity after the vibrational stimulus.

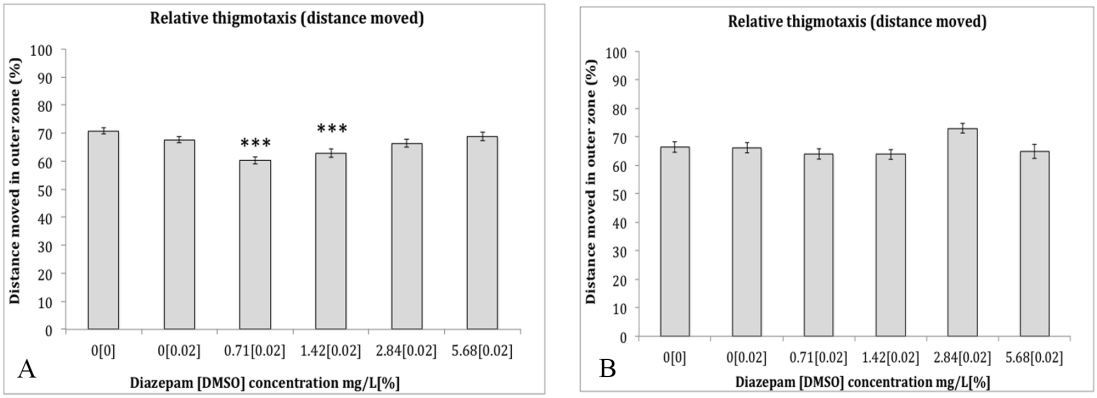


**Figure 4. Impact of diazepam on relative thigmotaxis level after acute and chronic exposure.** Relative thigmotaxis level was measured as *% TDM* in the outer zone as compared to the whole arena. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icon: *** p-value ≤ 0.001. Abbreviation: DMSO = dimethylsulfoxide and *% TDM* = percentage of total distance moved.

**Table 3. AMI and BUS concentrations that reduced larval burst activity significantly after vibrational stimulus.** Drugs were exposed chronically.

| Drugs | Comparison (Control⇔ Drug Dose) | Burst activity (maximum velocity in mm/s) Mean ± SEM, *n* | *p*-values | Test statistics |
|---|---|---|---|---|
| AMI | 0⇔10 | 15.07 ± 2.45, *n = 18* | ≤ 0.05 | $F_{(2,63)} = 7.313$ |
| BUS | 0⇔6.25 | 17.97 ± 1.91, *n = 23* | ≤ 0.05 | $F_{(4,87)} = 2.961$ |
| | 0⇔10 | 18.18 ± 1.77, *n = 24* | ≤ 0.05 | |

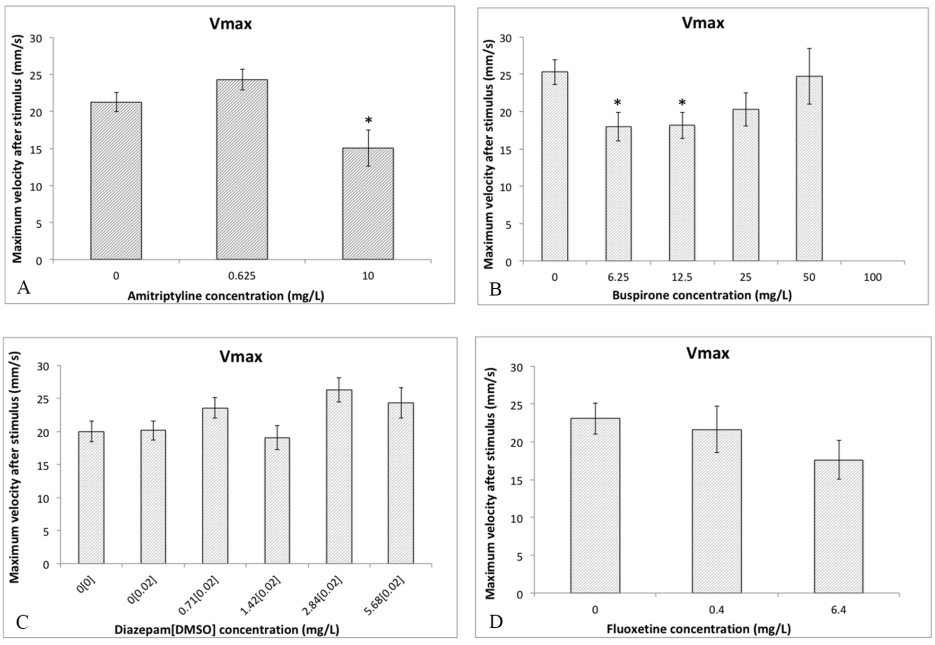


**Figure 5**. **Impact of tapping (vibrational) stimulus on larval zebrafish burst activity after chronic treatment with drugs.** Bar charts represent mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05. Abbreviation: DMSO = dimethylsulfoxide. Vmax = Maximum velocity. Chronic exposure to 10 mg/L resulted in survival rate of 78.26%. Larval survival rate after chronic treatment with buspirone at 6.25, 25 50 and 100 mg/L are 95.84%, 79.17%, 15% and 0% respectively.

# Discussion

## Serotonin toxicity-like phenotype

The goal of the current study was to identify the presence of phenotypes resembling serotonin toxicity in zebrafish larvae. A previous study showed that acute amitriptyline at 1 and 5 mg/L resulted in putative serotonin toxicity-like phenotypes in adult zebrafish, including hypolocomotion and ataxia (vertical swimming and falling on a side) at the bottom of the tank [41]. As of now, it is still not known if serotonin toxicity can be observed in larval zebrafish. Hence, this is the first behavioural study showing serotonin toxicity-like phenotypes in early developing larvae after chronic exposure to serotonergic psychotropic drugs.

Results from the dark challenge test indicate that acute treatment with all drugs did not alter absolute thigmotaxis level represented by increased distance travelled away from the periphery, although hypolocomotion was observed. Chronic exposure to the SNRI, amitriptyline, and SSRI, fluoxetine also caused severe hypolocomotion. In addition, these two drugs also impaired increased absolute thigmotaxis levels in the dark challenge compared to the untreated larvae.

Serotonin toxicity can result in movement and motor disturbances [17]. Larval burst activity measured after chronic treatment with amitriptyline and buspirone further corroborates the presence of impaired motor responses after. Zebrafish larvae respond to the vibrational stimulus by short latency C-bend responses (SLC) that occur within 15 milliseconds of the stimulus [60, 61]. In contrast to the dark challenge stimulus, vibrational stimulus induces a reflex behaviour, modulated by Mauthner cells, without the involvement of CNS [62]. Therefore, our results showing impaired larval burst activity after chronic treatment with amitriptyline and buspirone could indicate phenotypes of serotonin toxicity. Nonetheless, chronic fluoxetine treatment did not show impaired motor responses in behavioural test with vibrational stimulus.

We speculate that young larvae used in this present study have a plastic serotonergic system that may results in high individual variations in response to the treatment with serotonergic psychotropic drugs. Our suggestion could offer a possible explanation on larval responses to chronic fluoxetine (vibrational stimulus experiments) and buspirone (dark challenge stimulus experiments) treatments.

Previous studies show expression of 5-$HT_{1A}$ and 5-$HT_2$ receptors in larval zebrafish [37, 63, 64], which are implicated in serotonin toxicity [18, 65]. Several studies with larval zebrafish reported increased levels of extracellular serotonin levels with increased heart rate, suggesting resemblance tachycardia observed in clinical serotonin toxicity [18, 66]. A very recent study recapitulated rhabdomyolysis in 4 dpf zebrafish larvae after chronic treatment (48 h) with a psychoactive designer drug that acts through serotonin-2A (5-$HT_{2A}$) receptor [67]. Rhabdomyolysis is a condition that induces serious muscle injury [68] and often observed in serotonin toxicity.

## Pharmacological and physiological drug effects

A previous study shows that diazepam significantly attenuated thigmotaxis and therefore reduces anxiety-like behaviour in AB wild type zebrafish larvae after acute exposure with a concentration of 0.71 mg/L [46]. Our current results with the ABTL wild type zebrafish larvae are also in agreement with the previous study. In addition to this, all three serotonergic drugs used in the current study did not reduce anxiety-like behaviour in larvae since they failed to attenuate thigmotaxis.

Richendrfer *et al.* reported that acute fluoxetine (2 hours exposure) had no effect on 7 dpf larval (AB wild type) zebrafish thigmotaxis assay (retained baseline anxiety), but reduced avoidance behaviour (fear response) [38]. In our study, we did not see any pharmacological effects of fluoxetine after both acute and chronic exposure. Previous studies have shown that the choice of animal strain could influence the pharmacological effects antidepressants and this has been shown in both rodents and larval zebrafish. For example, chronic fluoxetine treatment of different mouse strains revealed that only a highly anxious strain (BALB/c) was sensitive to an SSRI [69]. Similar effects were seen in zebrafish larvae, where fluoxetine treatment attenuated startle response in $gr^{s357}$ mutants while it had no effect in the wild type strain [70]. These studies show that fluoxetine could induce its pharmacological effects on organisms with higher baseline anxiety level. Therefore, we suggest to evaluating the drugs used in this study with highly anxious strains such as the Wild Indian Karyotype (WIK), Nadia, and Leopard strains [71].

Another concern that we want to highlight is the optimal time frame for testing larval zebrafish is relatively constricted [72]. Very young larvae (< 3 dpf) show limited behavioural repertoire [72], while larvae from 3–4 dpf are relatively on a

critical time frame of development. For example, by 5 dpf, larval brain development occurs at a slower pace and is still immature [72]. We believe that testing serotonergic drugs on very young larvae could yield individual and batch-wise variations due to possible differential expression patterns of serotonin receptors. Moreover, movement disorders seen in larvae treated chronically with serotonergic drugs could be due to development defects in addition to resembling serotonin toxicity. A previous study [63] that compared the analysis of serotonin receptors and transporters gene expression in the larval and adult zebrafish further corroborates our suggestion that larval zebrafish is developmentally naïve. According to that study, 3-dpf larval brains represent a critical stage of neural development, with similar expression domains of neurogenic genes as embryonic day 12.5/13.5 mouse embryos.

# Conclusion

We report for the first time serotonin toxicity-like phenotype in zebrafish larvae treated chronically with serotonergic anxiolytic and antidepressants. This is based on the drugs capacity to impair larval swimming activity after dark challenge and vibrational stimulus. Moreover, we also want highlight that larval zebrafish used in this study is at a critical time point of neural development and this also could also be the reason for impaired locomotion after chronic treatment with serotonergic drugs. Collectively, the findings from the current study highlight the importance of serotonin neurotransmitter modulation in physiology and behaviour of early developing zebrafish larvae.

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## Supplementary materials

**Table S1** AMI, BUS, and FLU concentrations that caused significant effects on relative thigmotaxis level of zebrafish larvae after acute and chronic exposure. Abbreviations: *H* = Kruskal-Wallis chi squared values, df = degrees of freedom and % TDM = percentage of total distance moved.

| **Drugs (Exposure)** | **Comparison (Control⇔ Drug Dose)** | **Relative thigmotaxis level (*% TDM* in the outer zone)** | ***p*-values** | **Test statistics** |
|---|---|---|---|---|
| | | **Mean ± SEM, *n*** | | |
| AMI (acute) | 0⇔2.5 | 59.26 ± 2.06, *n = 48* | ≤ 0.001 | *H* = 37.032, df = 5 |
| | 0⇔10.0 | 79.36 ± 3.38, *n = 48* | ≤ 0.05 | |
| AMI (chronic) | 0⇔1.25 | 80.17 ± 2.27, *n = 47* | ≤ 0.01 | |
| | 0⇔2.5 | 83.27 ± 2.15, *n = 46* | ≤ 0.001 | *H* = 45.694, df = 5 |
| | 0⇔5.0 | 85.93 ± 4.18, *n = 38* | ≤ 0.001 | |
| BUS (acute) | 0⇔12.5 | 78.12 ± 1.40, *n = 48* | ≤ 0.01 | |
| | 0⇔25 | 84.02 ± 1.10, *n = 48* | ≤ 0.001 | *H* = 44.084, df = 5 |
| | 0⇔50 | 83.30 ± 1.77, *n = 48* | ≤ 0.001 | |
| BUS (chronic) | 0⇔6.25 | 73.73 ± 1.49, *n = 48* | ≤ 0.05 | |
| | 0⇔12.5 | 74.10 ± 2.58, *n = 48* | ≤ 0.05 | *H* = 17.271, df = 4 |
| | 0⇔25 | 74.32 ± 1.87, *n = 41* | ≤ 0.01 | |
| FLU (acute) | 0⇔1.6 | 75.70 ± 1.45, *n = 48* | ≤ 0.01 | *H* = 17.749, df = 5 |
| FLU (chronic) | 0⇔1.6 | 82.99 ± 1.42, *n = 48* | ≤ 0.001 | |
| | 0⇔3.2 | 85.38 ± 1.32, *n = 48* | ≤ 0.001 | *H* = 122.60, df = 5 |
| | 0⇔6.4 | 91.57 ± 1.36, *n = 48* | ≤ 0.001 | |

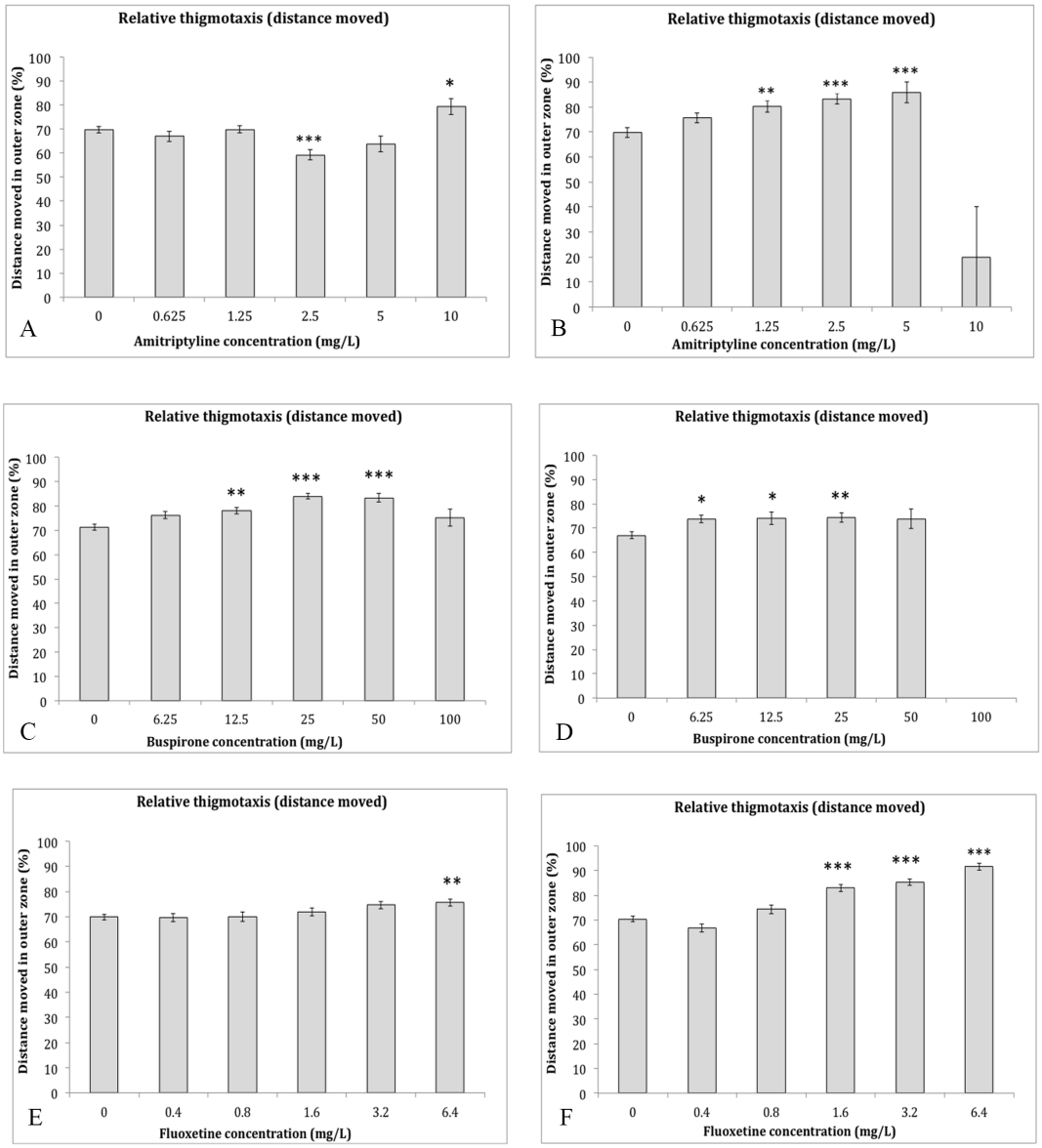


**Figure S1** Impact of amitriptyline, buspirone and fluoxetine on relative thigmotaxis level after acute and chronic exposure. Relative thigmotaxis level was measured as *% TDM* in the outer zone as compared to the whole arena. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001. Abbreviation: DMSO = dimethylsulfoxide and *% TDM* = percentage of total distance moved. Larval survival rate after chronic exposure to the drugs are same as reported in Fig. 3.

Chapter 5

# Indication of developmental toxicity in zebrafish embryos and larvae after treatment with synthetic and herbal-based psychotropic drugs

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*Manuscript in preparation*

# Abstract

Several studies have suggested that synthetic and herbal psychotropic drugs are commonly used during pregnancy. It is therefore important to have a good understanding of the potential adverse effects of these drugs on development. One way to assess developmental toxicity is by using animal models including zebrafish embryos and larvae. Here, we have assessed the developmental toxicity of eight psychotropic drugs by recording lethality ($LC_{50}$), and the incidence of 16 morphological abnormalities, in developing zebrafish. Exposure was done at 1-day post fertilisation (dpf) and the readout was at 2 and 5 dpf. We tested four synthetic drugs (amitriptyline, buspirone, diazepam, and fluoxetine) and four herbal extracts popularly used as psychotropic drugs (*Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Withania somnifera*). All drugs and extracts tested showed concentration-dependent lethality. However, the synthetic drugs showed higher lethality (lower $LC_{50}$) and were associated with a higher incidence of abnormalities compared to the herbal extracts. Among the synthetic drugs, amitriptyline had the lowest $LC_{50}$ and produced numerous abnormalities. *Hypericum perforatum* was associated with a much higher lethality than the other three extracts. Although *Valeriana officinalis* had a relatively low lethality it produced a pattern of multiple abnormalities comparable with the synthetic drugs. Circulatory-related defects were the commonest category of abnormality observed in larvae when embryos treated with amitriptyline, buspirone, and diazepam. We conclude that assays using zebrafish embryos and larvae have good predictivity for the developmental toxicity of synthetic and herbal psychotropic drugs. Given the popularity of the plant-based drugs and their easy availability without prescription, it might be useful to further characterise their pharmacology.


# Introduction

Anxiety disorders are characterized by severe and sustained feelings of fear[1], often accompanied by adverse physiological symptoms including fatigue, dizziness, chest pain, and sleeping problems [2]. Anxiety disorders cause significant disability across the life span in different areas of life such as health, income, education, and interpersonal relationships [3].

Several studies have shown an increased incidence of anxiety-related disorders among pregnant women. Moreover, a significant association between antenatal anxiety and postnatal depression have also been reported [4-6]. The prevalence of anxiety and anxiety-related disorders during pregnancy in developed and developing countries are 10% and 25% respectively [7-9]. Some common synthetic drugs used during pregnancy include anxiolytics such as benzodiazepines and antidepressants such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), serotonin-norepinephrine reuptake inhibitors (SNRIs) and selective serotonin reuptake inhibitors (SSRIs).

However, drug management during pregnancy poses diverse risks for women afflicted with anxiety and related disorders [10]. The risks include immediate reactions such as spontaneous abortion or premature labour [10]. Moreover, synthetic anxiolytics and antidepressants have been in the past reported to cause major adverse effects such as congenital abnormalities, withdrawal symptoms to the foetus, and birth defects (morphological teratogenicity) [10].

Due to the adverse effects of some synthetic drugs, some pregnant women, especially in developing countries, use plant-based medicines (herbalism) to treat anxiety [11, 12]. Many herbal medicines in the form of tinctures, herbal teas, and essential oils are available as over the counter medicines for various mood disorders. Although plant medicines have the reputation of being safer than synthetic drugs, their potential toxicity and teratogenicity have not been investigated thoroughly, if at all. It might, therefore, be valuable to be able to screen plant drugs for developmental toxicity (including teratogenicity).

The Federal Drug Administration (FDA) in the USA and the European Medicines Agency (EMA) require developmental toxicity of drugs to be screened thoroughly as part of the drug discovery process [13]. Often, pregnant animals (of two different species: usually rodents and rabbits) will be exposed to the candidate drugs during the ‘critical period’ of development when many organ systems are being specified (i.e. the period of organogenesis) [14]. The resultant offspring are monitored for different parameters including mortality rate, morphological abnormalities, and changes in growth rate [15].

While mammalian models have the advantage of being closely related to humans, they have some disadvantages. Work on mammals can be time-consuming, labour-intensive, expensive and typically requires the sacrifice of the mother. The latter can lead to heightened ethical concerns. With the thalidomide disaster, it became apparent that multiple species are necessary for detecting risk to humans. This is because thalidomide was screened on rodents and guinea pigs and was found not to produce malformations in the offspring [15]. The thalidomide disaster is one of the factors that led to animal experimentations being criticized for their lack of consistency in predicting developmental toxicity in humans [16, 17]. Zebrafish are increasingly being used as an alternative or complementary model for drug screening [18, 19] and have been shown to be sensitive to thalidomide and treatment during early development impaired proper development of embryonic fins [20].

Zebrafish (*Danio rerio*) is a small, tropical fresh-water teleost fish whose eggs are fertilised and develop externally, and are optically transparent throughout early development [21, 22]. At 5 days post-fertilization (dpf), the zebrafish larva shows complex multiple behavioural repertoires [23-27] with distinct tissues and organs [28, 29]. These include the brain, heart, liver, intestines, muscle and the nervous system [28, 29]. These organ and tissue systems show many homologies at the physiological and molecular levels with mammals, including humans [28, 30]. Furthermore, 70% of all human genes have counterparts in the zebrafish genome [31]. Despite having a discrete organ and tissue systems, the larva is nonetheless by no means complete in its development. Thus, although larval development starts at around 3 dpf it does not finish until around 45 dpf [32].

The zebrafish is increasingly being used in different areas of toxicological analyses, such as environmental, predictive and reproductive (developmental). The main reasons behind its use in these include its small size, rapid development, and crucially, its consumption of much smaller quantities of test compound than models [33, 34]. Previous studies have exploited different life stages of the zebrafish of to screen toxic effects of various compounds. For example, adults were used to screen lead[35], malathion[36], and metronidazole[37]; juveniles for testing agricultural biocides[38]; embryos and larvae for screening different types of small molecules and nanoparticles [19, 26, 39].

Earlier studies done in our laboratory and elsewhere have shown that zebrafish larvae can be used to screen different types of chemical compounds for developmental toxicity. We have reported on the developmental toxicity and teratogenicity of different classes of water-soluble compounds in this model [40]. The compounds included alkaloids, alcohols, amides, carboxylic acids, and glycosides. Moreover, we demonstrated the presence of phenotypes that resemble foetal alcohol syndrome (craniofacial abnormalities, microphthalmia, and growth retardation) in zebrafish embryos after acute ethanol exposure [41]. In addition, Bugel and colleagues compared the developmental toxicity of various flavonoids using 5 dpf zebrafish larvae [42]. Zebrafish embryos and larvae were also used to assess developmental neurotoxicity of several compounds, including atrazine, dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxyacetic acid (2,4-D), and dieldrin [43].

Despite the promising features of zebrafish embryos and larvae in developmental toxicity assays, there are important issues or limitations that need to be addressed before accepting the full potential of this model. For example, absorption, distribution, metabolism, and excretion (ADME) are important pharmacological factors that may affect the outcome of toxicity. Most zebrafish based developmental toxicity assays are based on waterborne exposure, whereby compounds uptake is by diffusion through the skin [44]. This may result in non-linear compound uptake; therefore internal concentration analysis is necessary to correlate toxic phenotypes with the actual concentration of the compounds within the larvae [15].

Our aim here is to compare the developmental toxicity of four types of a synthetic drug and plant extracts commonly used in the treatment of anxiety-related disorders. We assessed $LC_{50}$, mortality rate, and different phenotypic abnormalities after exposing the larvae to the drugs/ extracts. Abnormalities were scored on the basis of an assessment of various qualitative characters (**Table 1**). We chose qualitative characters because one of our objectives is to have a rapid method for assessing the toxicity of psychotropic drugs. The synthetic drugs tested were amitriptyline (SNRI), buspirone (serotonin receptor agonist), diazepam (a GABA agonist), and fluoxetine (SSRI). The four synthetic drugs used in the current study are commonly used as anxiolytics and/or antidepressants. Furthermore, during pregnancy, these types of drugs are commonly prescribed since they are a mainstay in the

management of panic disorders, anxiety disorders and depression [45-49]. The four plant species were *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis*, and *Withania somnifera*. According to the World Health Organization (WHO) *Monographs on Selected Medicinal Plants* and the references therein, these plants and their products (extracts, decoctions, tinctures etc.) have long been used in traditional medicine to treat various mood disorders and psychological disturbances, including anxiety, anxiety-induced sleep disturbances, depression, nervous excitation, and stress [50-52].

# Materials and methods

## Ethics statement

Animal experimental procedures conducted in this study were all carried out in accordance with the Dutch Animals Act (http://wetten.overheid.nl/BWBR0003081/2014-12-18), the European guidelines for animal experiments (Directive 2010/63/EU; https://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX:32010L0063&qid=1531309204564&from=N) and institutional regulations.

## Zebrafish husbandry

Male and female adult zebrafish (*Danio rerio*) of ABTL wild type strains were maintained in our facility according to standard protocols (zfin.org). Zebrafish eggs were obtained by random pairwise mating. Approximately 10 adult zebrafish (equal male to female ratio) were placed together in small breeding tanks the evening before eggs were required. The breeding tanks have mesh traps to prevent the eggs from being eaten by the adult fish. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (approximately 80 eggs per dish) containing 40 mL fresh embryo medium (EM). The procedure for the preparation of EM is based on a previously published protocol [40]. Unfertilized, unhealthy and dead embryos were identified and discarded using a plastic Pasteur pipette immediately after plating into Petri dishes.

At 1 dpf, the embryos were again screened and any dead or unhealthy embryos were removed. Live, healthy embryos were later dechorionated and transferred to 96

well plates together with 50 µl of EM, one embryo per well. Dechorionation was performed under a light microscope with a pair of watchmaker's forceps. We chose to dechorionate the embryos prior to the exposure to the drugs and herbal extracts since the chorion can act as a protective barrier [53-55]. Only several studies have used dechorionated embryos prior to exposure to the test compounds [42, 56], while most studies used non-dechorionated embryos in their toxicity studies [33, 41, 43, 57]. In studies that used non-dechorionated embryos, there could be an issue related to the exact dose of compounds that is uptaken by the embryos.

The outer wells of the 96 well plates were not used since a previous study in our laboratory showed high levels of evaporation in these wells [33]. Throughout all procedures, the embryos and the solutions were kept in an acclimatized room at 28 ± 0.5 °C, under a light-dark cycle of 14 hours light and 10 hours dark (lights on at 08:00).

## Exposure to synthetic drugs and plant extracts

At 1 dpf, after dechorionation, zebrafish larvae were exposed to a set of test solutions comprising either synthetic drugs or plant extracts. The synthetic drugs were amitriptyline (Sigma-Aldrich, catalogue number PHR1384), buspirone (Sigma-Aldrich, catalogue number B7148), diazepam (Duchefa Farma, catalogue number 5372) and fluoxetine (Sigma-Aldrich, catalogue number F132). The plant extracts used in this study were *Hypericum perforatum*, *Passiflora incarnata*, *Valeriana officinalis* and, *Withania somnifera*.

## Exposure of embryos to compounds or extracts

We used a series of concentrations for both synthetic drugs and plant extracts, whereby each concentration was double the next lowest value (i.e. a geometric range). The concentrations used are shown in Supplementary **Table S1**. In total we used four independent 96 well plates for each test compound or extract, with 24 embryos for each treatment group and 24 embryos for controls.

Amitriptyline, buspirone, and fluoxetine were dissolved directly in embryo medium. Diazepam and all plant extracts were dissolved in dimethylsulfoxide (DMSO). The highest concentration of diazepam (142.4 mg/L) was dissolved in DMSO at 1.0%. The highest concentration for *H. perforatum* (500 mg/L) was

dissolved in 0.16% DMSO, while 2% DMSO was used to dissolve the highest concentration used for the rest of extracts. All highest concentrations of DMSO described above also served as controls for the corresponding drug or extract. Since the DMSO concentrations used were different for diazepam and the plant extracts, we analysed the DMSO independently on zebrafish embryos and larvae. Exposure to the test solutions initiated at 1 dpf and the duration of exposure was 96 hours.

### Morphological assessment of larvae

The morphological assessment was done at 5 dpf using a dissecting stereomicroscope. We scored for mortality rate and also different types of abnormalities. The abnormalities were either physiological (such as poor peripheral blood circulation), or morphological (including various kinds of abnormalities). These abnormalities and their criteria are given in **Table 1**.

### Data analysis and interpretation

The abnormalities are presented as frequencies of occurrences in bar charts. The charts also show the mortality rate as a secondary line plot. $LC_{50}$ was determined based on mortality scoring of four independent experiments from geometric series using Regression Probit analysis. This was achieved by using the dose-response curve (drc) package in RStudio© (version 1.1.456). N was 24 for controls, synthetic drugs, and herbal extracts treated embryos and larvae.

## Results

We have examined the toxicity profiles of eight different psychotropic drugs commonly used in treating anxiety disorders (four synthetic and four herbal-based), in zebrafish larvae, after 48 and 96 h exposures. A normal 5-dpf larva is shown in **Figure 1** and larvae with selected abnormalities (BA, TF, SE, FD PO, YO, AP) after exposure to drugs are shown in **Figure 2.** The full classification and criteria for the various abnormalities are given in **Table 1**. We categorized all 16 abnormalities observed in the current study arbitrarily into four different groups of abnormalities: (a) circulatory-related defects (CD), developmental defects (DD), head defects (HD), and tissue defects (TD).

The description of our findings consider the incidence of abnormalities at the population level; it is beyond the scope of this study to look at the clustering of two or more abnormalities per larva since we are simply interested in comparing synthetic and herbal anxiolytics. A previous study done in our laboratory has reported clustering of abnormalities per larva [58].

**Table 1. Qualitative criteria of abnormalities scored in 2 dpf embryos and 5 dpf larvae.**

| Category of abnormalities | Abnormalities | Qualitative criteria |
|---|---|---|
| Circulatory-related defects (CD) | Cardiomegaly (CM) | The heart appears abnormally bigger compared to the control groups |
| | Pericardial sac oedema (PO) | Pericardium is abnormally swollen with accumulation of pellucid fluid |
| | No circulation (NC) | No sustained blood flow in the peripheral circulation; only occasional heart contractions |
| | Impaired circulation (IC) | Minimal blood flow in the peripheral circulation, circulation in the tail absent |
| | Yolk sac oedema (YO) | Yolk sac is abnormally swollen due to the accumulation of pellucid fluid |
| Developmental defects (DD) | Bent body axis (BA) | Primary axis is abnormally flexed either dorsoventrally or laterally |
| | Growth retardation (GR) | Larvae appear smaller and less developed compared to the controls* |
| | Incomplete pigmentation (IP) | Larvae have dermal hypo-pigmentation compared to the control groups* |
| | Absence of pigments (AP) | Larvae have no dermal melanin |
| | Posterior truncation (PT) | The posterior part of larva (from cloaca to apex of caudal fin) appears truncated* |
| | Enlarged swim bladders (SB) | Swim bladder is abnormally distended compared to the control groups |
| Head defects (HD) | Facial defect (FD) | Larval jaws are malformed compared to the control groups |
| | Small eyes (SE) | The eyes appear abnormally small compared to the control groups |
| Tissue defects (TD) | Tail fin defect (TF) | The tail fin is either absent or truncated at the tip |
| | Necrosis head (NH) | Tissue necrosis of the head of the larvae** |
| | Necrosis body (NB) | Tissue necrosis of body of the larvae** |

*Key:*
*These abnormalities were not quantified and only recorded qualitatively
**In these abnormalities, tissue appeared opaque and amorphous.

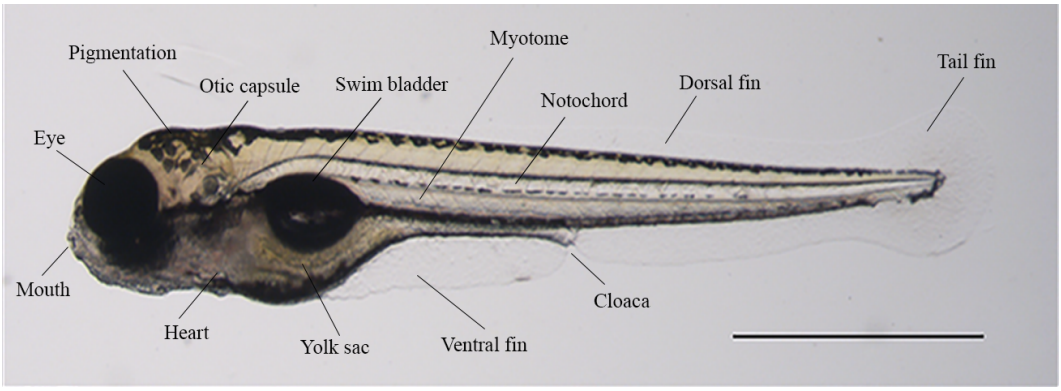


**Figure 1. Photomicrograph of a normal (untreated) zebrafish larva at 5 dpf.** Left lateral aspect. Rostral is to the left. Scale bar = 1 mm.


**Figure 2. Selected phenotypic abnormalities observed in zebrafish larvae at 5 dpf.** This figure shows some of the abnormalities scored after exposure to synthetic drugs or plant extracts. All images depict left lateral views. Rostral is to the left. Each larva is shown to the same scale, bar in A = 1 mm). A: Larva shows bent body axis (BA), small eyes (SE), facial defect (FD), and pericardial oedema (PO). B: Larva shows caudal fin abnormality (TF). C: This showing absence of pigment abnormality (AP) and BA. D: larva with yolk sac oedema (YO) and BA. Larvae in A, B and C come from a batch treated with amitriptyline (7.813 mg/L). Larva in D comes from batch treated with diazepam treated with 17.8 mg/L. The abnormalities shown here are representative of some of the individual abnormalities in Table 1.

All synthetic and herbal-based psychotropic drugs tested here show concentration-dependent mortality (**Figure 3 and Figure 4**). Interestingly, we noticed a higher incidence of abnormalities among larvae exposed to the four synthetic drugs than in those exposed to herbal extracts. For example, amitriptyline-treated larvae, in general, showed 12 different abnormalities in surviving individuals at both 2 dpf and 5 dpf stages (**Figure 3**). The 12 different abnormalities included pericardial oedema (PO), facial defect (FD), small eyes (SE), bent body axis (BA), yolk sac extension (YO),

enlarged swim bladder (SB), necrosis of body (NB), necrosis of head (NH), impaired circulation (IC), tail fin defect (TF), growth retardation (GR), and absence of pigments (AP). Moreover, most of the 12 abnormalities occurred at a higher frequency. Specifically, among 24 larvae, treatment with 7.813 mg/L amitriptyline resulted in PO, FD, and YO occurring at frequencies of 16, 9, and 7, respectively at 5 dpf.

Similar to the synthetic drugs, a group of multiple abnormalities occurred among surviving embryos and larvae at 312.5 mg/L after *Valerian officinalis* exposure (**Figure 4E** and **F**). The other three herbal extracts (*H. perforatum*, *P. incarnata* and *W. somnifera*), by contrast, showed little or no evidence of multiple abnormalities either at the highest concentrations or among surviving individuals (**Figure 4A-D**, **G** and **H**). To take one example, *H. perforatum* exposure was associated with only two types of abnormalities (PO and AP) at 2dpf (15.625 and 31.25 mg/L); and four types of abnormalities (PO, BA, GR, and AP) at 5 dpf (concentration range 3.906 to 62.5 mg/L). Moreover, the number of embryos and larvae that showed these abnormalities occurred were very low (**Figure 4A and B**).

Since diazepam and all four herbal extracts were dissolved in dimethylsulfoxide (DMSO), we decided to assess the potential toxicity of this solvent. We found that similar to the synthetic anxiolytics, DMSO also produced different types of abnormalities at higher frequencies than controls after both 48 and 96 h of exposure. DMSO caused six different abnormalities among surviving embryos after 48 h of exposure and seven different abnormalities among surviving larvae after 96 h of exposure (**Supplementary Figure S1**). The number of larvae with these abnormalities was high at both stages. Thus, in one example, all 24 larvae exposed to 56.64 g/L DMSO showed the TF abnormality at 2 dpf whereas the controls showed no abnormalities. Furthermore, in the same experiment, 12 larvae also showed BA, IC, no circulation (NC), and incomplete pigmentations (IP) abnormalities.

The $LC_{50}$ values for the synthetic and herbal drugs at 2 dpf and 5 dpf are shown in **Table 2**. For all synthetic drugs, the $LC_{50}$ values were dependent on the duration of exposure, such that longer exposure (96 h) resulted in lower $LC_{50}$ values than shorter exposure (48 h). To give an example, the $LC_{50}$ value for diazepam is 100.65 ± 246.83 mg/L after 48 h exposure; while after 96 h exposure, the $LC_{50}$ value was 37.09 ± 5.94

mg/L. In contrast to synthetic drugs, two herbal extracts had similar $LC_{50}$ values at 2 and 5 dpf. The $LC_{50}$ value for *H. perforatum* was 45.49 ± 5.21 mg/L and 44.19 ± 5.0 mg/L at 2dpf and 5 dpf respectively. *V. officinalis* produced similar $LC_{50}$ values after both 48 h and 96 h, which is 416.07 ± 34.03 mg/L.

Comparison of $LC_{50}$ values between synthetic and herbal-based drugs revealed that the synthetic drugs had low $LC_{50}$ values compared to *P. incarnata*, *V. officinalis*, and *W. somnifera*, consistent with their being more toxic than the herbals. The exception to this generalisation was *Hypericum perforatum*, which had relatively high toxicity (i.e. a low $LC_{50}$) more comparable with that of the four synthetics (**Table 2**).

**Figure 5** depicts the incidence (as a percentage) of clustering of morphological abnormalities, arranged in four categories, observed after exposure to amitriptyline, buspirone and diazepam. We chose to show the results for these three synthetic compounds since they were associated with most of the 16 abnormalities scored in the current study. In addition, concentrations of drugs chosen to represent the clustering are shown in the legend of **Figure 5**. The outer ring represents the abnormalities by category, while the inner ring represents the 16 individual abnormalities.

Circulatory defects (CD) are the main category of abnormality observed in 2 dpf larvae after treatment with buspirone (77.14%) and diazepam (87.10%) (For a full list of the abbreviations used for abnormalities, see **Table 1**). This category of abnormality has the highest incidence at 5 dpf after exposure to each of the following three synthetic drugs: amitriptyline (45.01%), buspirone (55.71%), and diazepam (60%). Tissue defects (TD) scored the highest percentage incidence at 2 dpf in larvae treated with amitriptyline, with a percentage incidence of 65.39%. This category of abnormality was reduced dramatically in incidence at 5 dpf (19.99%).

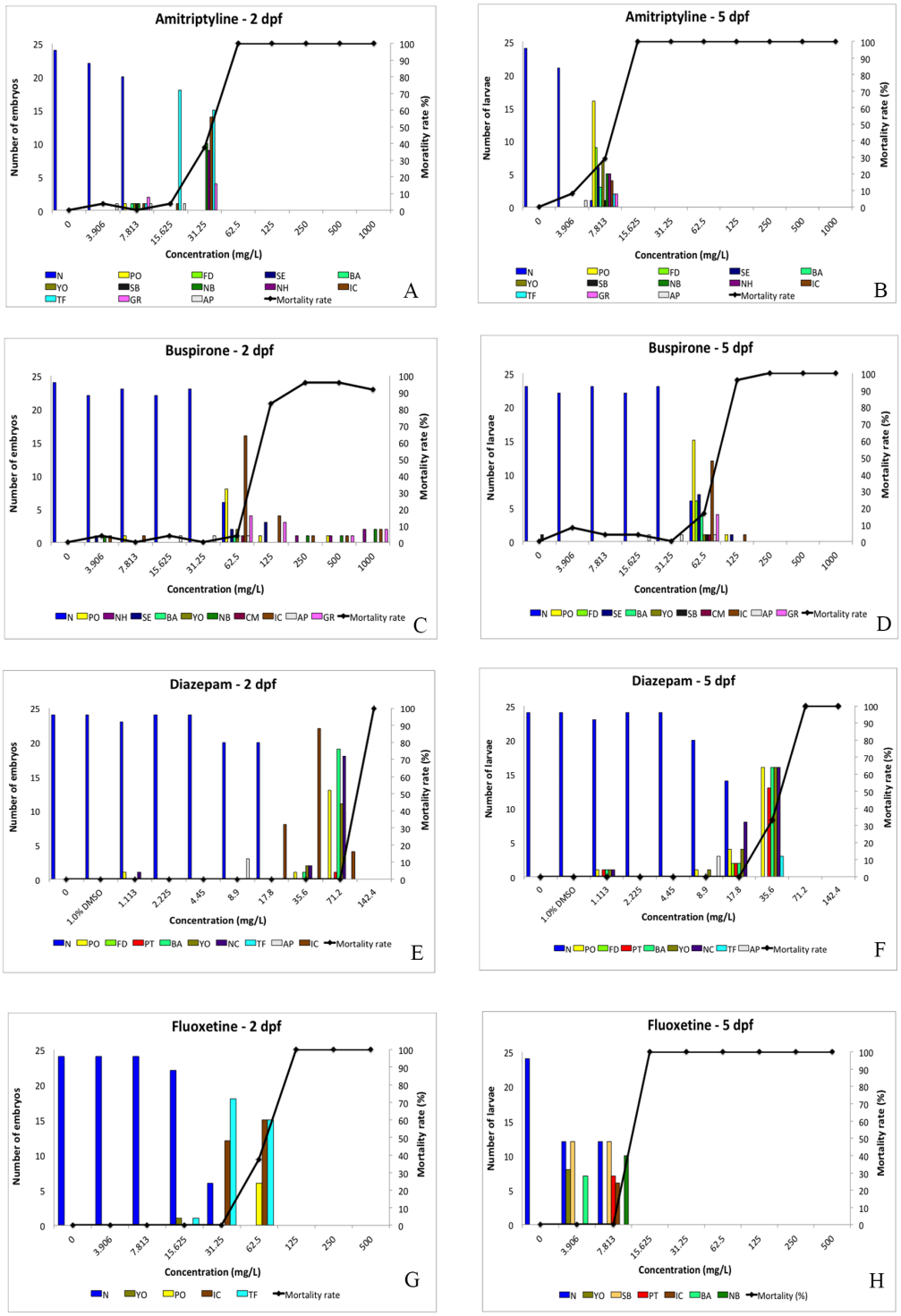


**Figure 3. Incidence of abnormalities in zebrafish embryos and larvae after exposure to synthetic psychotropic drugs.** A, C, E, and G: incidence of abnormalities at 2 dpf after exposure to amitriptyline, buspirone, diazepam and fluoxetine, respectively. B, D, F, and H: incidence of abnormalities observed at 5 dpf after exposure to amitriptyline, buspirone, diazepam and fluoxetine respectively. Secondary line chart: mortality rate. Diazepam was dissolved in 1% dimethylsulfoxide (DMSO). Refer to Table 1 for descriptions of the abbreviations used to describe the abnormalities.

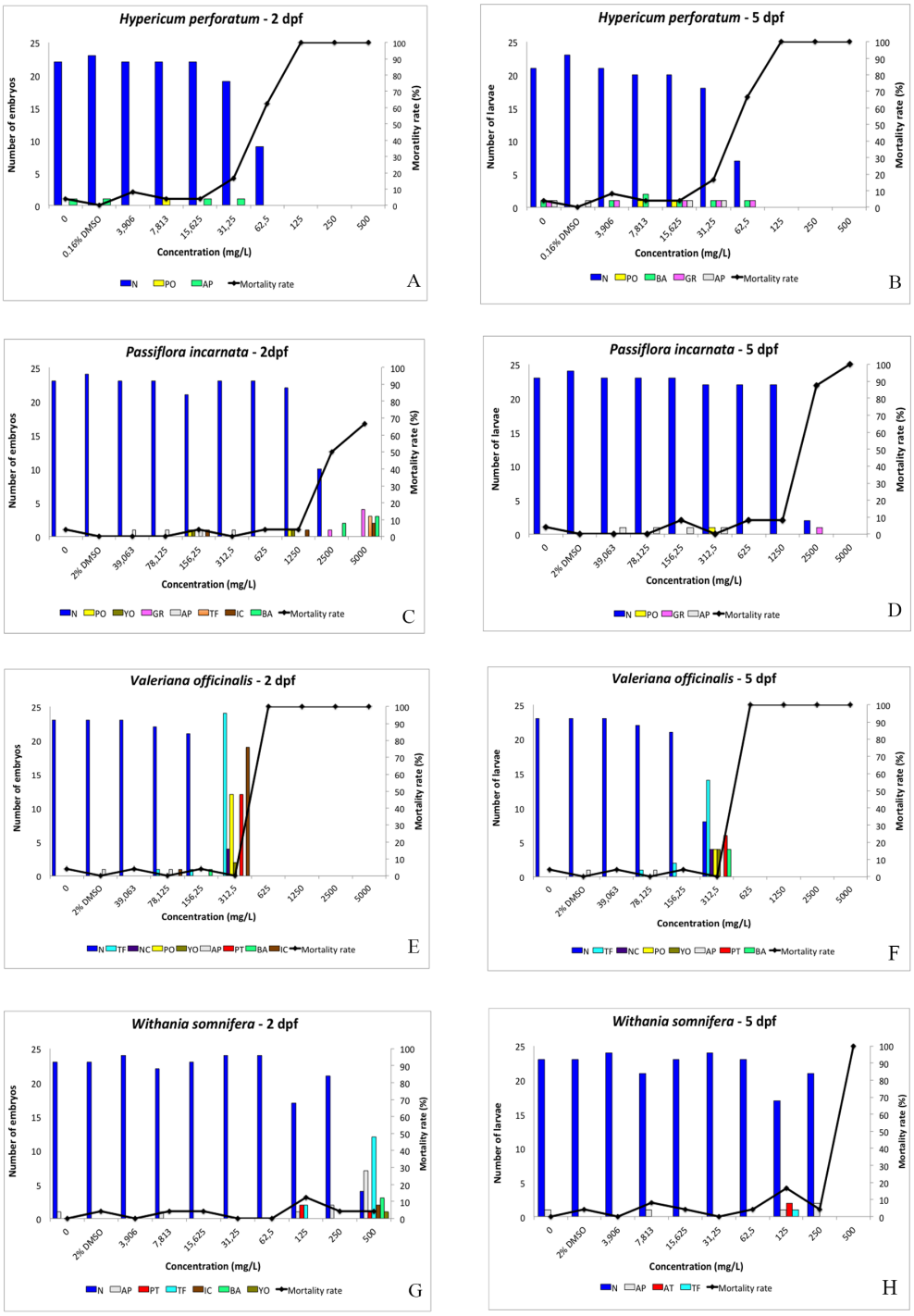


**Figure 4. Incidence of scored abnormalities in zebrafish larvae after exposure to herbal extracts.** A, C, E, and G: incidence of abnormalities observed at 2 dpf after exposure to *H. perforatum*, *P. incarnata*, *V. officinalis*, and *W. somnifera* respectively. B, D, F, and H: incidence of abnormalities observed at 5 dpf after exposure to *H. perforatum*, *P. incarnata*, *V. officinalis*, and *W. somnifera* respectively. Secondary line chart: mortality rate. Refer to Table 1 for descriptions of the abbreviations used to describe the abnormalities.

**Table 2. $LC_{50}$ values of synthetic and herbal based psychotropic drugs.**

| $LC_{50}$ (mg/L) | Developmental stage of assessment | |
|---|---|---|
| | **2 dpf (48 hpf)** | **5 dpf (120 hpf)** |
| Amitriptyline | 32.15 ± 2.81 | 8.48 ± 0.65 |
| Buspirone | 102.26 ± 12.14 | 72.22 ± 8.11 |
| Diazepam | 100.65 ± 246.83 | 37.09 ± 5.94 |
| Fluoxetine | 64.35 ± 9.14 | 11.06 ± 45.27 |
| *Hypericum perforatum* | 45.49 ± 5.21 | 44.19 ± 5.02 |
| *Passiflora incarnata* | 3232.31 ± 498.58 | 1625.2 ± 174.5 |
| *Valeriana officinalis* | 416.07 ± 34.03 | 416.07 ± 34.03 |
| *Withania somnifera* | n.a | 322.86 ± 57.67 |

*Key:*
hpf = hours post fertilization
n.a = Not applicable; $LC_{50}$ could not be calculated as the concentrations used did not hit 100% lethality

**Table 3. $LC_{50}$ values of dimethylsulfoxide (DMSO).**

| Developmental stage of assessment | Dimethylsulfoxide (DMSO) $LC_{50}$ | |
|---|---|---|
| | **$LC_{50}$ (g/L)** | **$LC_{50}$ (%)** |
| 2 dpf (48 hpf) | 76.48 ± 58.29 | 6.91 ± 0.53 |
| 5 dpf (120 hpf) | 29.184 ± 37.65 | 2.64 ± 0.32 |

*Key:*
hpf = hours post fertilization

In amitriptyline treated larvae, CD incidence increased from 26.92% at 2 dpf to 45.01% at 5 dpf. In contrast, buspirone and diazepam showed decreased incidence between 2 dpf and 5 dpf for this category of abnormalities. For example, in buspirone-treated larvae, the incidence of CD decreased from 77.14% to 55.77%. Furthermore, the incidence of CD in diazepam-treated embryos/larvae dropped from 87.10% to 60%. All three of these synthetic drugs showed an increased incidence of developmental defects (DD) from 2 dpf to 5 dpf, with diazepam showing the highest difference in incidence between the two ages (33.02%). Both amitriptyline and buspirone showed a slight increase in the incidence of DD from 2 dpf to 5 dpf (2.31% and 2.08%, respectively).

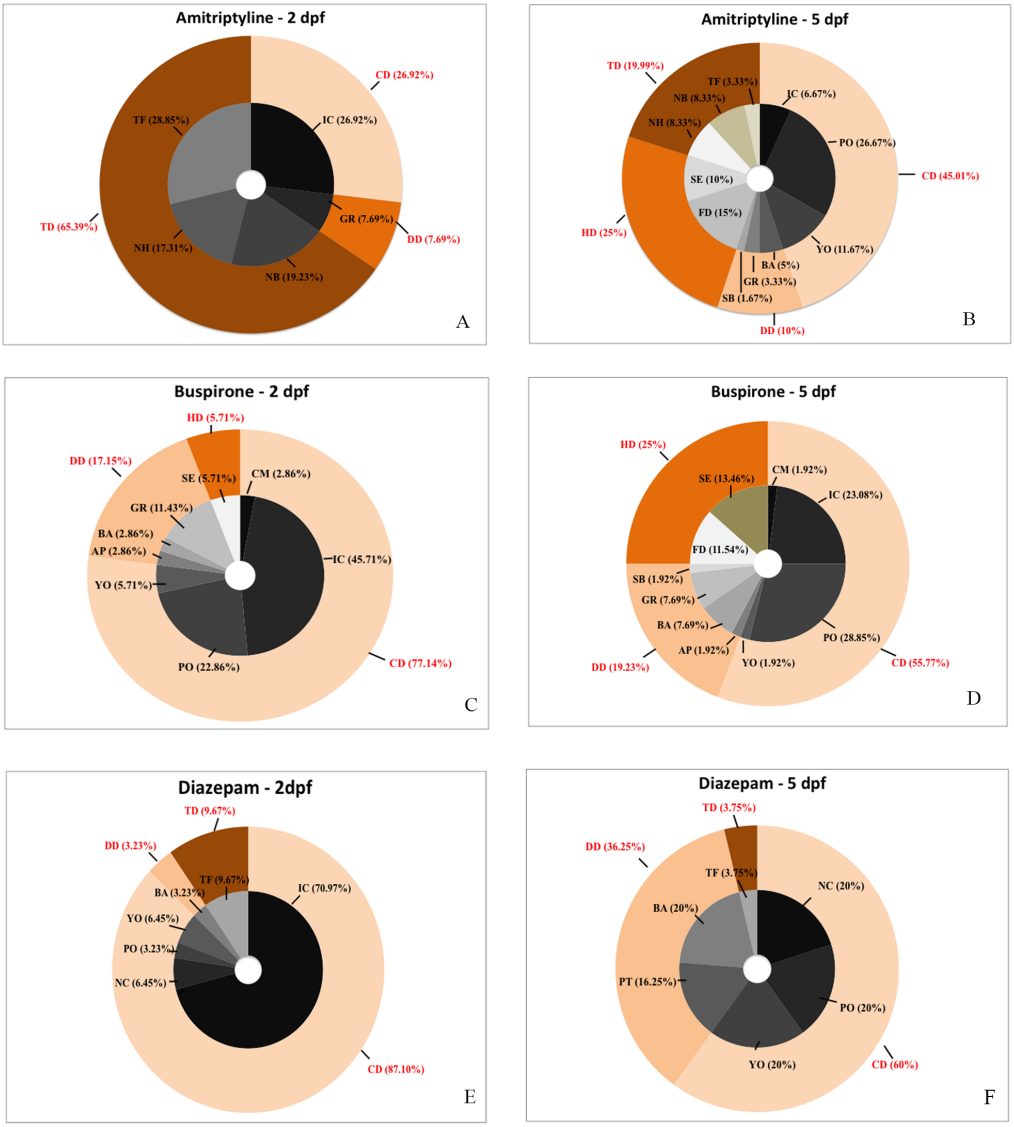


**Figure 5. Doughnut chart representing the clustering of morphological abnormalities (according to four categories) after exposure to amitriptyline, buspirone, and diazepam.** A, C, and E: abnormalities at 2 dpf. B, D, and F: abnormalities at 5 dpf. Outer ring, category of abnormalities; inner ring, individual abnormalities. Concentrations of drugs: amitriptyline (2 dpf = 31.25 mg/L; 5 dpf = 7.813 mg/L), buspirone (62.5 mg/L for both 2 and 5 dpf), and diazepam (35.6 mg/L for both 2 and 5 dpf). Refer to Table 1 for abbreviations. The data for fluoxetine have not been shown because the number of abnormalities was low.

# Discussion

The potential developmental toxicity of psychotropic drugs, whether synthetic or herbal, is of considerable importance due to the fact that these drugs may be commonly used during pregnancy [12, 48, 59-61]. One striking finding from our study is that multiple abnormalities start to appear in the larvae only when the

concentration of synthetic or herbal drugs approaches lethal range. This could suggest that the abnormalities recorded here are the immediate phenotypic consequences of high toxicity in a dying embryo or larva. We did not observe these lethality-induced peaks of abnormalities in treatments with *Hypericum perforatum*, *Passiflora incarnata*, and *Withania somnifera* extracts. This finding of a relation between lethality and abnormalities was also found in a previous study from our laboratory. According to that study, among 43 water-soluble compounds, there was a strong correlation between teratogenicity and $LC_{50}$ values [58].

Findings from our current study show that all four synthetic drugs are associated with some form of developmental toxicity in zebrafish embryos and larvae. Among the four drugs tested, amitriptyline showed the lowest $LC_{50}$ and produced abnormalities at the lower concentration. Amitriptyline, a first generation tricyclic antidepressant (TCA) that has long been on the market, has already been linked with increased risk of congenital malformations with first-trimester exposure in humans [59, 62].

In addition, our findings on the four synthetic drugs are comparable with the numerous published reports of the developmental toxicity of these compounds in animal and clinical studies. For example, Beyer et al. found in hamsters that there was an increase in the foetal incidence of encephalocele[63], and bent tail[64], after the pregnant dam was exposed to amitriptyline on day 8 of pregnancy. Furthermore, a meta-analysis of case-controlled studies showed that benzodiazepine use during the first trimester of pregnancy was associated with orofacial clefts in new-borns born humans [65]. The findings on the developmental toxicity of fluoxetine are more of a mixed outcome than the other three. Numerous studies have found no convincing association between fluoxetine ingestion and perinatal abnormalities in humans, rabbits, and rats (for references see[66]). On the other hand, in other studies, this drug was reported to cause higher rates of prematurity and miscarriage in humans [67, 68].

Our findings with the herbal-based psychotropic drugs are interesting because very little has been reported in the literature about their toxicity [50-52]. We have shown here that zebrafish embryos and larvae treated with *H. perforatum* had lower $LC_{50}$ values (higher lethality) than the other three extracts, which were comparable with the synthetics drugs. *V. officinalis* extract was associated with multiple

abnormalities at the concentrations close to its relatively high $LC_{50}$. Previous studies using animal models found no evidence that *Valeriana officinalis* extract, or its active constituents (valepotriates), were teratogenic after oral administration [69, 70]. However, there have been some concerns expressed about the use of this herbal drug during pregnancy due to variations in its composition between manufacturers [71, 72]. A very recent study highlighted pregnancy outcomes in psychiatric patients who had used *P. incarnata* [12]. A variety of adverse outcomes were seen in these pregnancies including neonatal death and various congenital anomalies, including premature rupture of membranes, pulmonary hypertension, and meconium aspiration syndrome.

Abnormality in pigmentation is interesting because various stressors and stimuli can disrupt pigmentation. A developing zebrafish can undergo aggregation or dispersion of pigments in response to different types of stimuli, including environmental, physical or chemical [41]. Hormonal mechanisms are thought to regulate these physiological changes [73]. Dispersion of melanocytes has been linked to activation of the stress mechanism in Arctic char (*Salvelinus alpinus*) species fish [74]. One of the two pigmentation abnormalities screened (AP, absence of pigment) was not actually found either in the case of the four synthetic or the four herbal extracts. We had included this abnormality on the screening list because it is commonly observed in various published studies. The other pigmentation abnormality that we did observe, however, was incomplete pigmentation (IP). This abnormality showed a marked increase in incidence in DMSO treated larvae. This is interesting because previous studies have shown that DMSO at subtoxic concentrations can increase heat-shock protein 70 (hsp 70, a marker for stress response [75]) levels in zebrafish larvae [76]. Thus, it is possible that DMSO induces pigmentation abnormalities through disruption of the stress pathway.

We used DMSO to dissolve the plant extracts and diazepam. The highest concentration used for herbal extracts was 2% and for diazepam was 1%. When we tested DMSO alone, we only found evidence of toxicity at $\geq$ 28.32 g/L ($\geq$ 2.56% v/v). Therefore, it is reasonable to assume that toxicity observed with the herbal extracts and diazepam in this study is not due to the presence of DMSO itself. Our findings relating to DMSO are comparable with previous studies, which showed zebrafish embryos and larvae to be tolerant to DMSO up to concentrations of 2% [76-78]. They are also in alignment with another study, which showed higher $LC_{50}$ values (lower

lethality) of DMSO at earlier life stages in zebrafish [77]. Some abnormalities (BA, IP, IC, and PO) due to DMSO-exposure reported in our study were also found in previous studies [76, 78].

Our study indicates that the four synthetic psychotropic drugs examined here are capable of causing circulatory defects. Furthermore, such defects have the highest incidence among the four categories of abnormality for amitriptyline, buspirone, and diazepam treated embryos at 5 dpf. Interestingly, the clustering of abnormalities also showed that larvae with impaired circulation or no circulation have a higher incidence of pericardial and yolk sac oedema. In view of these findings, it would be interesting to look cardiotoxicity in more depth. Zebrafish larvae are good candidates for this type of analysis because their heart develops rapidly, with a beating heart formed by 22 hpf [28]. By 48 hpf, the cardiovascular system of zebrafish larvae is fully functional [79, 80]. It would be interesting to investigate if the synthetic drugs used in our study could affect the heart rate of developing zebrafish larvae, given the fact that some compounds has produced of arrhythmias and bradycardia in zebrafish larvae [43, 76, 81, 82]. Lee *et al.* recommended counting the heartbeat for a 30-second period beginning from 48 hpf when the heart is fully functional [83].

In addition, there are some concerns that we would like to highlight regarding the comparison of lethality between zebrafish and other species. Previous studies [33, 84] have examined the correlation between larval $LC_{50}$ and rodent $LD_{50}$ values and found that the toxicity of compounds in zebrafish embryos and larvae correlated well with values reported from rodent studies. Hence, zebrafish embryos and larvae could be used as a predictive model for the developmental toxicity of compounds. However, one of the two studies [33] (see above) has suggested the presence of various methodological factors that may affect the outcome of such studies. The factors include differences in exposure time, developmental stage, and route of administration. Therefore, correlating larval $LC_{50}$ with rodent $LD_{50}$ is not conclusive. Furthermore, in rodents, the amount of drug used is determined by the weight of the animal ($LD_{50}$ expressed as mg/kg), while this is not the case in zebrafish larvae (where $LC_{50}$ is expressed as mg/L or mmol/L of swimming water). Hence, there remains an issue regarding extrapolation of data acquired from the zebrafish model to humans [85].

Another major limitation of zebrafish embryo and larval-based toxicity assays is that there is no consensus on optimal protocol [15]. Elements of the protocol that can vary between workers include the scoring system for abnormalities, the duration of exposure and age of embryos/larvae at which the abnormalities were scored [15]. However, there are common interests among scientists to harmonize zebrafish-based developmental toxicity assays so that concordance with mammalian data and inter-laboratory reproducibility are ensured [86].

## Future directions

Our results show that assays using zebrafish embryos and larvae are capable uncovering developmental of synthetic and herbal psychotropic drugs. Nevertheless, it is necessary to include some further analyses that can yield in-depth understanding of how the psychotropic drugs can induce developmental toxicity. The current study did not characterise the abnormalities observed in detail or examine their mechanism of action at the cellular or molecular level. In addition to reporting $LC_{50}$ values, it is also would be interesting to evaluate the teratogenicity of the pure compounds at every developmental stage. This information could be very useful in determining whether specific toxicity is due to general developmental toxicity or was specific to the biological system.

A previous study reported teratogenicity index (TI) as the ratio of $LC_{50}/EC_{50}$ values and this ratio was used to rank teratogenic compounds, with most teratogenic compounds showing higher TI values [57]. Since we could not determine $EC_{50}$ values in the present study, teratogenicity index could not be determined. Several studies have reported the use of larval zebrafish in assessing teratogenicity of small molecules. One study demonstrated that 36/41 mammalian teratogens were teratogenic in zebrafish embryos [87].

In addition, a study done previously in our laboratory showed that among 43 water-soluble compounds tested, there was a variable correlation between teratogenicity $LC_{50}$ values [58]. Some compounds were relatively teratogenic but had low lethality and other compounds only showed abnormalities near the lethal dose. We previously reported that amitriptyline was teratogenic at doses well below the lethal dose. It would be interesting to test the synthetic drugs and herbal extracts on

the embryos and larvae using linear concentration ranges. This could be useful in determining $EC_{50}$ and TI values.

Zebrafish larvae develop rapidly, especially at early stages (< 5 dpf) [88]. To make future studies more robust, it might be interesting to collect the readout (that is, of screening or scoring of abnormalities) at a different time point of development. Moreover, it might be also desirable in the future to use a more finely tuned series of exposures regime within this crucial 5-dpf range in order to more closely resolve the teratogenic and lethal exposure ranges.

# Conclusion

In conclusion, we have demonstrated that zebrafish embryos (2 dpf) and larvae (5 dpf) are good models for assessing the developmental toxicity of synthetic and herbal-based psychotropic drugs. The assay performed in the current study has potential as a high-throughput screening assay. It could, in principle, be implemented during the early drug development stage for the assessment of safety/toxicology of candidate psychotropic drugs. This could reduce or complement the usage of mammalian models. In addition, it is also essential to know the ADME properties of these compounds and extracts; these would provide scientific information on the stages of development most sensitive to the toxic effects of drugs. Due to our incomplete knowledge of the developmental toxicity of plant extracts such as *Hypericum* and *Valerian* products, which are widely available over the counter, we recommend more studies into the pharmacology of these plants.

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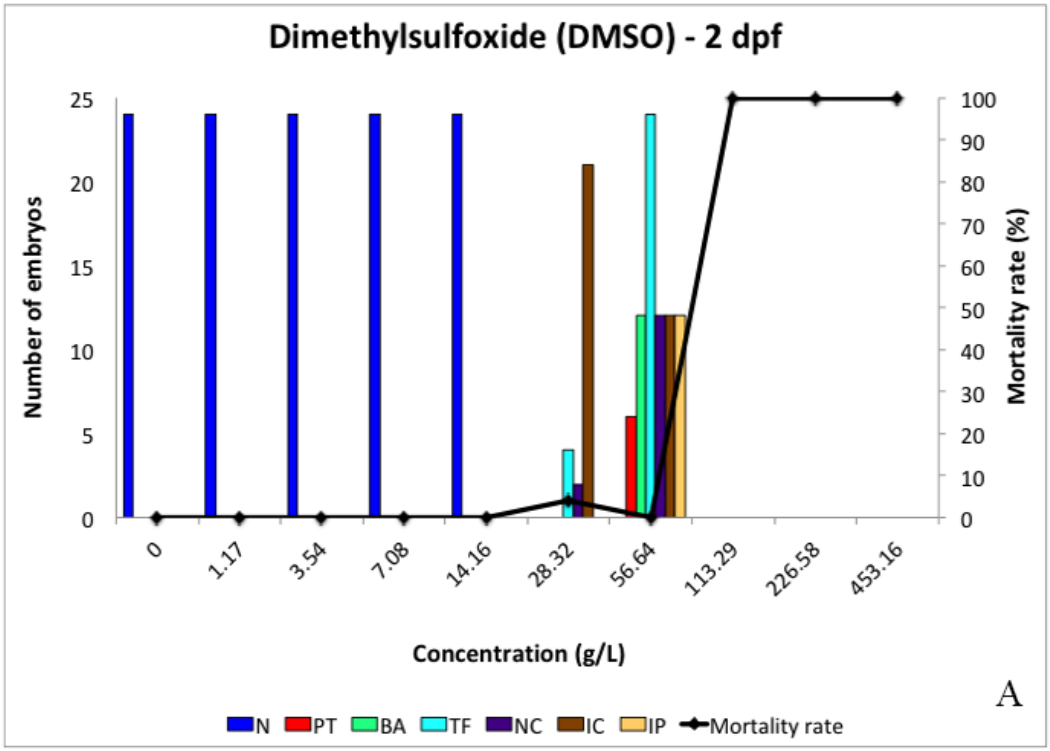
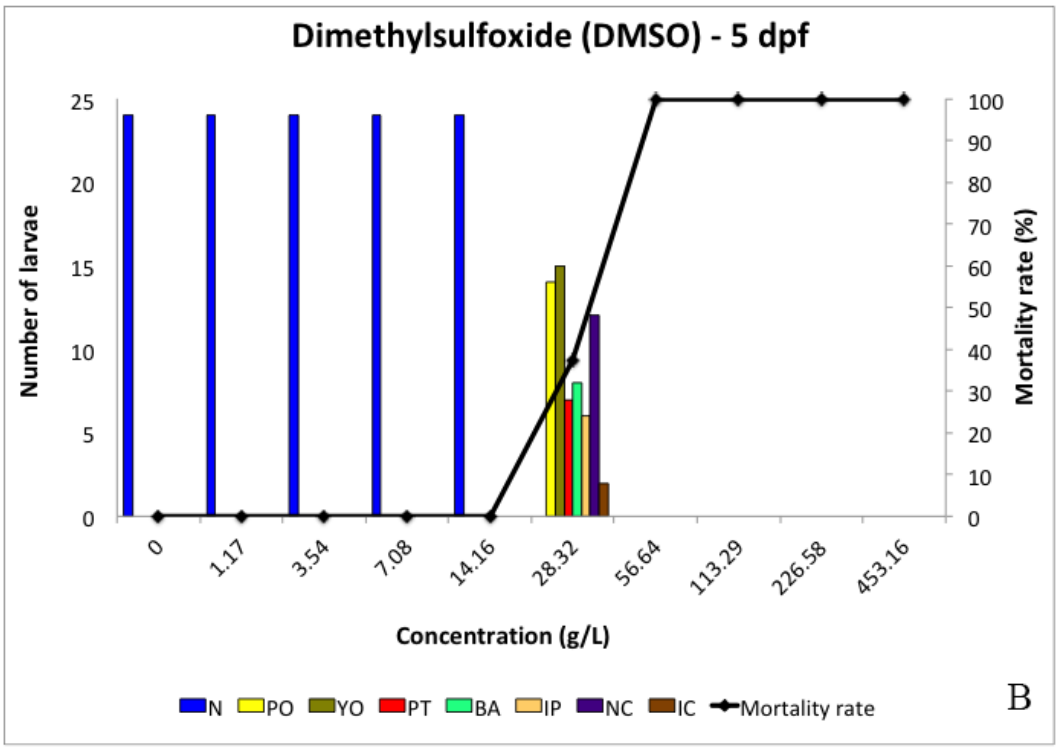
## Supplementary materials:

**Supplementary Table S1**. Concentrations used in geometric series in the current study for compounds and plant extracts. N = 24 for both controls and untreated larvae.

| **Compounds/ Extract** | **Concentrations in geometric series (mg/L)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***C0*** | ***C1*** | ***C2*** | ***C3*** | ***C4*** | ***C5*** | ***C6*** | ***C7*** | ***C8*** | ***C9*** |
| Amitriptyline | 0 | 3.906 | 7.813 | 15.625 | 31.25 | 62.5 | 125 | 250 | 500 | 1000 |
| Buspirone | 0 | 3.906 | 7.813 | 15.625 | 31.25 | 62.5 | 125 | 250 | 500 | 1000 |
| Diazepam | 0 | 1.0% DMSO | 1.113 | 2.225 | 4.45 | 8.9 | 17.8 | 35.6 | 71.2 | 142.4 |
| Fluoxetine | 0 | 3.906 | 7.813 | 15.625 | 31.25 | 62.5 | 125 | 250 | 500 | n.a |
| *H. perforatum* | 0 | 0.16% DMSO | 3.906 | 7.813 | 15.625 | 31.25 | 62.5 | 125 | 250 | 500 |
| *P. incarnata* | 0 | 2.0% DMSO | 39.063 | 78.125 | 156.25 | 312.5 | 625 | 1250 | 2500 | 5000 |
| *V. officinalis* | 0 | 2.0% DMSO | 39.063 | 78.125 | 156.25 | 312.5 | 625 | 1250 | 2500 | 5000 |
| *W. somnifera* | 0 | 2.0% DMSO | 3.906 | 7.813 | 15.625 | 31.25 | 62.5 | 125 | 250 | 500 |
| Dimethylsulfoxide* (in g/L) | 0 | 1.17 (0.16) | 3.54 (0.32) | 7.08 (0.64) | 14.16 (1.28) | 28.32 (2.56) | 56.64 (5.12) | 113.29 (10.24) | 226.58 (20.48) | 453.16 (40.96) |

*Key:*
*Concentration of DMSO is also show in percentage inside parentheses; DMSO concentration represented in g/L unit
n.a = not applicable




**Supplementary Figure S1. Incidence of abnormalities in zebrafish embryos and larvae after exposure to dimethylsulfoxide (DMSO).** A and B: incidence of abnormalities observed after exposure to DMSO at 2 dpf and 5 dpf respectively. Secondary line chart: mortality rate. Refer to the Table 1 for descriptions of the abbreviations used to describe the abnormalities. Figure only shows concentrations in g/L unit, for the corresponding concentration in percentage, refer to the Supplementary Table S1.

## Chapter 6

# Summary and discussion

## State vs. trait anxiety

In 1966, Charles Donald Spielberger developed the concept of State-Trait Anxiety Inventory (STAI), in which he classified anxiety into state and trait anxiety [1]. According to Spielberger, trait anxiety is an individual's general propensity to be anxious while state anxiety is a temporary emotional change characterized by physiological arousal with consciously perceived feelings of worry and tension [2]. Often, an individual with state anxiety is anxious at a particular moment of time when there is an increased presence of anxiogenic stimuli [3]. In contrast, trait anxiety is considered to be an enduring feature of an individual, where anxious feelings do not vary from time to time [3].

According to the diathesis-stress model of anxiety, some individuals have predisposed vulnerabilities to develop anxiety disorders after experiencing a traumatizing event [4]. Inter-individual variations in anxiety have also been reported in animal studies [5, 6]. Coping styles could explain the presence of inter-individual variations in anxiety, where reactive and proactive individuals respond in different ways towards a stressful event [7-9]. Deciphering the complexities of state and trait anxiety could explain why some individuals are more susceptible to developing anxiety disorders; this, in turn, could be very useful in personalized medicine.

The difference between state and trait anxiety has received wide recognition in the field of psychology since the mid-sixties [10-12]. By contrast, in the field of behavioural pharmacology, most research (perhaps, unfortunately) only focuses on drug-induced changes in state anxiety, where a particular model organism is exposed to an anxiogenic stimulus and the effect of the drug is measured [3]. Although this approach seems convenient and rapid, it ignores important factors that may contribute to high trait anxiety, which could underlie the potentials of anxiety being a developmental disorder [3]. Therefore, it is crucial to design animal studies that include both trait and state anxiety. Zebrafish could be a good model in such experiments. In addition to the general advantages of using zebrafish, which has been reported extensively in the literature (for references see [13, 14]), coping styles have been also shown to be present in both adult and larvae [15, 16].

## Anxiety as a developmental disorder

It is increasingly being recognized that many psychiatric disorders such as anxiety disorders are neurodevelopmental in their origins and aetiology [17]. Approaching anxiety disorders as being developmental disorders is relevant since they manifest earlier in life compared to other psychiatric disorders. For example, the mean age for the onset of depression is during young adulthood, while the mean age for anxiety is much earlier, which 11 [18]. This suggests that the neural circuits that mediate anxiety disorders [17] are established early in life [19, 20].

Genetic predisposition and environmental effects are thought to be the underlying cause of anxiety disorders. However, these two factors are not capable of providing full clarifications for anxiety disorders on their own. In addition, factors related to age also play an important role in gene-environment interactions, with some times of life being more vulnerable towards environmental manipulations, resulting in intensely different outcomes [17].

In their review, Leonardo and Hen integrated data from both animal and human studies and postulated potential age susceptibilities to adverse events during development [17]. This age is characterized by heightened brain plasticity and any adverse events during this time frame lead to the manifestation of anxiety disorders later in life. In humans, children by the age of 2 have already established consistent patterns of response to novel environments characterized by behavioural inhibition that are stable over longer periods [21-24]. Therefore, this age group is helpful in predicting future risk of anxiety disorders [19].

This evidence from humans is also consistent with the results of rodent studies, where the second and third postnatal weeks are critical in shaping the behavioural response of rodents later in life (for references see [17]). This time period is characterized by the rapid development of neurocircuitry and the animals begin to explore their own environment on their own (for references see [17]).

## Behavioural assays with older zebrafish larvae

Compared to the adult zebrafish, only a few studies evaluated the effects of psychotropic drugs on anxiety-like behaviour using larval zebrafish. I summarize these studies in **Table 1**. Diazepam was the only psychotropic drug tested in multiple studies. Brief exposure of larvae to diazepam earlier than 7 dpf was associated with

anxiolytic-like effects. Longer duration of exposure (2 h) at 7 dpf had similar effects. The other three psychotropic drugs (amitriptyline, buspirone, and fluoxetine) were not extensively studied in larvae younger than 6 dpf.

**Table 1. Behavioural assays that have evaluated psychotropic drugs using zebrafish larvae.** Except for amitriptyline, all behavioural assays were performed on larvae ≥ 6 dpf.

| Drug | Strain | Exposure | Behavioural assay | Reference |
|---|---|---|---|---|
| Amitriptyline | AB | Exposure at 1 dpf for 96 h without wash-out before behaviour analysis | Visual motor response (VMR) assay: amitriptyline suppressed locomotion in both basal and challenge phase | [25] |
| Buspirone | AB | Exposure at 6 dpf for 3 min followed by wash-out before behaviour analysis | Scototaxis assay: buspirone attenuated dark avoidance at 25 mg/L | [26] |
| | AB | Exposure at 8 and 30 dpf for 10 min followed by wash-out prior to behaviour analysis | Swimming plus maze assay: buspirone attenuated preference for deep arms over shallow arms at 50 mg/L in both 8 dpf larvae and 30 dpf juvenile zebrafish | [27] |
| Diazepam | AB | Exposure at 6 dpf for 7 min followed by wash-out before behaviour analysis | Scototaxis assay: diazepam attenuated dark avoidance at 0.71 mg/L | [26] |
| | AB | Exposure at 5 dpf for 10 min without wash out before behaviour analysis | Thigmotaxis assay: diazepam attenuated wall preference at 0.71 mg/L | [28] |
| | Wild type | Exposure at 7 dpf for 2 h without wash out before behaviour analysis | Thigmotaxis two-fish assay: diazepam attenuated wall preference at 0.05 and 5 mg/L. Five-fish bouncing ball assay: diazepam failed to attenuate avoidance behaviour | [29] |
| | AB | Exposure at 6 dpf for 45 min without wash-out before behaviour analysis | Scototaxis and thigmotaxis assays: diazepam failed to attenuate wall preference and dark avoidance at 0.71 mg/L | [30] |
| Fluoxetine | Wild type | Exposure at 7 dpf for 2 h without wash-out before behaviour analysis | Thigmotaxis two-fish assay: fluoxetine failed to attenuate wall preference at 0.2 and 2 mg/L. Five-fish bouncing ball assay: fluoxetine attenuated avoidance behaviour at 2 mg/L | [29] |

To the best of my knowledge, the two behavioural studies (**Chapter 3** and **4**) in this thesis are the first to evaluate anxiety-like behavioural effects in zebrafish larvae ≤ 5 dpf after exposure to different types of psychotropic drugs (shown in **Table 1**). In addition, these chapters also investigated both acute (short-term) and chronic (long-

term) exposure to psychotropic drugs simultaneously. Richendrfer *et al.* suggested that chronic exposure might interfere with neural development [29]. However, it would be interesting to check both short-term and long-term exposure of psychotropic drugs on a developing larva.

At 5 dpf, zebrafish larvae already show a wide range of behavioural repertoires (**Chapter 1**) including the visual motor response (VMR) and thigmotaxis. Both VMR and thigmotaxis assay can be used to study anxiety-related behavioural responses and pharmacological responses after treatment with psychotropic drugs. Diazepam, a gold-standard anxiolytic (that acts through the GABA-ergic system) produced the expected effects in the thigmotaxis assay after acute exposure (**Chapter 4**). The three serotonergic psychotropic drugs (amitriptyline, buspirone, and fluoxetine) are capable of reducing larval locomotion in the basal phase of the behavioural assays (when there is no stimulus; **Chapter 3** and **4**). In addition, buspirone and fluoxetine failed to produce pharmacological effects in the thigmotaxis assay both after acute and chronic exposure (**Chapter 4**). Finally, the serotonergic drugs tested in **Chapter 4** caused phenotypes that resembled symptoms of serotonin toxicity after chronic exposure. This inference is based on observations from behavioural (visual startle response stimulus) and physiological (vibrational stimulus) response of zebrafish larvae after exposure to these drugs.

Based on the findings from this thesis, my suggestion is that the serotonergic system in larval zebrafish ≤ 5 dpf is not fully developed to the extent where pharmacological effects could be seen. This suggestion is also supported by behavioural studies that showed pharmacological effects of serotonergic drugs in older zebrafish larvae (for example, ≥ 7 dpf; see **Table 1**). Therefore, using older larvae (≥ 7 dpf) or juvenile zebrafish (30 dpf), in behavioural assays that evaluate anxiety disorders, could be more effective. The age of zebrafish that I suggested here (≥ 7 dpf) could also be practical in the study of psychotropic drugs with complex pharmacological mechanisms, such as those used in this thesis.

## Proposed zebrafish model for studying early-life stressors on the development of anxiety disorders

Efforts to improve the application of animal studies in the drug development pipeline often concentrate on high-end models that more faithfully mimic human biology [31]. As technologies advance, the zebrafish might become increasingly representative of human diseases and disorders. Here, I would like to suggest a model (**Figure 1**) that can be potentially useful for studying trait anxiety and not just the state anxiety, which is what was mainly studied in the past. Moreover, this model could be very practical for the assessment of anxiety as a developmental disorder. The brain-gut axis and gut microbiome play an important role in the development of anxiety disorders [32]. This model also explores how early chronic stressor modulates gut microbiome later in life in zebrafish. This would allow us to study the role of early-life stressors and their contribution to the later development of anxiety disorders. This is currently a highly topical issue in human psychiatry and personalised medicine.

This model includes zebrafish from different age groups: early larvae (≤ 7 dpf), late larvae (≥ 7 dpf), juvenile (30 dpf), and adults (90 dpf). The model presented here can also be translated into different strains. Some zebrafish strains that can be potentially useful in studying anxiety-like behaviours are Nadia, Wild Indian Karyotype (WIK), Leopard, and wild-caught. These strains are highly anxious strains and are particularly sensitive to anxiogenic stimuli [13]. In addition, tests using fully mature adult zebrafish are also more suitable than larval for studying anxiety disorders because they have a functionally mature central nervous system. For example, the 'novel tank test' with adults has relevance to the clinical conditions of generalized anxiety disorder, as well as agoraphobia [14, 33-35]. The shoaling test can be useful in mimicking social anxiety disorder [14, 36]. Overall, the model that I have outlined here offers a comprehensive approach to the study of early-life effects, including exposure to psychotropic drugs, in the development of anxiety disorders. As Kalueff has pointed out, the main strength of zebrafish models resides in their 'dual' nature in brain research, where both adult and larval assays complement each other and are equally important for different research purposes [37].

## The serotonergic system in developing zebrafish

Neurotransmission plays a critical role in normal brain development, behaviour, memory, and learning [38]. These functions are maintained by the nervous system, which in turn is modulated by the numerous enzymes, receptors, and transporters

involved in neurotransmission[38]. Exposure to drugs, food additives, and environmental toxicants can alter neurotransmission [39-43], and this has been linked to many diseases and disorders. Some examples include movement disorders, and neuropsychiatric

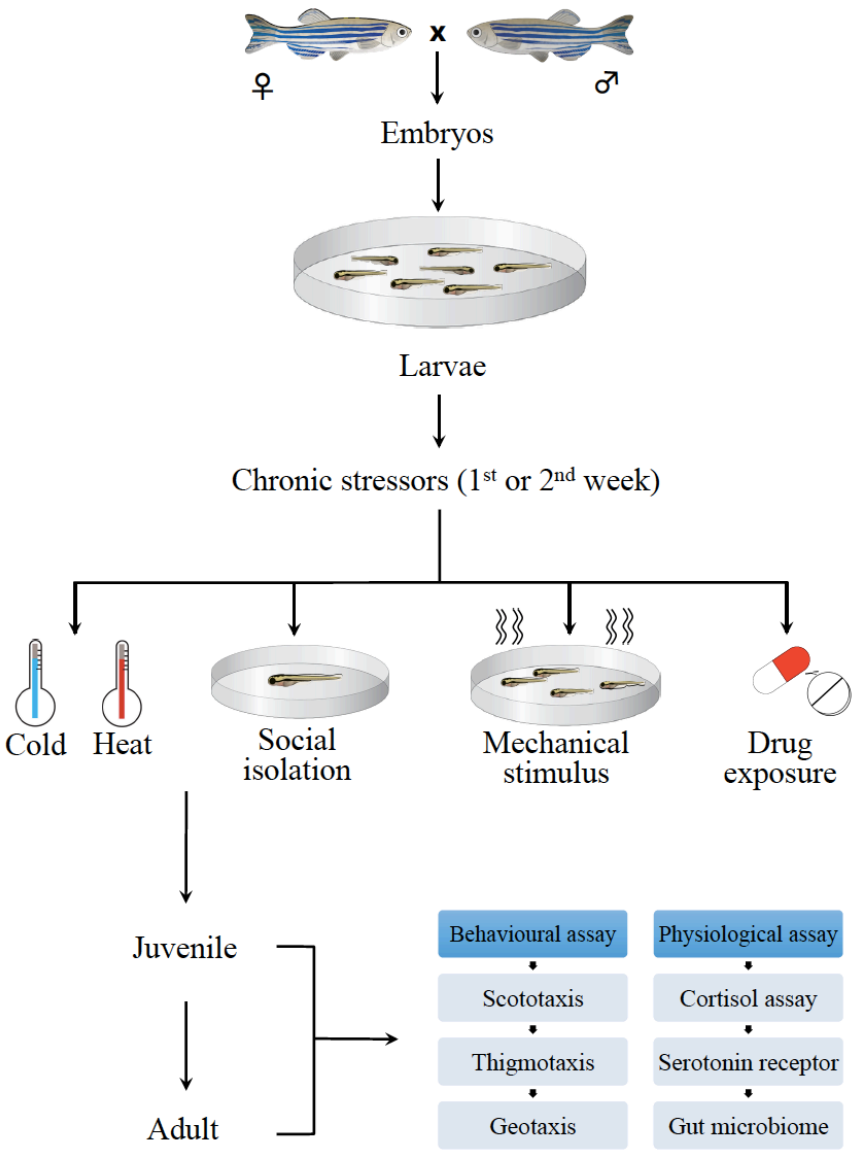



**Figure 1. Schematic diagram showing the experimental design for the proposed model.** Treatment with stressors at first week: 3 – 7 dpf. Treatment with stressors at the second week: 7 – 14 dpf. In this experimental design, only three physiological assays were included; this can be expanded based on specific research questions. Diagram does not represent actual dimensions. Adult zebrafish image: courtesy of Togo picture gallery of Database Center for Life Sciences. Larval zebrafish image: courtesy of Lizzy Griffiths. Thermometer and Petri dish images: courtesy of Integration & application network (IAN). All images obtained after permission.

disorders including depression (reviewed by [44-46]). The neurotransmitter systems of zebrafish and mammals are highly conserved [38], and so the zebrafish can be useful in studying mechanisms of neurotransmission and they are modulated by psychotropic drugs.

Here, I will focus my discussion on the serotonergic neurotransmitter system in relation to the developing zebrafish. This is because three of the four synthetic drugs used in the experiments in **Chapter 3 - 5** exert their pharmacological effects by modulating the serotonergic system. Moreover, the behavioural assays in **Chapter 3** and **4** show that these three serotonergic drugs can reduce larval locomotion at 5 dpf (see above) without having any detectable anxiolytic effect. The importance of the serotonin neurotransmitter in the embryonic development of motor neurons in zebrafish embryos has been shown in previous studies [47, 48]. Another study showed that the treatment of embryos with fluoxetine inhibited the expression of multiple serotonin receptors in larval zebrafish [49]. Overall, there is a possibility for psychotropic drugs to alter serotonergic pathways during development. This alteration could also result in serotonin toxicity (syndrome). This has been shown in 4 dpf zebrafish larvae after treatment with a psychoactive designer drug (25-NBOMe), which acts as an agonist at the serotonin-2A (5-$HT_{2A}$) serotonin receptor [50].

## Zebrafish larvae in developmental toxicity analysis

The rising incidence of mental disorders from adolescence onwards could be due to adverse early-life events [51]. These events may include early exposure of the foetus to psychotropic drugs *in utero* [49]. As many as 10-16% of pregnancies are estimated to be associated with depression [52] and the use of psychotropic drugs by the pregnant mother is common [53, 54]. It is important to understand any potential adverse effects of these drugs. Human foetal risk-assessment studies are extremely limited due to stringent ethical rules [49].

Developing zebrafish larvae are very useful in studying the potential developmental toxicity of psychotropic drugs (**Chapter 5**). Studies in **Chapter 5** clearly show that both synthetic and herbal-based psychotropic drugs are capable of causing developmental toxicity in zebrafish embryos and larvae. The synthetic drugs were associated with high lethality (low $LC_{50}$) and a higher incidence of

morphological abnormalities compared to the herbal extracts, except for *Hypercium perforatum*. It would be interesting in future studies to explore the abnormalities in detail and examine their cellular and molecular mechanism of action.

Studying pharmacological aspects of drug absorption, distribution, metabolism, and excretion (ADME) using mammalian models is extremely laborious, expensive, and requires large quantities of test compound (which is a big problem if the compound is scarce or very expensive) [55]. Although zebrafish embryos and larvae offer solutions for these problems, a major hurdle in using the embryonic and larval zebrafish model in studying developmental toxicity is the difficulty in determining the effective concentration in the zebrafish embryos and larvae [56]. Although it is in principle possible to use liquid chromatography-mass spectrometry (LC-MS) technology to determine the concentration of a drug in the larva or embryo [56, 57] very few studies have done this. Chromatographic techniques such as LC-MS could be useful for ranking different drugs according to their potency based on body burden (uptake of a compound) and also avoiding potential false-positives [56]. Understanding the body burden of in zebrafish embryos and larvae will facilitate further investigation on translating zebrafish results into mammalian toxicity data [56].

# Conclusion

The experimental studies in this thesis address the fundamental role of zebrafish larvae in the investigation of anxiety-like behaviours and also in evaluating the pharmacological effects of psychotropic drugs. Behavioural analyses in this thesis show that developing zebrafish larvae are susceptible to adverse physiological reactions (e.g. locomotor suppression) that may hinder the desired pharmacological effects of psychotropic drugs. The zebrafish embryo/larva model has the capability for high-throughput phenotyping; this ability is difficult or impossible to achieve with most other animal models.

A major conclusion of this thesis is that there is a trade-off in using developing organisms to study complex affective disorders. Namely, that the young stages do not have a sufficiently mature nervous system to fully recapitulate what mainly adult disorders in humans. At least, this is the clear conclusion from our behavioural assays. We have therefore identified a potential limitation in using the young stages of

zebrafish larvae to study adult disorders such as anxiety. Despite this reservation, it would nonetheless be interesting to expand what I have reported in this thesis by using a range of zebrafish stages up to and including adults, to gain in-depth knowledge on the physiology and pharmacology of psychotropic drugs.

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