Understanding the effect of protocol variations in the zebrafish light/dark transition test

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Declaration

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Abstract

Anxiety disorders have devastating individual and societal costs, and are a major contributor to years lost to disability worldwide. They appear to be increasing in prevalence, both in South Africa and the world at large. Although there are medications to treat anxiety, there is a need for more treatment options. Anxiety is often not treated effectively due to treatment resistance or non-compliance to medication due to side effects. One of the best options for identifying novel anxiolytics is to use animal models. Zebrafish are useful for screening of potential anxiolytic treatments because they are the most well-studied vertebrate model that shares the size, cost and fecundity benefits of invertebrate models.

The light/dark transition test (LDTT) is the most widely used zebrafish larvae behavioural test. It has many applications in pharmacology, particularly in toxicology and screening for potential pharmaceuticals, including treatments for anxiety disorders. It is likely to be one of the first tests used when screening for neuroactivity in zebrafish larvae. During the test, zebrafish larvae are exposed to a period of light followed by an abrupt transition to darkness which produces a hyperlocomotion response that responds to anxiolytics and anxiogenics. The design of the LDTT varies between studies, but it is unclear how common protocol variations affect the comparison of results and contextualisation of data generated using slightly varied protocols.

Through both prospective experiments and retrospective data analysis, the effect of age (from 2 dpf to 5 dpf), lighting conditions during rearing (standard or continuous darkness), capture order, repeated light/dark cycles, repeated light/dark transition tests, duration of the light period (1 minute or 10 minutes), light intensity during the light period, and breeding stocks on the response to the light/dark transition test was measured. All experiments consisted of an acclimation period, and at least one cycle consisting of a light period and a dark period. Experiments were recorded using the DanioVision system and activity was measured automatically using the EthoVision XT software.

Variations in age, time of day, light period and breeding stock had a significant impact on the response to the light/dark transition test and should therefore be carefully controlled. Light conditions during rearing did not have a statistically significant effect, but more research is needed to confirm that variations in light-rearing do not affect response to the light/dark transition test. Finally, capture order, repeated cycles, repeated light/dark transition tests and light/dark transition intensity did not have a significant effect, suggesting that they can vary according to logistical requirements without affecting results. This opens up the use of repeated measurements that facilitate identifying neuroactivity when the amount of time it will take for the onset of action is unknown. This informs both experimental design, and which studies are comparable. It will also facilitate the use of the light/dark transition test to screen for potential anxiolytics.

Opsomming

Kostes verwant aan angsversteurings is astronomies vir die individu, maar ook die ekononomiese kostes verwant aan ongeskikteheid, soos mediese kostes en produktiwiteitsverlies in die werkplek. Ons sien steeds ‘n styging in die tendens, nie net in Suid Afrika nie, maar wereld wyd. Medikasie vir die behandeing van angstoestande bestaan, maar behandeling vir angsversteurings is nie altyd bekostigbaar nie en huidige behandelings het dikwels newe effekte. Daar is dus steeds ruimte vir verbetering. Een van die beste opsies om nuwe medikasie te ontwikkel is om na diere modelle te kyk. Die zebravis is veral geskik vir hierdie doeleinde omdat hulle die mees bestudeerde gewerwelde model is en die grootte, koste en vrugbaarheidsvoordele van ongewerwelde modelle ewenaar.

Die lig/donker oorgangstoets (LDOT) is een van die mees wydgebruikte toetse om die gedrag van die sebravis larwe te bestudeer. Dit het baie toepassings in farmakologie, veral in toksikologie en die skandering vir potensiële farmakologiese bates vir angsversteurings. Dit is waarskynlik die eerste toets wat gedoen sal word om die neuroaktiwiteit van die sebravis te bepaal. Gedurende die toets word die sebravis larwe aan lig blootgestel vir ‘n periode en dan skielik oorgeskakel na donker, wat ‘n hiperaktiwiteitsreaksie veroorsaak wat deur op angswekkers en kalmeermiddels reageer. Die protokol van die LDOT wissel tussen studies, maar dit is onduidelik hoe algemeen die protokol variasies is en hoe dit die uitslae, of vergelyking van data verkry deur verskillende protokolle, beïnvloed.

Prospektiewe eksperimente en retrospektiewe data analise is gebruik om die effek van ouderdom, ligtoestande tydens groei (standaard of deurlopende donker), volgorde van vangs, herhaalde lig/donker siklusse, herhaalde lig/donker veranderings, periode van die lig periode (een of tien minute), die intensiteit van beligting, en die broei kolonie op die LDOT te bepaal. Alle eksperimente het ‘n aanpasperiode en ten minste een siklus van lig en donker ingesluit. Eksperimentele data is vasgevang met die DanioVision sisteem en EthoVIsion XT sagteware.

Variasies in ouderdom, tyd van die dag, periode van beligting en teelvoorraad het ‘n merkbare impak gemaak op die resultate van die LDOT en behoort aan streng kontrole onderhewig te wees. Toestande tydens die lig groeiperiode het nie ‘n statisties betekenisvolle effek getoon nie, maar verdere navorsing is nodig om te bevestig dat variasies in die lig tydens groeifases nie ‘n effek op die LDOT reaksie het nie.

Ten slotte, die volgorde van vangs van die larva, herhaaldelike siklusse, herhaalde toetsing en die intensiteit van die lig/donker veranderinge het nie ‘n merkwaardig impak gehad op die kwaliteit van die resultate van die LDOT nie. Daarom kan daar verskille werwant aan die logistieke uitleg wees sonder om resultate te beïnvloed. Dit beteken dat verskillende toets formate en herhaaldelike toetsing gebruik kan word om die neuroaktiwiteit te bepaal wanneer ons nie weet wat die spesifieke reaksietyd van medikasie is nie. Dit beïnvloed die eksperimentele formaat en ook watter studies

vergelykbaar is. Dit beïnvloed weer die potensiaal vir die gebruik van die LDOT in die soektog na effektiewe opsies vir die behanding van angstoestande.

Dedication

To all of my friends and family who are struggling with mental illness, especially those who suffer from anxiety.

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List of Abbreviations

BNST	bed nucleus of the stria terminalis
BLA	basolateral amygdala
dpf	days post fertilisation
DALYs	disability-adjusted life years
DSM-5	Diagnostic and Statistical Manual 5
E3	embryo medium
GABA	gamma-aminobutyric acid
HPA	hypothalamic-pituitary-adrenal
hpf	hours post fertilisation
HPI	hypothalamic-pituitary-interrenal
ICD-11	International Classification of Diseases, 11th Revision
LC	locus coeruleus
LDTT	light/dark transition test
SANS	South African National Standards
SAVC	South African Veterinary Council
SNRI	selective serotonin and norepinephrine reuptake inhibitors
SSRI	selective serotonin reuptake inhibitor
TL	Tupfel long-fin
TU	Tübingen TU
WHO	World Health Organisation
YLDs	years of healthy life lost due to disability or ill-health

Chapter 1: Introduction

Anxiety disorders cause significant distress and can cause substantial impairment in the personal, family, social, educational and occupational domains of people's lives (World Health Organisation, 2019). It is estimated that in 2017 alone, ~27 million years of healthy living were lost due to anxiety disorders (Chin *et al.*, 2018). In South Africa, anxiety is having a major impact, and during 2019, anxiety disorders comprised the seventh greatest contributor to years lost due to disability or ill-health (YLDs) in the country (Achoki *et al.*, 2022). Anxiety disorders are more common in women, and the ratio of women to men for anxiety disorders is approximately 2:1 (American Psychiatric Association, 2013). Globally, anxiety disorders are a major cause of the non-fatal disease burden of women (Chin *et al.*, 2018). It is clear that anxiety disorders need to be prioritised at both global and national levels. Anxiety also has economic effects: it decreases productivity (Bouwmans *et al.*, 2014; Marciniak *et al.*, 2004; Sado *et al.*, 2013) and increases healthcare costs, particularly at the individual level (Marciniak *et al.*, 2004; Konnopka & König, 2020). The prevalence of anxiety disorders means that these problems cause a large, although often hidden, economic burden.

Furthermore, the prevalence of anxiety disorders is increasing (Santomauro *et al.*, 2021). It is estimated that the COVID-19 pandemic resulted in a 25.6% increase in these disorders. In 2020, anxiety disorders caused 44.5 million disability-adjusted life years (DALYs). Women and men of younger age were the groups most affected by the increase in anxiety disorders during this period (Santomauro *et al.*, 2021). During the COVID-19 pandemic, infection with COVID-19, fear of infection and the disruption caused by the measures put in place to manage the pandemic all contributed to increased anxiety on a global scale (Guo *et al.*, 2020; Jansen van Vuren *et al.*, 2021; Özdin and Bayrak Özdin, 2020; Salari *et al.*, 2020; Santomauro *et al.*, 2021; Shah *et al.*, 2021; Wang *et al.*, 2020). In addition, there was a substantial interruption to the provision of mental health care (World Health Organisation, 2020), which exacerbated existing anxiety disorders. The incidence of anxiety has also been increasing in South Africa (Achoki *et al.*, 2022; Santomauro *et al.*, 2021), with a nationwide study indicating the largest increase occurred in the Northern Cape (Achoki *et al.*, 2022). This is alarming, as this means that even less urbanised areas of the country are now increasingly burdened by anxiety.

In well-resourced environments, when patients are diagnosed with an anxiety disorder they are offered psychotherapy, medication or a combination of the two approaches (National Department of Health, 2020). Medications used to reduce anxiety (anxiolytics) typically include benzodiazepines (diazepam, lorazepam and alprazolam), antidepressants (imipramine, paroxetine, escitalopram and venlafaxine), buspirone and the beta-blocker propranolol (Neal, 2016). Generally, the first line of medication used is a selective serotonin reuptake inhibitor (SSRI), such as fluoxetine or citalopram (National Department of Health, 2020). Access to treatment for anxiety disorders varies, and there is a wide disparity between the availability and allocation of mental health resources in high- and

low-income countries. Many low- and middle-income countries have low expenditure on mental health. Low-income countries have fewer than two mental healthcare workers per 100 000 of the population, while high-income countries may have over 60 per 100 000 of the population. These disparities can also be seen between regions within a country (World Health Organisation, 2020). These disparities have significant consequences for the availability of psychotherapy, making low-cost pharmaceutical treatment options more accessible and therefore, increasingly important.

Although treatment options are available, as mentioned above, the varied symptomology of anxiety disorders suggests a complex mechanistic disease aetiology, with the result that there is still a pressing need for more effective anxiolytics with more desirable profiles in terms of side-effect risk. The main treatments currently used in anxiety disorders either risk aggravating symptoms for the first 2-4 weeks (SSRIs and the selective serotonin and norepinephrine reuptake inhibitors; SNRIs) or they carry a high risk for dependence (e.g. benzodiazepines). It is thought that an increase in serotonin release from the dorsal raphe nucleus binding to 5-HT_{2C} receptors on a subpopulation of corticotropin-releasing factor neurons located in the bed nucleus of the stria terminalis (BNST) which stops anxiolytic signals from the BNST area from being sent to the ventral tegmental area and lateral hypothalamus, potentially causing fear and anxiety associated with the first period of treatment with SSRIs (Marcinkiewcz *et al.*, 2016). In addition, the SSRIs and SNRIs must be used for a minimum of 12 months to reduce the risk of relapse (National Department of Health, 2020). SSRIs and SNRIs must also be decreased gradually to prevent antidepressant discontinuation syndrome (which includes symptoms similar to flu symptoms, insomnia, nausea, imbalance, sensory disturbances and hyperarousal) (Harvey & Slabbert, 2014). Moreover, many anxiolytics also have sedative effects that must be controlled through dosage or accepted as unavoidable side effects (Gray, 2018; Neal, 2016).

Additionally, another factor adding to the complexity of managing anxiety in patients is the fact that as many as 40% of those who suffer from anxiety disorders are resistant to the currently available medications (Bystritsky, 2006). This resistance to the existing treatments makes it clear that there is still a need for better anxiolytic treatment options.

In the context of new drug discovery, pre-clinical research on the mechanisms of anxiety and potential treatments often utilizes animal models (Bourin, 2015; Egan *et al.*, 2009; Kalueff & Cachat, 2011; Steimer, 2011). Although rodents are typically used for this work (Bourin, 2015; Steimer, 2011), they represent a comparatively costly and time-consuming model (Sieber *et al.*, 2019). Zebrafish larvae (*Danio rerio*) provide a practical alternative to bridge the gap between *in vitro* experiments and rodent models. However, the number of behavioural tests that can be used with zebrafish to investigate anxiety is growing (Blaser *et al.*, 2010; Fontana *&* Parker, 2022; Jesuthasan *et al.*, 2021; Lucon-Xiccato *et al.*, 2020; Sabadin *et al.*, 2022). Despite their widespread use, the protocols for tests such as the light/dark transition test vary across studies (López & Smith, 2021; MacPhail et al., 2009; Maphanga et al., 2022; Padilla et al., 2011; Sveinsdóttir et al., 2022; Velki et

al., 2017). As the consequences of these variations have not been fully investigated, the validity of a comparison of the results generated by different research groups remains unclear. This thesis therefore aims to determine the limits beyond which different studies cannot be compared.

This thesis aims to investigate the effect of protocol variations in the light/dark transition test, and thereby facilitate the contextualisation and comparison of the results of different studies. The general structure of the thesis is as follows: the next chapter will provide a literature review of the most pertinent literature. First, the causes of anxiety and the physiological basis of anxiety responses will be outlined. Next, the advantages and disadvantages of using zebrafish larvae as models for anxiety will be described, including a description of the different models of anxiety employed with zebrafish larvae, with an emphasis on the potential for the light/dark transition test to be used in drug discovery. Finally, existing research on the effects of protocol variations in the light/dark transition test will be reviewed in detail. Chapter 3 will describe the methods and results pertaining to a series of experiments conducted. Chapter 4 will describe the data interpretation, contextualise it within existing literature, and make recommendations. It will also include concluding remarks. This will be followed by a reference list in Chapter 5.

Chapter 2: Literature Review

2.1 Anxiety

2.1.1 Definition and clinical profile

The Diagnostic and Statistical Manual 5 (DSM-5) defines anxiety as “the apprehensive anticipation of future danger or misfortune accompanied by a feeling of worry, distress, and/or somatic symptoms of tension” (American Psychiatric Association [APA], 2013, p. 818). This is almost identical to the definition of anxiety in the International Classification of Diseases, 11th Revision (ICD-11), which states that anxiety is “characterized by excessive fear and anxiety and related behavioural disturbances, with symptoms that are severe enough to result in significant distress and/or significant impairment in personal, family, social, educational, occupational, or other important areas of functioning” (World Health Organisation [WHO], 2019). Both the DSM-5 and the ICD-11 agree that the focus of anticipated danger may be internal or external (APA, 2013; WHO, 2019). Anxiety can be viewed as a transdiagnostic trait (Demetriou et al., 2021; Fontana & Parker., 2022; Newby et al., 2015), and it is therefore present in many disorders, but it is of greatest significance in anxiety disorders.

Fear and anxiety are closely related and at times, overlap. The main difference is the proximity of a real or a perceived threat: fear is the response to an imminent threat, while anxiety is the anticipation of a future threat (APA, 2013; WHO, 2019). Common symptoms of anxiety and fear-related disorders include physiological symptoms of excessive arousal, apprehension and avoidance behaviour (WHO, 2019). These behaviours also form the basis of the zebrafish larval models of anxiety.

Anxiety is individualized: not all individuals will display exactly the same symptoms (National Department of Health, 2020). Thus, neuropsychiatric disorders such as anxiety are difficult to model in animals, and even to standardise in human studies. This underlines the importance of having standardised animal models which, if we understand how to use them optimally, can support the investigation of specific anxiety phenotypes and of responses to compounds which modify anxiety.

2.1.2 Understanding the disease aetiology of anxiety

2.1.2.1 Genetics

Anxiety disorders are considered moderately heritable (Gottschalk & Domschke, 2017; Meier & Deckert, 2019). They show familial aggregation, due both to genetic factors and a shared environment. In the case of generalised anxiety disorder and panic disorder, the role of genes has been confirmed, with generalised anxiety disorder showing a heritability of 0.32 and panic disorder showing a heritability of 0.43 (heritability refers to the proportion of phenotypic variation that is due to genetic factors). In addition, the odds ratio of panic disorder (5), of generalized anxiety disorder (6.1), and of phobias (4.1) show that the first-degree relatives of probands (usually the first person in a family to bring the disorder to the attention of healthcare professionals) are ~ 4 to 6 times more likely to be diagnosed with that disorder (Hettema et al., 2001). This shows that genetic factors play a role in anxiety disorders.

2.1.2.2 Neurotransmitters

Neurotransmitters can have different physiological effects when they bind to different receptors, or when they are present at different binding sites in the nervous system. Dopamine, serotonin, gamma-aminobutyric acid (GABA), glutamate, epinephrine, norepinephrine, endocannabinoids and adenosine are all thought to play a role in anxiety (Alia & Petrunich-Rutherford, 2019; Bandelow *et al.*, 2017; Basnet *et al.*, 2019; Bowery & Smart, 2006; Daviu *et al.*, 2019; Gottschalk & Domschke, 2017; Jakubovski *et al.*, 2019; Kaur & Singh, 2017; Khalifeh *et al.*, 2021; Maximino *et al.*, 2011; Möhler, 2012; Murrough *et al.*, 2015; Nasir *et al.*, 2020; National Department of Health, 2020; Neal, 2016; van Calker *et al.*, 2019; Yang *et al.*, 2016; Zhong *et al.*, 2022).

Serotonin deficit is thought to increase anxiety, and increased serotonin appears to be anxiolytic. Many of the treatments for anxiety modulate serotonin, including selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors and buspirone. In terms of the involvement of specific receptors, there is strong evidence for the $5HT_{1A}$ receptors and some evidence for $5HT_{2C}$ (Bandelow *et al.*, 2017; Gottschalk & Domschke, 2017; Jakubovski *et al.*, 2019; Kaur & Singh, 2017; Khalifeh *et al.*, 2021; National Department of Health, 2020; Neal, 2016).

GABA and glutamate, the main excitatory and inhibitory neurotransmitters, are thought to play a role in anxiety (Bandelow *et al.*, 2017; Bowery & Smart, 2006; Kaur & Singh, 2017; Khalifeh *et al.*, 2021; Möhler, 2012; Nasir *et al.*, 2020; Neal, 2016; Yang *et al.*, 2016; Zhong *et al.*, 2022). Agonism of glutamate receptors is thought to have an anxiogenic effect, and increased glutamate is associated with increased arousal and hyperactivity (Kaur& Singh, 2017; Khalifeh *et al.*, 2021; Nasir *et al.*, 2020; Yang *et al.*, 2016). Several medications that affect GABA are used to treat anxiety. These include benzodiazepines and barbiturates. These treatments have anxiolytic and sedative effects. There is good evidence that the $GABA_A$ (anionic ligand-gated ion channel) is involved in anxiety (Bowery & Smart, 2006; Bandelow *et al.*, 2017; Kaur & Singh, 2017; Khalifeh *et al.*, 2021; Möhler, 2012; Nasir *et al.*, 2020; Neal, 2016; Zhong *et al.*, 2022).

Norepinephrine and epinephrine function as both hormones and neurotransmitters and tend to have an anxiogenic effect. Epinephrine primarily functions as a hormone, and beta blockers are used to counteract the physiological symptoms of arousal that it triggers in anxiety disorders such as panic disorder (Khalifeh *et al.*, 2021; Neal, 2016). Norepinephrine, on the other hand, functions primarily as a neurotransmitter. Antagonism of the α1-adrenergic receptor is an important mechanism in treatments to reduce anxiety (Khalifeh *et al.*, 2021). Moreover, serotonin and norepinephrine reuptake inhibitors (SNRIs), some of the most important medications used to treat anxiety, modulate both serotonin and norepinephrine to reduce anxiety (Bandelow *et al.*, 2017; Daviu *et al.*, 2019; Gottschalk & Domschke, 2017; Jakubovski *et al.*, 2019; Khalifeh *et al.*, 2021).

Agonism of dopamine receptors can increase anxiety-like behaviour (Simon *et al.*, 1993). Although the anxiolytic effects of antipsychotics are often attributed to the modulation of other

neurotransmitters, dopamine receptor antagonism could be a contributing mechanism (Juza *et al.*, 2022). The role of dopamine in anxiety disorders needs to be explored further.

Adenosine is a metabolite that serves a signalling function that can be considered a neurotransmitter (Liu *et al.*, 2019). Caffeine, an adenosine receptor antagonist, can induce anxiety and panic attacks in susceptible humans (Nardi *et al.*, 2009; Yang *et al*., 2010) and is often used to induce anxiety-like behaviour in animal models (Lopez-Luna *et al.*, 2017; Pellow *et al.*, 1985; Richendrfer *et al.*, 2012). Although antagonism of adenosine receptors in general appears to have an anxiogenic effect, A_{2A} appears to be particularly significant in anxiety disorders (van Calker *et al.*, 2019).

2.1.2.3 Brain Regions and Networks

There appears to be an overlap in the brain regions that function in anxiety and stress. Areas of the brain that play an important role in both states and as well as in anxiety disorders include the hypothalamus, amygdala, (basolateral amygdala [BLA]), prefrontal cortex (medial prefrontal cortex [mPFC]) and the nuclei of the brainstem (especially, the locus coeruleus [LC]) (Azevedo *et al.*, 2020; Khalifeh *et al.*, 2021; Liu et al., 2018; Zarrindast & Khakpai, 2015). The globus pallidus and superior colliculus are associated with anxiety (Azevedo et al., 2020). The nucleus accumbens (NAc) is another important region that plays a role in both fear and reward processing (Daviu et al., 2019).

The amygdala plays an important role in assigning emotional valence to stimuli (Daviu et al., 2019; Herrington *et al*., 2017; Pignatelli & Beyeler, 2019). Positive emotional valence encourages approach, while negative valence encourages avoidance (Pignatelli & Beyeler, 2019). Individuals with anxiety show increased attentional bias, usually in the form of increased vigilance in response to minor threats.

Alterations in connectivity between these regions play an important role in anxiety disorders (Daviu *et al.*, 2019). For example, norepinephrine signalling from the LC to the BLA plays a significant role in acute anxiety (Daviu *et al.*, 2019). Comparing the brain activity of anxious humans with animal models of anxiety is an avenue by which to investigate their validity.

2.1.2.4 Stress, cortisol and the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis plays an important role in both stress and anxiety (Daviu *et al.*, 2019). Stress, or the physiological and/or behavioural response to a real or perceived threat to homeostasis, has long been associated with anxiety disorders. Hyperactivity of the HPA axis is thought to play a role in both anxiety disorders and mood disorders. Pharmacological treatments already available for treating these disorders, such as the benzodiazepines, clonazepam and alprazolam, and the SSRI escitalopram, all of which decrease the activation of the HPA axis. This suggests that modulation of the HPA axis plays an important role in current and future treatment of anxiety disorders (Tafet & Nemeroff, 2020).

When humans are exposed to a stressor, the amygdala can trigger the release of the corticotropin releasing hormone from the neurons in the paraventricular nucleus of the hypothalamus into the hypophyseal portal blood directly, and indirectly by causing noradrenergic signalling from the LC. Corticotropin releasing hormone causes the pituitary release of adrenocorticotropic hormone into the circulation. It travels to the adrenal cortex where it triggers the synthesis and release of cortisol. The hormone cortisol binds to the mineralocorticoid and glucocorticoid receptors, resulting in structural changes that allow for the translocation to the nucleus. Once in the nucleus, the receptor-cortisol complex will bind to the glucocorticoid response elements in the DNA to modify the expression of target genes (Mommsen *et al*., 1999; Tafet & Nemeroff, 2020). Cortisol levels are often measured as an indication of stress (Yeh *et al.*, 2013), which is important for models of anxiety.

2.1.2.5 Oxidative stress and inflammation

Oxidative stress may be both a cause and a consequence of anxiety disorders: animal models provide evidence for both (Fedoce *et al*., 2018). Oxidative stress is an imbalance between pro-oxidants and antioxidants. This imbalance is caused when reactive oxygen species exceed antioxidant capacity, allowing for excessive oxidation, which damages macromolecules such as proteins, lipids and nucleic acids. Accumulation of damage to cellular components can cause functional impairment and even the death of cells (Fedoce *et al.*, 2018; Katerji *et al*., 2019; Lichtenberg & Pinchuk, 2015). Cell damage and death are particularly concerning when they occur in the brain, because the brain is an organ with limited regenerative capacity in mammals (Fedoce *et al*., 2018; Kizil *et al.*, 2012).

Many lines of evidence suggest that inflammation plays a role in anxiety disorders. Signals of increased inflammation have been found in fear- and anxiety-based disorders, including posttraumatic stress disorder, generalized anxiety disorder, panic disorder and phobias (Hou *et al.*, 2017; Michopoulos *et al.*, 2017). Inflammation consistently influences the activity of anxiety-associated brain regions, such as the amygdala, insula and anterior cingulate cortex. It can also influence neurotransmitters that play a role in anxiety disorders, such as glutamate, serotonin and dopamine (Felger, 2017). Inflammation has been used to induce anxiety-like behaviour in rodent models, indicating that inflammation can play a role in the development of anxiety disorders (Yang *et al.*, 2016; Zhong *et al.*, 2022).

Some of the most effective treatments for anxiety, such as fluoxetine, show an anti-inflammatory effect, suggesting that reduction of inflammation forms part of their anxiolytic effect (Fedoce *et al.*, 2018; Liu *et al.*, 2011).

2.1.2.6 Childhood Trauma, Environmental Adversity and Stressful Life Events

Childhood trauma is an important risk factor in the development of anxiety disorders (Heim & Nemeroff, 2001; Kuzminskaite *et al*., 2021). Childhood trauma is also associated with hyper-reactivity to stress, neuroticism, inflammation, increased amygdala reactivity, elevated corticotropin releasing hormone and cortisol, glucocorticoid resistance and decreased medial prefrontal cortex

and hippocampal volume (Gottschalk & Domschke, 2017; Tafet & Nemeroff, 2020). Gene-environment studies also support the role of childhood trauma and stressful life events in the development of generalised anxiety disorder (Gottschalk & Domschke, 2017).

Neuroticism is one of the main five personality domains in the five factor model of personality (McCrae & John, 1992). Neuroticism has been associated with the internalising of disorders such as anxiety disorder and major depressive disorder, and it may play a role in the high level of comorbidity (Wang *et al.*, 2022). There is a high genetic correlation between neuroticism and anxiety disorders (especially generalised anxiety disorder) (Gottschalk & Domschke, 2017; Hettema et al., 2004, 2006; Mackintosh *et al.*, 2006).

2.1.3 Limitations of current anxiety treatments

Both non-adherence and non-response to treatment must be addressed to ensure effective treatment of individuals suffering from anxiety disorders. A fifth of patients in studies on treatments for anxiety disorders drop out prematurely, potentially due to lack of response or to side effects (Taylor *et al.*, 2012). Around a third of participants who complete treatment do not respond to the treatments (Bystritsky, 2006; Taylor *et al.,* 2012). Benzodiazepines reduce symptoms of anxiety rapidly, but they carry a high risk of dependence and are therefore only recommended for short term use (a maximum of two weeks of treatment with tapering off completed within six weeks) in cases of severe, acute distress (Department of Health, South Africa, 2019). SSRIs and SNRIs may aggravate symptoms for the first 2-4 weeks, must be taken for 12 months to prevent risk of relapse and must be discontinued gradually to prevent antidepressant discontinuation syndrome (Harvey & Slabbert, 2014; National Department of Health, 2020). In addition, those who do adhere and respond to treatment may have to contend with side effects. Side effects of benzodiazepines may include cognitive impairment and sedation (Department of Health South Africa, 2019). Side effects of SSRIs and SNRIs may include sexual dysfunction (an important cause of non-compliance) (Rothmore, 2020). More treatment options, with better side effect profiles or rapid action without a high risk of dependence, are needed for the successful treatment of individuals with anxiety disorders. Therefore the discovery of drugs to treat anxiety disorders should be made a priority. Zebrafish models are commonly used in the study of anxiety and can contribute significantly towards new drug discovery in this context.

2.2.1 Benefits and limitations of the zebrafish as a model organism

Preclinical research is the link between drug discovery and clinical trials in humans, and includes testing for the safety and efficacy (Steinmetz and Spack, 2009). The light-dark transition test that this project ultimately investigates can be used to indicate efficacy and toxicity, making it a valuable part of preclinical development. Preclinical testing involves both *in vitro* and *in vivo* testing (Avila *et al.*, 2020). Before clinical trials take place, treatments must be tested in two mammalian species: a rodent species and a non-rodent species (Son *et al.*, 2020). Zebrafish currently have a place in the early stages of preclinical research, and can be considered new approach methodologies because

they can be used to shift experiments to phylogenetically lower animals (Avila *et al.*, 2020). Zebrafish are unlikely to fully replace the other animal models at later stages of drug development, but they can reduce the number of mammalian organisms required for preclinical research (Avila *et al.*, 2020). They are already playing an important role in chemical hazard identification and candidate drug screening (Avila *et al.*, 2020). Therefore, they are an important bridge between *in vitro* and *in vivo* preclinical research, and play an important role in investigating the safety and efficacy of candidate treatments.

Zebrafish can be used to model neuropsychiatric disorders. More specifically, they are useful for linking specific signalling events to behavioural outcomes. They can also be used as screening tools for treatments that influence vertebrate behaviour. Therefore, zebrafish can play a valuable role in the search for treatments for neuropsychiatric disorders, including anxiety disorders.

Although zebrafish are vertebrates, they share many of the advantages of invertebrate models such as *Drosophila melanogaster* and *Caenorhabditis elegans* (low cost, ease of husbandry, fecundity, ease of genetic manipulation, small size, transparency and drug delivery through immersion) (Bai *et al*., 2016; Baraban *et al*., 2005; Brugman, 2016; Wagle *et al.,* 2017; Wang *et al*., 2016; Wronikowska *et al.,* 2020). Moreover, due to the fact that they are vertebrates, they have a high homology to humans in terms of their genome, nervous system and endocrine system (Maximino *et al.,* 2013). Therefore, findings with zebrafish have a higher likelihood of being translatable to humans.

Zebrafish play a particularly valuable role in bridging the gap between *in vitro* experiments and *in vivo* experiments using mammalian models (Wronikowska *et al*., 2020). The more established rodent models for neuropsychiatric disorders have limitations that make them less suitable for large-scale drug screening. These include ethical concerns, high costs as well as time-consuming experimental protocols (Fontana *et al.,* 2018). Furthermore, zebrafish have comparatively simple brains, which contain approximately 100 000 neurons. This makes it easier to understand the neural circuits that control their behaviours (Bai *et al.,* 2016; Lau *et al.,* 2011). The relative ease of experimental, genetic and pharmacological manipulation (Khan *et al.,* 2017) makes zebrafish—and the larval models in particular—a valuable tool for research on brain disorders, such as anxiety.

Zebrafish can be used for physiologically complex phenotyping, which includes behaviour. Aggression, anxiety, long- and short-term memory, object discrimination, colour and place preference have all been characterized in zebrafish (Fontana *et al.,* 2018). They are particularly suitable for studying stress and anxiety (Maximino *et al.,* 2013). In part, this is due to the homology of their hypothalamic-pituitary-interrenal (HPI) axis to the human HPA axis (Solomon-Lane *et al.,* 2013).

Larval zebrafish exhibit anxiety-like behaviour, which can be coupled with probing specific neuronal mechanisms. It is the only model where physiological changes can be directly associated with changes in behavioural outcomes, even in real time. Models that look at the effects of stress,

particularly in larval zebrafish, can be considered to be relevant to stress-related neuropsychiatric disorders more broadly, rather than to anxiety specifically. Although this could be construed as a disadvantage, it can also benefit appropriately designed research that focuses on the early stages of the aetiology of these disorders and on medications that could address more than one of these frequently comorbid disorders.

As in humans, anxiety and fear in animals can be adaptive responses. In general, animals respond to threat by fighting, fleeing or freezing, or in the case of a potential threat, by risk assessment. These responses become maladaptive when they interfere with other important processes such as feeding or mating (Lovick & Zangrossi, 2021). Although research with animals should be limited for ethical reasons, cell lines cannot replace the use of animals for complex research at the tissue, organ, system and behavioural level (Ogungbemi *et al*., 2019), necessitating the use of *in vivo* animal models. However, it is important to be aware of both the benefits and limitations of the model chosen for research.

In general, when animals are used to model anxiety disorders, it is important to consider whether adaptive states to stress or anxiety are being measured, and also whether the psychopathology of anxiety disorders is being modelled. It is also important to note that baseline levels of activity and anxiety in model organisms may differ from those in humans. Understanding the natural niche and behaviour of the organism in question is vital to the accurate use of these organisms. The ethological relevance of tests for anxiety-like behaviour should also be considered (Lovick & Zangrossi, 2021). In other words, the ecological niche that the model organism evolved to fill should be considered when designing tests. This approach assists in differentiating between adaptive and pathological behaviour within the animal model, as well as in preventing confounding due to the use of tests that are not appropriate for the model organism (Peters *et al.,* 2015).

Despite their usefulness as a model, it is important to recognise the limitations of investigating neuropsychiatric disorders in zebrafish. As with any animal model, it is impossible to recapitulate the full neuropsychiatric disorder (Yang *et al.,* 2020). In particular, the inability to interview animals to learn more about their cognition presents a challenge in the investigation of neuropsychiatric disorders (Lovick & Zangrossi, 2021). On the other hand, in zebrafish it is possible to link specific neuronal activation in real-time with a behavioural outcome in ways that are not possible to do with humans. Research using zebrafish thus has great potential to contribute to the understanding and treatment of neuropsychiatric disorders.

2.2.1.1 Comparing the central nervous system of zebrafish and humans

There is conservation between the neurological system, the different neuron types and the neurotransmitters of zebrafish and humans (Legradi *et al.,* 2015). The hindbrain, retina and spinal cord of zebrafish and humans are clearly anatomically similar (Horzmann & Freeman, 2016), but the developmental paths of the telencephalon and the mesodiencephalon differ between zebrafish and

humans (Panula *et al.*, 2010). This is because the zebrafish brain develops by eversion rather than inversion. Although eversion makes the zebrafish telencephalon appear very different, recent work indicates that it is far more similar to the mammalian forebrain in structure than was previously thought. There appears to be conservation of function despite the structural differences caused by development through eversion (Ganz *et al.*, 2015; Mueller & Guo, 2009; Mueller *et al.*, 2011; Porter & Mueller, 2020). This similarity extends to the zebrafish amygdaloid complex, which is critical for emotion (Porter & Mueller, 2020).

Zebrafish share their main neurotransmitter systems with humans, including glutamate, GABA, catecholeamines (epinephrine, norepinephrine and dopamine), acetylcholine, histamine and glycine (Horzmann & Freeman, 2016; Panula *et al.*, 2006, 2010). Moreover, the architecture and function of these neurotransmitter systems in zebrafish is similar to that of humans (Panula *et al.*, 2010). This plays a role in their sensitivity to neurotropic pharmaceuticals and in the similarity of their responses to these agents to those of humans and other vertebrates (Kalueff *et al*., 2014). In addition, zebrafish can and have been used to investigate the relationship between the nervous system, microorganisms and the immune system. This is of growing importance as the role of the microbiome in the development of neuropsychiatric disorders is becoming increasingly evident (Lee *et al.*, 2021; Levraud *et al*., 2022). Microbial products or particular strains of microorganisms are also being explored as potential treatments for neuropsychiatric disorders such as anxiety.

Over 70% of proteins found in humans have zebrafish orthologues. More importantly, more than 80% of the disease-causing genes in the human genome have orthologs in the zebrafish (Howe *et al.*, 2013; Varga *et al.*, 2018). Nonetheless, it should be noted that the zebrafish genome underwent a duplication event. Although many of the duplicated genes have been lost, there are often two paralogues of human genes that may exhibit subfunctionalisation or neofuntionalisation (Alsop & Vijayan, 2008; Horzmann & Freeman, 2016). This also affects genes in systems related to stress and anxiety, such as the glucocorticoid receptors and the tyrosine hydroxylase gene (Alsop & Vijayan, 2008; Panula *et al.*, 2010). Sometimes, zebrafish also have fewer copies of a gene: for example, zebrafish have one copy of the monoamine oxidase gene (Panula *et al.*, 2010). The gene duplication event that the ancestors of zebrafish underwent makes it unsurprising that they have more G-protein coupled receptors than mammals. Despite differences between the genomes of zebrafish and humans, the patterns of expression, binding and signalling of receptors in the central nervous system of zebrafish are very similar to those found in mammals (Panula *et al.*, 2010).

Due to the usefulness of zebrafish as a model organism, there are many resources that aid the study of their brains, including a cellular-resolution atlas of the larval zebrafish brain at 6 days post fertilisation (dpf) (Kunst *et al*., 2019) and a detailed atlas for adult zebrafish brains (Kenney *et al*., 2021). Larval atlases are facilitated by the transparency and genetic tractability of zebrafish larvae, and they are useful because of the level of complexity and typical vertebrate architecture of the larval

zebrafish brain (Kunst *et al.,* 2019). Moreover, it is possible to image the brains of zebrafish larvae while they are responding to stimuli (Portugues *et al.,* 2014).

2.2.1.2 HPI Axis

As already stated, the zebrafish hypothalamic-pituitary-interrenal (HPI) axis is homologous to the human hypothalamic-pituitary-adrenal (HPA) axis (Solomon-Lane *et al.*, 2013). As is the case in rodent strains, stress hormone levels may differ significantly between zebrafish strains. For example, cortisol levels at 5 dpf differ significantly between the AB and TL strains. This, together with expression of various marker genes, suggests that HPI axis activity in AB larvae is higher than in TL larvae (Van Den Bos *et al.*, 2017).

As in humans, activation of the HPI axis in zebrafish results in the release of cortisol (Van Den Bos *et al.*, 2017), while activation of the HPA axis in rodents results in the release of corticosterone (Miller *et al.*, 2022). This shows that although zebrafish do not release cortisol from adrenal glands, the hormone produced by their HPI axis is more similar to the stress hormone of humans than that of rodents (Solomon-Lane *et al*., 2013).

Zebrafish have also been used to model glucocorticoid resistance. These models have included mutants with non-functional glucocorticoid receptors and also glucocorticoid receptor knockouts. These models have potential in both anxiety and depression research (Sireeni *et al.*, 2020). For example, the grs357 mutant has glucocorticoid receptors that are transcriptionally inactive due to mutations in the DNA-binding domain. In the larvae, the grs357 mutants show lower spontaneous activity, which can be alleviated by fluoxetine (Griffiths *et al.*, 2012). Whole body cortisol is often used as a measure of stress in larvae (Alia & Petrunich-Rutherford, 2019; Bai *et al*., 2016; Yang *et al*., 2020; Yeh *et al.*, 2013).

2.1.2.3 Zebrafish development

The development of the visual system, nervous system and musculoskeletal system are critical for larvae to respond to the light/dark transition test, which is the specific topic of investigation in this thesis. (The test will be explained in detail later, but briefly, controlled transitions between light and darkness are used to induce an anxiety-like response, which is quantified by analysis of the zebrafish larvae's movement.) Zebrafish larvae are able to detect large changes in lighting conditions from an early stage, even though their visual system is undergoing major development until 5 dpf. The visual system is important for the detection of threats and prey, and it starts early. At 2 dpf, distinctions between light and dark changes over time are possible (Easter & Nicola, 1997). At 4 dpf, zebrafish larvae show a robust response to light and dark conditions (Basnet *et a*l., 2019). At 5 dpf, the zebrafish visual system is considered fully functional (Legradi *et al*., 2015), allowing for hunting in addition to the predator avoidance that larvae engage in from an early stage. This rapid development of the visual system facilitates the use of tests using visual stimuli in the early stages of development.

In order to respond to these visual stimuli, the musculoskeletal system must be sufficiently developed. The first movement that zebrafish show is spontaneous coiling in the egg. From 27 hours post fertilisation (hpf), dechorionated eggs may be able to swim briefly in response to tactile stimulation (Drapeau *et al*., 2002; Legradi *et al.,* 2015; Saint-Amant & Drapeau, 1998). They are also able to distinguish between head and tail touches and are able to adjust their avoidance behaviour to these stimuli (Legradi *et al.,* 2015). Newly hatched larvae (2 dpf and 3 dpf) show spontaneous burst swimming as well as burst swimming in response to tactile stimulation (Brustein *et al.,* 2003; Drapeau *et al.,* 2002). Brief 'beat and glide' swimming first emerges in 3 dpf larvae, while 4 dpf and 5 dpf larvae show sustained 'beat and glide' swimming (Buss & Drapeau, 2001; Brustein *et al*., 2003). Their swim bladders inflate between 4 dpf and 5 dpf (Strähle *et al.,* 2012). Larvae at 5 dpf have fully functional swim bladders and sensory systems and therefore swim more frequently (Buss & Drapeau, 2001; Muto & Kawakami, 2013). Zebrafish larvae locomotion can be broken down into motor elements such as C-starts, routine turns, J-turns, O-bends, slow scoots, burst swims, and capture swims (Colwill & Creton, 2011). Differences in muscle function may affect swimming behaviour in the absence of physical abnormalities (Christou *et al.,* 2020). Both obvious musculoskeletal malformations and mild malformations have a significant effect on activity that can be detected in the light/dark transition test (Padilla *et al.,* 2011). Obvious malformation should be excluded when trying to detect either neuroactivity or neurotoxicity using the light/dark transition test (Padilla *et al.,* 2011).

The development of the nervous system is important for coordinating movement, and also the capacity to exhibit anxiety-like behaviour. For example, the development of the serotonergic system is important for sustained swimming (Drapeau *et al.,* 2002). Zebrafish neurogenesis starts during gastrulation at 6 hpf and neurulation, or formation of the neural tube, occurs by 9-10 hpf. At 24 hpf, axons connect the first neurons. At this stage, the forebrain, diencephalon, telencephalon, midbrain, hindbrain and spinal cord have all formed. At 48 hpf, formation of the ventricles has taken place. At 96 hpf all catecholaminergic neuron clusters, glial cell subtypes, oligodendrocytes, Schwann cells and astrocytes are present (Legradi *et al.,* 2015). The zebrafish blood-brain barrier starts developing from 3 dpf and has matured by 10 dpf (Fleming *et al.,* 2013). This has important implications for the timing of different pharmaceutical treatments: some studies may want to start treatment before the development of the blood brain barrier so that the treatment can affect the nervous system, while others may prefer starting after the development of the blood-brain barrier to avoid toxicity. Therefore the stage of development is an important consideration when using the light/dark transition test to screen for neuroactivity.

The importance of the development of many different systems for the typical response to the light/dark transition test to occur means that perturbations of the light/dark transition test response can be used to detect developmental abnormalities and developmental toxicity. At the early stages of development, when a few hours can represent a large change in the functionality of important

systems, the choice of the timing of tests is very important. Age has been reported to have a significant effect on the response to the light/dark transition test (Padilla *et al.*, 2011), but little is known about the earliest stage at which the light/dark transition test can be used.

2.2 Modelling Anxiety in Zebrafish

Zebrafish provide a useful model that, enables researchers to probe particular pathways and systems *in vivo*. Behavioural tests in zebrafish can be used both to test the effect of chemicals on anxiety-like behaviour and to investigate the role of specific genes in anxiety-like behaviour by using genetically modified fish (Han *et al.*, 2021). Larval tests can be executed rapidly and ethically, therefore, although adult zebrafish are frequently used in anxiety-related research, this review will focus exclusively on research into anxiety using zebrafish larvae. In this section, the most-used larval zebrafish tests for anxiety research are described briefly. More importantly, there will be an in-depth discussion of the light/dark transition test. Zebrafish larvae prefer moderately light conditions. Both bright light and complete darkness are aversive to them, thus the transition from bright light to complete darkness, as used in the light/dark transition test, causes hyperlocomotion, whose extent can be related to anxiety. The light/dark transition test is the most widely used zebrafish larval behavioural test and the focus of this project.

2.2.1 Thigmotaxis and the open field test

Thigmotaxis is the degree to which animals avoid open spaces (Bouwknecht & Paylor, 2008). It is an anxiety-related behaviour that can be observed in both adult and larval zebrafish (Maciąg *et al.*, 2020; Nema *et al.*, 2016; Peng *et al.*, 2016; Schnörr *et al.*, 2012). Reverse-thigmotaxis is the reduction in thigmotaxis observed after treatment with anxiolytic compounds (Maphanga *et al.*, 2020). Thigmotaxis has therefore been used to screen for the efficacy of potential anxiolytic treatments in zebrafish (Maphanga *et al.*, 2020). Moreover, thigmotaxis is highly conserved across the animal kingdom (Maciąg *et al*., 2020; Peng *et al*., 2016; Schnörr *et al.*, 2012). Specifically, it is present in both rodents (Telonis & Margarity, 2015) and humans (Kallai *et al.*, 2007).

Thigmotaxis can be measured when an organism is introduced to an open field, as well as when an anxiogenic stimulus is used. The open field test will provoke an “approach/avoidance” conflict. A less exposed area is generally preferred to an open area because the subjects perceive it to be safer. Fish have an advantage when they are near a wall and stationary, as this makes them less visible to sighted predators (Sharma *et al.,* 2009). Open field tests are linked to the exploration of a novel environment, therefore immediate measurement is useful if an anxiogenic stimulus is not being used. Anxiogenic stimuli, including abrupt changes in lighting conditions, can also be used in thigmotaxis assays. In normal zebrafish, locomotion and thigmotaxis increase in the context of elevated anxiety levels (Peng *et al.*, 2016). Thigmotaxis can be elicited after the stimulus of a light/dark transition (Schnörr *et al.*, 2012), therefore measuring thigmotaxis can potentially be combined with the light/dark transition assay.

Measuring thigmotaxis requires a larger well size which would have made the arena size differ from most use ofthe light/dark transition test. In addition, there are strong indications that well size (either due to radius or circumference) significantly impact the results (Padilla et al., 2011). Moreover, with our set up of one plate being read at a time, it would have lowered our throughput and required testing at many times of day. Throughput would have been three times lower, before we knew what the effect of repeated testing or different time of day was. Therefore, for this initial investigation we made the decision to focus on the more common variation of the light/dark transition test, with efficient and common measurement in a 96-well plate.

2.2.2 Startle response in zebrafish larvae

The startle response is related to anxiety. For, example, humans with generalised anxiety disorder exhibit a greater startle reflex (Girirajan *et al.*, 2011). In zebrafish, the startle reflex refers to a C-shaped bending of the body in response to an unexpected stimulus. It is thought to be an escape response to a potentially threatening stimulus (Beppi *et al.*, 2021). Startle responses can be elicited by tactile, auditory and visual stimuli in zebrafish larvae (Beppi *et al.*, 2021; Easter & Nicola, 1997). Touch elicits a startle response in zebrafish at an earlier age than visual stimuli. Visual stimuli are able to elicit a startle response from 68-79 hpf (Easter & Nicola, 1997). Both abrupt changes in illumination (such as those found in the light/dark transition test) and looming stimuli can elicit a measurable startle response (Beppi *et al.*, 2021). This makes the startle response a potential tool for anxiety-related research.

2.2.3 Light/dark preference test

The light/dark preference test is one of the most widely used tests for screening for anxiety-related effects (Perez-Rodriguez *et al.*, 2019; Shi *et al.*, 2022; Yang *et al.*, 2021). Light/dark preference is associated with anxiety in both larval and adult zebrafish, but whereas larvae display dark avoidance, adults display light avoidance (Sireeni *et al.*, 2020; Steenbergen *et al*., 2011; Wagle *et al.*, 2017). Dark-aversion is an anxiety-associated trait that shows heritable variation. Larvae with a strong dark-aversion also show more avoidance of the centre of the arena, or thigmotaxis, which is another anxiety-related behaviour (Wagle *et al.*, 2017). The light/dark preference test has been used in organisms from *Drosophila melanogaster* to rodents, and appears to be evolutionarily conserved (Bourin & Hascoët, 2003; Gong *et al.*, 2010; Wagle *et al.*, 2017).

2.2.4 The light/dark transition test in zebrafish larvae

The light/dark transition test is widely used, including in pharmacology, toxicology, ecotoxicology, cognition and neurobiology (Basnet *et al.*, 2019; Perez-Rodriguez *et al.*, 2019). Although the assay is usually used in larval zebrafish, the response of adult zebrafish to light/dark transition has also been measured in the past (Vignet *et al.,* 2013). It is thought that that light/dark transition evokes anxiety-like behaviour in zebrafish (Basnet *et al.*, 2019; Maphanga *et al.*, 2020, 2022). Due to the consistent responses of zebrafish larvae to transitions from light to darkness, perturbations of the response caused by developmental disruptions, neurotoxicity or neuroactivity can be identified

(Haigis *et al.*, 2022; Jarema *et al.,* 2022; Maphanga *et al.,* 2020). Despite its potential for screening for neuroactive substances (including those with anxiolytic and anxiogenic-like effects) (Basnet *et al.*, 2019; Maphanga *et al.*, 2020, 2022), the light/dark transition test is most often used during neurotoxicity testing (Haigis *et al.,* 2022; Jarema *et al.,* 2022; Legradi *et al.,* 2015). It is the most widely used assay for studying early life stage behaviour in zebrafish larvae (Haigis *et al.,* 2022).

The light/dark transition test consists of an acclimation period (usually under dark conditions), a light period, and a dark period (Figure 2.1). During the acclimation period, the activity level gradually decreases as the zebrafish larvae recover after handling. When the light is switched on for the light period, there will be a rapid startle response followed by a low level of activity (Irons *et al.,* 2010; MacPhail *et al.,* 2009; Van Den Bos *et al.,* 2017). In larvae that are old enough, there will be a gradual recovery in activity over a longer light period (Macphail *et al.,* 2009; Van Den Bos *et al*., 2017). When the light is switched off again for the dark period, there will be hyperlocomotion that exceeds the baseline activity level prior to the light period, and which will gradually decrease throughout the dark period (Irons *et al*., 2010; Macphail *et al*., 2009; Van Den Bos *et al*., 2017).

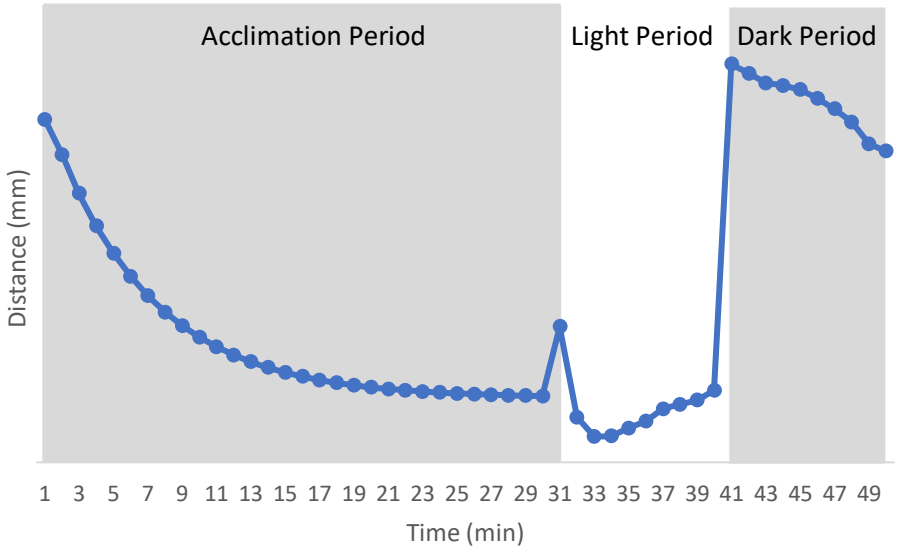


Figure 2.1. Line graph showing the distance moved per minute over the course of the light/dark transition test

The activity during the light/dark transition test is different from the activity under light or dark conditions when there has not been a recent transition. When there are frequent transitions, activity is higher during the dark period than during the light period, while activity during continuous illumination is higher than activity under continuous darkness (Burgess & Granato, 2007; Kristofco *et al.*, 2016; Macphail *et al.*, 2009; Vignet *et al.*, 2013). This means that when the periods of different light conditions are too long, the test will decrease in sensitivity because the signal from the period directly after the transition will be lost (when response is measured in terms of total activity during the dark period).

Study design for the light/dark transition test varies. The use of different protocols without the knowledge of how these protocol changes may impact the data generated complicates the integration of results. To address this, some studies have examined the effects of changing the parameters of the light/dark transition test. For example, MacPhail *et al*. (2009) found that the length of the light period altered the activity level in the dark period, with a longer light period resulting in a greater increase in activity after the transition from light to darkness (MacPhail *et al.,* 2009). Although the activity in the dark period was greater when the preceding light period was longer, the rate of decrease was similar (approximately 50% in 10 minutes) (Macphail *et al.,* 2009). This illustrates how variations in protocol can have significant impacts on the results of this test.

2.2.5 Linking light to dark transition response to anxiety

Zebrafish larvae show nondirectional dark photokinesis after a light to dark transition (Burgess & Granato, 2007; Emran *et al*., 2008; Fernandes et al., 2012; Fitzgerald *et al.,* 2019; Horstick *et al.,* 2017). Nondirectional dark photokinesis refers to hyperlocomotion under dark conditions after a light to dark transition, and it is not aimed in a particular direction (it usually consists of increases in routine turns and swimming bout length). Even zebrafish larvae lacking eyes and a pineal gland show this response (changes in light conditions are detected by deep brain photoreceptors and will trigger the hyperlocomotion). The period of brief hyperlocomotion following a transition to darkness has been referred to as the visual motor response (VMR) (Fernandes *et al.,* 2012).

There are several hypotheses for why there is such a rapid increase in activity after a light to dark transition. The first is that the larvae see a light to dark transition or decrease in illumination as a threat, possibly because it may be a sign of a predator overhead (Basnet *et a*l., 2019). Like looming stimuli, which evoke an escape response (Temizer *et al.,* 2015), the response to light to dark transition may represent visually invoked predator avoidance behaviour (Colwill & Creton, 2011; Steele *et al*., 2018) and in the literature, it is widely associated with anxiety (Basnet *et al.,* 2019; Irons *et al.,* 2010; Li *et al.,* 2015; MacPhail *et al.,* 2009; Peng et al., 2016; Sireeni *et al*., 2020; López & Smith, 2021; Vignet *et al.,* 2013). The hypothesis that a sudden decrease in illumination is a stressful stimulus is supported by the successful use of a light/dark transition after a light acclimation period to elicit thigmotaxis (Schnörr *et al.,* 2012).

Zebrafish larvae also show an aversion to the darkness itself, as shown in the light/dark preference test (Wagle *et al.,* 2017). Adult zebrafish exhibit scototaxis (preference for darkness), while larval zebrafish exhibit scotophobia (fear of or aversion to darkness) (Maximino *et al.,* 2014; Wagle *et al.,* 2017). It is logical that this dark aversion would cause an anxiety-like response after a sudden transition from light to darkness.

It is also possible that the larvae find bright light after acclimation to darkness aversive. Although zebrafish larvae will prefer the lighter part of an arena in a light/dark preference test, larvae do not always move towards a light spot when there is a change from uniform illumination to darkness with

one light spot: the direction of their movement is dependent on the relative intensity of the uniform illumination and the target light. They will swim quickly towards a low intensity light spot but will swim slowly away from a very high intensity light spot. Swimming towards the light is referred to as positive phototaxis and swimming away from the light is referred to as negative phototaxis (Burgess *et al*., 2010). Larvae avoid lights that are brighter than their pre-adapted light level. This suggests that a sudden transition to bright light after acclimation to dark conditions may be a stressful stimulus. This would be consistent with the extremely low activity observed during the light period being due to a freezing response.

Regardless of the precise part of the test that is the anxiogenic stimulus, alternating light/dark cycles are considered a stressful condition for zebrafish larvae (Peng *et al.,* 2016). There are several studies (Irons *et al*., 2010; López & Smith, 2021; MacPhail *et al*., 2009; Peng *et al*., 2016; Sireeni *et al*., 2020; Vignet *et a*l., 2013) that attribute the increase in activity after a light to dark transition to anxiety. The foundational work of MacPhail *et al*. (2009) found that a longer light period elicited a larger response in the dark period, which is consistent with bright light being aversive (MacPhail *et al*., 2009). Later work by Peng *et al.* confirmed that increased anxiety results in increased locomotor activity (Peng *et al*., 2016).

Another explanation for the hyperlocomotion following a light/dark transition is that larvae increase their activity to find an area that is as brightly lit as possible to facilitate feeding: zebrafish larvae require vision for successful predation (Fernandes *et al*., 2012; Horstick *et al.*, 2017). However, this is an unlikely scenario, because larvae show a response to light/dark transitions before 4 dpf. Before 4 dpf, larvae do not have all the physical requirements for feeding and do not display prey capture behaviour (Muto & Kawakami, 2013; Strähle *et al.*, 2012). However, avoidance of predators would be relevant throughout development. The thigmotaxis elicited by light/dark transitions is consistent with avoidance behaviour (Schnörr *et al.*, 2012), making this a more likely explanation. The thigmotaxis elicited by a light/dark transition indicates that anxiety-like behaviour is present during the dark period, even if searching for light conditions where prey could be detected does contribute to the hyperlocomotion response.

Gauthier and Vijayan (2018) proposed a different anxiety-related explanation for the increase in activity after a light to dark transition. They suggest that increased activity after a light to dark transition could, in fact, represent increased boldness and therefore decreased anxiety-like behaviour. They based this on a risk model where brightly lit environments make larvae more visible to predators and suggested that dark environments would therefore be perceived as safe. This is not supported by the results of larval light/dark preference assays, where larvae tend to choose the lighter part of an arena due to innate scotophobia (Bai *et al.,* 2016; Chen *et al.,* 2015; Wagle *et al.,* 2017). This is also not supported by the robust phototaxis exhibited by larvae when a dim light (10-fold less intense than preadapted illumination levels) is extinguished following a uniform field of illumination (Burgess *et al*., 2010; Chen & Engert, 2014; Horstick *et al.,* 2017). Although for adult

zebrafish scototaxis is an indicator of anxiety, larvae exhibit scotophobia: the zebrafish preference for lighting conditions switches between the larval stage and adulthood (Bai *et al.,* 2016). It is therefore unlikely that increased activity after a light to dark transition is due to decreased anxiety-like behaviour and increased boldness. The relationship between boldness and response to light to dark transitions should be investigated further, but there are problems with the arguments put forward to justify these contrasting explanations for activity patterns in response to light to dark transition.

Overall, the light/dark transition test is a test that can be used to detect neurotoxicity, toxicity or neuroactivity. Although it has potential for identifying anxiety-related effects during the initial screening, it should be followed by other tests relating to anxiety. Although it is not specific to anxiety, it is often the first behavioural test that larvae being exposed to a treatment are subjected to. Understanding how the different stages of this test are linked to stress, fear and anxiety could benefit the use of this test to identify anxiolytic treatments.

2.3 The effect of protocol variations on zebrafish behaviour during the light/dark transition test and other measures of anxiety-like behaviour

Many studies have recognised that lack of standardisation in the light/dark transition test hampers the comparison of results across laboratories (Christou *et al.*, 2020; Haigis *et al.*, 2022; Jarema *et al.*, 2022). The sources of variation across laboratories and studies need to be identified so that the full benefits of the wide application of this test can be realised. When comparisons across studies can be made within a context, it will improve the identification and comparison of potential neuroactive compounds, including those with the potential to modulate anxiety.

2.3.1 Intrinsic differences

2.3.1.1 Strain

Strain, and therefore genetic background, affects anxiety-like behaviour (Robison & Rowland, 2005). Zebrafish have several different wild type strains, which include the AB, Tübingen (TU), TE, Tüpfel long fin (TL), WIK, EK and TM1 strains (Coe *et al.*, 2009; Legradi *et al.*, 2015; Liu *et al.*, 2014; Vignet *et al.*, 2013). Sometimes commercially available fish are used with little information about their history and genetic background. Fish that come directly from the wild have also been used in zebrafish studies. They may come from regions such as Nadia, Gaighatta and Bangladesh, and they are often significantly different to the laboratory strains (Vignet *et al.*, 2013). Wild populations harbour more genetic diversity (Coe *et al.*, 2009) and could therefore be used for the establishment of new strains with particular phenotypes, including high or low levels of anxiety-like behaviour. Using strains with different basal levels of activity or anxiety-like behaviour, could be leveraged to improve the sensitivity of screens for potential anxiolytic and anxiogenic compounds. Many studies have compared the behaviour of wild zebrafish and established laboratory strains (e.g. Vignet *et al.*, 2013). For example, there is evidence of heritable variation in anxiety-like behaviours, such as avoidance of dark areas in the larval light/dark preference test (Wagle *et al.*, 2017). This shows that naturally

heritable variation in anxiety-like behaviour occurs in zebrafish, which could provide invaluable material for future research. Lines with different levels of anxiety could be developed through selective breeding.

Laboratory strains undergo selection for zootechnical requirements, such as fertility and robustness. However, in an environment without predators, selection pressure for many behavioural traits may be reduced. This is one of the reasons that laboratory strains are more likely to develop behavioural differences, both from other laboratory strains and also from wild zebrafish (Vignet *et al.*, 2013). Other factors, such as genetic isolation, genetic drift, inbreeding and founder effects, could also play a role. On the other hand, some of the more common behavioural changes that occur in the process of domestication may be present (Robison & Rowland, 2005). Chin *et al.* investigated the effect of another environment that lacked predators (cave environment) on behaviours associated with anxiety in fish. This research compared the responses of blind cavefish and sighted fish of the same species and showed that after adapting to caves, where the fish had very few predators, blind cavefish showed reduced anxiety-like behaviour. Treatment with anxiolytics abolished the differences between the blind cavefish and the sighted fish (Chin *et al.*, 2018). This research provides support for a reduction in predation resulting in a reduction in baseline levels of anxiety-like behaviour. It should be noted that this change may not have been due to a reduction in anxiety-like states, but changes in how the behaviour related to anxiety is expressed after generations of exposure to a different environment. This may also have occurred in laboratory strains of zebrafish where selective pressures caused by predation have been relaxed. This provides support for the need for zebrafish lines that have been selectively bred to have higher or lower baseline levels of anxiety-like behaviour. Models with a higher baseline level of anxiety can be leveraged to support research.

It is useful to characterise the responses of fish of different strains to different tests of anxiety. There are significant differences in the light/dark transition response of the TL and AB strains of zebrafish larvae (Van Den Bos *et al.,* 2017). Larvae from both strains show the expected hyperlocomotion response to a light/dark transition, but AB larvae show a larger hyperlocomotion response than the TL strain (Christou *et al.*, 2020; de Esch *et al.*, 2012; Van Den Bos *et al.*, 2017). These differences in light/dark transition response are accompanied by an elevated baseline HPI-axis activity in the AB strain as compared to the TL strain, differences in expression of (neuro)development and immune system-related genes (the AB has increased expression relative to the TL strain) and in habituation to acoustic/vibrational stimuli (Van Den Bos *et al.,* 2017). Genetic background can influence response to treatment. For example, the increase in activity caused by a fixed dose of ethanol was higher in the AB strain than in the WIK strain (Lockwood *et al.*, 2004). Strain differences between the response of TU and AB to treatments have been observed in other studies even when differences prior to treatment were not significant. When treated with the NMDA receptor antagonist MK-801, the TU strain had both reduced travelling

distance and reduced activity response, while the AB strain did not show a difference in travelling distance and had the opposite reaction in their activity counts. These differences were accompanied by differences in neurotransmitter levels. For example, the TU strain had significantly higher levels of norepinephrine, 3-methoxy-4- hydroxyphenylene glycol (norepinephrine metabolite), 3,4- dihydroxyphenylacetic acid (dopamine metabolite) and serotonin (Liu *et al.*, 2014).

The term “wild type” can include larvae from specific strains. Despite the importance of strain, many studies, including that of MacPhail *et al.* (2009), do not report the strain that they used (Vignet *et al.*, 2013). Not only could the baseline response to anxiety and activity assays affect the utility of different strains for testing, but their responses to particular types of treatments may be important. The baseline level of anxiety-like behaviour in different strains should be determined, and the source of the zebrafish used in any study should be reported. Although specific strains may not be used due to lack of availability or because a more diverse genetic background will be useful for the study being carried out, the breeding stock should be described and the fact that one of the main strains was not used should be confirmed.

2.3.1.2 Age

Age influences the basal locomotor activity of larval zebrafish (Colwill & Creton, 2011; Ingebretson & Masino, 2013; Padilla *et al.*, 2011) and also their response to the light/dark transition test (Padilla *et al.*, 2011). Several experiments show that older fish are more active and that they adapt rapidly to changes in lighting (Colwill & Creton, 2011; Padilla *et al.*, 2011; Thirumalai & Cline, 2008). The impact of age on the locomotor activity of larval zebrafish is widely recognised and is consequently generally tightly controlled in testing.

For example, Padilla *et al.* (2011) examined the effect of age (4 dpf, 5 dpf and 6 dpf) on the activity of larvae undergoing the light/dark transition test and found that age affected activity during both the light period and the dark period of a light/dark transition test (Padilla *et al.*, 2011). The impact of age was particularly high in the light period, which exhibited lower overall activity. The locomotion during the 20-minute light period (following a rest in darkness) increased with age. This difference was seen because, after all groups showed similar activity at the beginning of the light period, the activity of the 5 and 6 dpf larvae increased gradually throughout the 20-minute light period. The larvae at 6 dpf increased their activity more rapidly and appeared to reach an asymptote, while the larvae at 5 dpf had lower activity and their activity levels had not reached a plateau by the end of the light period (Padilla *et al.*, 2011). This suggests that older larvae are able to adapt to lighting conditions rapidly.

Larvae between 3 dpf and 4 dpf consistently show lower activity than larvae between 5 and 10 dpf (Colwill & Creton, 2011; Padilla *et al.*, 2011; Thirumalai & Cline, 2008). These differences can be observed during the light/dark transition test, but the ability to observe these differences may depend on the exact design of the light/dark transition test (Padilla *et al.* used a long 20 minute light period and 20 minute dark period, which was not the most common experimental design—often the light

periods and dark periods are shorter) (Padilla *et al.*, 2011). The differences observed during the light period, in particular, may however be due to intrinsic differences in locomotor activity under light conditions rather than to differences caused by the stressful stimuli of the light/dark transition test per se (Colwill & Creton, 2011; Padilla *et al.*, 2011; Thirumalai & Cline, 2008). Despite several studies comparing the light/dark transition response of different ages, it is not clear how early the light/dark transition tests can be performed effectively.

In addition to the effects of age on how control larvae respond to light/dark transitions, age can influence the absorption of treatments that are being screened during the light/dark transition test. For example, age can affect whether it is the body or the yolk sac that absorbs more of the treatment (Souder & Gorelick, 2017). Differences in absorption are only one of several reasons that the same treatment could affect larvae of different ages differently. Neurotoxicity and metabolic activation of treatments may also be affected by age (Kristofco *et al.*, 2016).

These age dependent differences in response to treatments have been observed experimentally (Kristofco *et al.*, 2016). Kristofco *et al*. (2016) measured both intrinsic activity and photomotor response at 4 dpf, 7 dpf and 10 dpf. Their protocol for measuring photomotor response involved 20 minutes of darkness followed by 20 minutes of light. They observed an age- and dose-dependent response for one of their treatments (diphenhydramine [DPH]) but not for the other (diazinon). The larvae at 10 dpf were significantly more sensitive to DPH than either the 4 dpf or 7 dpf larvae (Kristofco *et al.*, 2016). This altered sensitivity at 10 dpf is mirrored by the increased sensitivity seen in toxicity endpoints, such as mortality (Kristofco *et al.*, 2016). This difference in response shows how the effect of age on activity is also related to the specific treatment being tested.

Age affects both the intrinsic activity of zebrafish larvae and their response to the light/dark transition test. Older larvae tend to have higher intrinsic levels of activity as well as larger responses to the light/dark transition test. Older larvae may also recover their normal level of activity under light conditions faster. The age of the larvae can affect both their absorption of treatments and their metabolism, resulting in different interactions between age and various treatments. The age of the larvae should therefore be carefully considered when locomotor activity is being used as an important end point measurement.

2.3.1.2 Temperature

Although this was not investigated experimentally, temperatures must be kept constant for both welfare and standardisation purposes. Temperatures of 10°C can be lethal (Ren et al., 2021), and milder temperature affects rate of development (Kimmel et al., 1995). Therefore, it follows that temperature could also affect locomotor activity due to acceleration or delay of development (Padilla et al., 2011). This means that caution should be exercised when comparing larvae reared at different temperatures, even if they were tested at the same age and time of day.

Development at 28.5 °C can be used as a reference. Kimmel et al. developed the formula HT=h/(0.055 T−0.57) calculate the relative stage of development of larvae reared at different temperatures (Kimmel et al., 1995).

2.3.2 Test design

2.3.2.1. Time of day

There is evidence that time of day consistently affects locomotor activity in zebrafish, whether they are kept under normal lighting conditions or in continuous darkness. It should also be noted that using light stimuli directly modulates fish behaviour throughout their circadian cycle and light/dark cues can be used to override their endogenous clock (Burgess & Granato, 2007). A number of studies have therefore explored how both total activity and variability in activity are impacted by the time of day and also how this impacts responses to light/dark transitions.

Although many studies have investigated the effect of time of day on activity under constant lighting conditions (MacPhail *et al.*, 2009; Vignet *et al.*, 2013; Kristofco *et al.*, 2016), only one has investigated the effect of time of day on the response to the light/dark transition test (Fitzgerald *et al.*, 2019), while another looked at the effect of time of day on a dark/light transition (photomotor response) (Kristofco *et al.*, 2016). The studies that looked at activity without a light condition change found that the level of activity changed over the course of the day. They agreed that activity was higher and more variable in the morning and decreased until it reached a plateau with low variability in the early afternoon (Kristofco *et al.*, 2016; MacPhail *et al.*, 2009; Vignet *et al.*, 2013). The study that continued longest found that there was a slight increase towards the end of the day (Vignet *et al*., 2013). Kristofco *et al.'s* (2016) study investigated the effect of time of day on photomotor response, but only at some ages. This study observed lower activity at 9:00. This result differs from that of Vignet *et al.* (2013), the only other study that started measuring activity early (Vignet *et al.*, 2013). The exact time of day that is most variable may be related to the time at which the incubator lights are switched on daily, which is seldom reported. Low activity in the afternoon could also be because the larvae are tired.

However, the study that investigated the effect of time of day on the response to the light/dark transition test (Fitzgerald *et al.*, 2019) didn't find any effect for time of day. On the other hand, they only measured the response to the light/dark transition test at two time periods: 9:00 and 14:00. Given the variation in activity seen throughout the day, and the lack of a study investigating whether the light/dark transition test's response is different at any point during the work day, more research is needed. Age (Kristofco *et al.*, 2016) and strain (Vignet *et al.*, 2013) can affect activity level at different times of day, but this study will start with the basic question of whether and how time of day needs to be standardised for the light/dark transition test and future studies can investigate how a combination of age and strain affect the outcomes of the test.

2.3.2.2. Habituation to repeated testing

There is a need to determine whether repeated measurement of the same larvae on the same day has an effect on their activity. Fitzgerald *et al*. (2019) did compare the intra-indivudual variability

Light/dark transition test response of larvae that had been tested twice a day at different ages. They found that the intra-individual variablity decreased with increasing age. In addition, they did not find that the correlation between the responses to tests at different times of day increased when the larvae had been exposed to tests on more consecutive days. They did not compare the response of larvae exposed to different numbers of light/dark transition tests directly. Moreover, they used radial index and activity index rather than more widely used measurements of light/dark transition test response. In this study, we will therefore investigate how repeated testing affects the response to the light/dark trasnition test. It should be noted significant differences in responses to the light/dark transition test across different age groups have been found (Padilla *et al.*, 2011). These findings suggest that a pre-post exposure test design that is performed across different days should be avoided. Instead, a comparison against a control group is more appropriate.

2.3.2.3 Photoperiod

The photoperiod (length of daily light exposure) applied to both adult zebrafish and larvae varies across studies (Legradi *et al.*, 2015). Continuous darkness starting from 2 dpf or earlier had a large effect on the presence and consistency of a circadian rhythm in the activity of zebrafish larvae (Hurd & Cahill, 2002), suggesting that photoperiod, particularly early in development, may affect locomotor activity.

Rearing larvae under consistent darkness affects hatching, survival, growth and the percentage of malformations, although the effects on survival and growth recover by 30 dpf if larvae are transferred to a normal photoperiod by 5 dpf (Villamizar *et al.*, 2014).

Despite the need for caution when rearing larvae under continuous darkness, there are situations where being able to rear larvae under dark conditions would be useful. Light can affect the lability of compounds (Wang *et al.*, 2019), and as young larvae are usually treated through immersion (Irons *et al.*, 2010; Singer *et al.*, 2016; Wang *et al.*, 2019) the compound of interest is usually exposed to light throughout treatment. Rearing larvae under dark conditions would improve the precision and accuracy of exposure concentrations and make it easier to distinguish between the effects of the compound of interest and the secondary effects produced by exposure to light. As larvae are sometimes reared under dark conditions when they are being treated with compounds whose lability are affected by light (Wang *et al.*, 2019), it is important to determine what the effect of rearing larvae under dark conditions is on their response to the light/dark transition test.

2.3.2.4 Light period length

MacPhail *et al*. (2009) found a significant difference in activity during the dark period following light periods of 5 minutes versus 15 minutes (MacPhail *et al.*, 2009). A longer light period resulted in greater locomotion following the light to dark transition (MacPhail *et al.*, 2009). The rate of habituation after the transition (i.e. a decrease in activity after reaching a peak) was similar, regardless of the light period length (MacPhail *et al.*, 2009). Over the course of longer light periods, a gradual increase in activity can be observed. MacPhail et al. (2009) suggested that this could be due either to

habituation or to dissipation of the aversive properties of the transition from darkness to bright illumination (MacPhail *et al.*, 2009). Despite these initial results, MacPhail *et al*. (2009) called for more studies to investigate a broader range of light period lengths (MacPhail *et al.*, 2009). This is particularly important due to the lack of a standardisation of light period lengths in the literature.

2.3.2.5 Dark period length

Activity during the dark part of alternating light and dark periods is higher than activity under continuous dark conditions (MacPhail *et al.*, 2009). A short dark period length may prevent the detection of changes in return to baseline activity levels. Detecting changes in how quickly larvae return to baseline levels of activity could be particularly useful in cases where ceilings on locomotor activity or anxiety-like behaviour have been reached. For example, MacPhail *et al.* (2009) observed a difference in the rate at which activity returned to baseline after a light to dark transition after treatment with 1% ethanol (Macphail *et al.*, 2009).

2.3.2.6 Transition intensity

A sudden decrease in illumination can cause a transient increase in activity, even if the illumination doesn't decrease to complete darkness. Padilla *et al.* (2011) found that a larger difference in the amount of illumination resulted in a larger increase in activity after the transition (Padilla *et al.*, 2011). If a transient increase in activity is smaller in magnitude than the change caused by a transition to complete darkness, this can be leveraged to increase the sensitivity of the light/dark transition assay to anxiogenic compounds. A similar strategy has been used in thigmotaxis assays when a ceiling for this anxiety-like behaviour has been reached (Schnörr *et al.*, 2012). Depending on whether the aim is to detect anxiogenic effects, anxiolytic effects or both, the intensity of the light to dark transition could be optimised accordingly.

2.4 Hypothesis, Aims and Objectives

Anxiety disorders are a major contributor to the years of healthy life lost due to disability globally, and more research is needed to expand the treatment options available for this complex set of disorders. Zebrafish are a small, vertebrate model with potential for high throughput screening. Several tests can be used in anxiety related research at the larval stage, including the light/dark transition test. Despite the widespread use of the light/dark transition test, the effects of common protocol variations are not well understood. Investigation of these variations will support the effective use of this test and may reveal ways that it can be optimised for use in anxiety related research.

After a thorough review of the relevant literature, the hypothesis that common protocol variations have significant effects on the response to the light/dark transition test in zebrafish larvae was formulated.

Therefore, this study aimed to investigate the effects of protocol variations on responses to the light/dark transition test.

In order to achieve the aims of this study, the following objectives were formulated:

- To assess the effect of larval age shortly after hatching on performance during the light/dark transition test.
- To assess the effect of lighting conditions during rearing on performance in the light/dark transition test.
- To assess whether the first larvae captured and transferred to the plates used for activity tracking have a different activity level during the light/dark transition test than the last larvae captured.
- To assess the effect of time of day on activity level during the light/dark transition test.
- To assess the effect of repeated light/dark cycles on activity during the light/dark transition test.
- To assess the effect of repeated light/dark transition tests on activity during the light/dark transition test.
- To assess the effect of duration of the light period on the activity during the light/dark transition test.
- To assess the effect of the magnitude of the light intensity fluctuation during the light to dark transition on activity during the light/dark transition test.
- To assess the effect of different breeding stocks on activity during the light/dark transition test.

Chapter 3: Methods and Results

This thesis aimed to elucidate the effects of subtle changes to the protocol used in the light/dark transition test in zebrafish larvae on behavioural outcomes. In order to improve clarity and avoid confusion, this thesis deviates from the conventional thesis format to present together the methods and findings for each individual experiment after a description of the general methods as applied in all experiments.

3.1 General Methodology

3.1.1 Ethical clearance

For all protocols, wild type *Danio rerio* (zebrafish) eggs, obtained from the Zebrafish Research Unit at Stellenbosch University, Tygerberg campus, Clinical Pharmacology, were used. Prospective studies were performed, but in order to reduce the animal numbers used, existing behavioural data from previous studies with a different focus were used where possible. All data were generated in accordance with South African National Standards (SANS) guidelines for animal use. For research on zebrafish larvae aged between five- and ten-days post fertilisation (dpf), ethical clearance was obtained from the Animal Research Ethics Committee: Animal Care and Use of Stellenbosch University (protocol # ACU-2021-21677; Appendix A). For experiments employing larvae used before 5 dpf, ethics exemption was obtained from the same ethics committee (exemption protocol # ACU-2019-11820; Appendix B).

3.1.2 Zebrafish husbandry

The adult zebrafish in the breeding stock were maintained at 28°C with a light/dark cycle of 14:10. They were housed in 60 litre tanks at a density of less than one fish per litre, with appropriate filter and heating systems to maintain a consistent environment. The temperature and pH were monitored twice daily. Temperature was maintained at between 27-30°C and pH was maintained in the range pH 6.8-7.50. The tanks were cleaned regularly to reduce waste and algae, and the water level was replaced at a rate of at least 10% per week. Tank water had a minimum depth of 25 cm.

3.1.3 Maintenance of eggs and larvae

Eggs were maintained in Petri dishes covered with 8 to 10 mm embryo medium (E3) at a maximum density of 50 eggs per Petri dish. The Petri dishes were kept in an incubator at 28.5°C with a light/dark rhythm of 14:10. The eggs and larvae were refreshed with E3 once a day. This involved removal of the unfertilised eggs (recognisable by their white discolouration) at 0 dpf and 1 dpf and removal of fragments of the chorion after hatching. If any larvae died, they were removed. After the removal of any dead biological material that could encourage the growth of fungus or paramecium, half of the media was replaced. The E3 50X Stock Solution was made using 1L water, 14.6g NaCl, 0.65g KCL, 1.77g $CaCl_2$, 1.87g $MgSO_4$ pH = 7.4. Methylene blue (0.05%) was added to reduce fungal growth. Zebrafish can be fed from 5 dpf, but doing so modulates their behaviour (Clift *et al.*, 2014). In addition, dietary change influences the composition of the zebrafish gut microbiome, and the gut microbiome is increasingly being recognised as a modulator of behaviour (Stephens *et al.*,

2016). External feeding also increases the risk of contamination of the culture medium and may introduce differences due to variability in food intake (Clift *et al.*, 2014). Therefore, the zebrafish were used before they needed to be fed to avoid this potential confounder. Before 8 dpf, zebrafish larvae are able to feed on their yolk sac and there is no negative effect on their subsequent survival and growth (Hernandez *et al.*, 2018). Therefore, the larvae used were not fed during the experiments.

3.1.4 Euthanasia

A South African Veterinary Council (SAVC) authorised para-veterinarian euthanised the larvae after the experiments through immersion exposure to 0.16 mg/mL Tricaine and subsequent freezing.

3.1.5 Light/dark transition test

Noldus DanioVision activity tracking equipment and EthoVision XT analysis software were used to measure distance (mm) moved over the course of the light/dark transition test. This provided an automated, observer-independent approach to measuring behaviour.

Each zebrafish larva was pipetted into its own well in a 96-well plate in a volume of 250 µL. Once placed in the observation chamber of the DanioVision system, larvae were maintained in a water bath kept at a constant temperature of 28.5°C. Lighting conditions were controlled using the on-board lighting in the observation chamber. At 100%, the illumination equated to ± 10 500 lux. Infrared light (with wavelength 960 nm) and built-in cameras enabled the tracking of activity under both light and dark conditions. The infrared illumination is referred to as darkness, as the 950 nm wavelength is above the threshold of 910 nm at which zebrafish are able to detect infrared illumination (Shcherbakov *et al.*, 2013). Unless stated otherwise, during light periods and conditions, the white light stimulus was used at 100% intensity.

Figure 3.1 represents the activity profile of a standard light/dark transition test. The first period of this test is an acclimatisation period under dark conditions. This is followed by one or more cycles consisting of a period of exposure to bright light (light period), followed by an instantaneous transition to darkness (dark period). Although the duration of each period varies across studies (MacPhail *et al.*, 2009; Padilla *et al.*, 2011; Sveinsdóttir *et al.*, 2022; Velki *et al.*, 2017), a 10-minute light period followed by a 10-minute dark period is common (Irons *et al.*, 2010; MacPhail *et al.*, 2009).

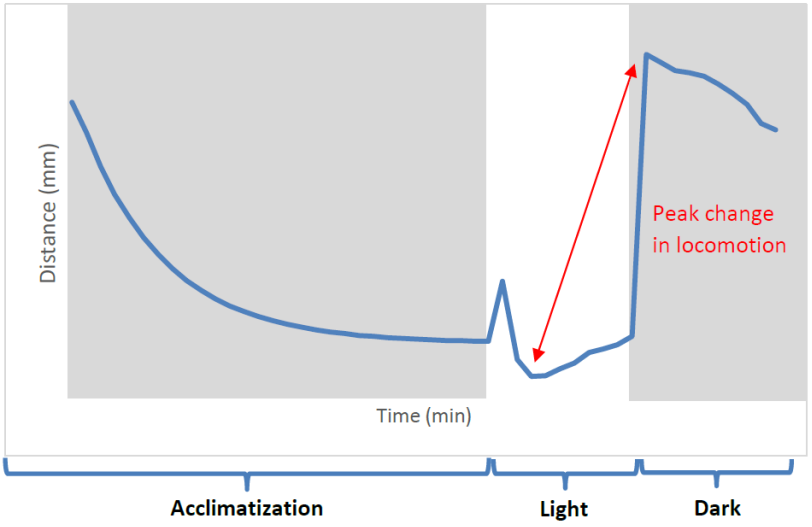


Figure 3.1. Activity profile of a standard light/dark transition test

3.1.6 Quality control and statistical analysis

For data processing, data acquired using the EthoVision XT software were exported and formatted in Microsoft Excel. Statistical analysis was executed in SPSS. Graphs were generated and formatted in Excel.

The first step of quality control was using the tracking visualisation in the EthoVision Software to check that tracking was successful. Where tracking was unsuccessful (i.e. where the camera failed to detect individual fish), the individual track data were excluded. Outliers in terms of total distance moved over the light/dark transition test as a whole were removed as a part of quality control. (These losses are reflected by different n values provided in the methods vs results sections for some experiments.)

After quality control, the assumption of normality of data distribution was tested using the Shapiro-Wilk test and the assumption of homogeneity of variance was tested using the Levene statistic. If neither of these assumptions were violated, a one-way ANOVA or a student t-test was performed, depending on the number of protocols compared. When a significant difference was found for an ANOVA, it was followed by Tukey's test (SPSS automatically changed this to a Tukey-Kramer test when unequal sample sizes were being used). When the assumptions of normality or homogeneity of variance were violated, the independent-samples Kruskal-Wallis test or the independent-samples Mann-Whitney U test were used instead. Where significant differences were found during the independent-samples Kruskal-Wallis test, this was followed up by pairwise comparisons of the groups. Bonferroni correction was used to correct for multiple comparisons.

The main parameters measured were extent of hyperlocomotion (total distance moved) during the dark period(s) following immediately after bright light exposure, as well as the peak change in locomotion (as indicated in Fig. 3.1) from the light to the dark period. The total distance moved

represented the total distance moved during the entire dark period. The dark period for each experiment is specified. Prospective experiments used a 10 minute dark period, but the retrospective experiments and the experiment using younger larvae used a shorter (3 min) dark period. The peak change in locomotion per larva was the difference between the 1-minute time bin with the highest activity during the dark period and the 1-minute time bin with the lowest activity during the light period. Unless stated otherwise, only the first light/dark cycle of the first light/dark transition test that larvae were exposed to was analysed. The graphs represent means and standard errors of the means. A significance threshold of $p < 0.05$ was used.

3.2 Specific Experiments

All experimental protocols described below are permutations of the light/dark transition test. As the general methodology is described above, only the protocol relating to the specific light/dark transition test is described in each case.

3.2.1 Experiment 1: Effect of age

Two experiments were conducted to investigate the effect of larval age on general activity levels, as well as responsiveness in the light/dark transition test (LDTT).

3.2.1.1 Protocols used

Age comparison between 2 dpf, 3 dpf and 4 dpf: Data were gathered from a retrospective experiment that had measured the activity levels of larvae over the light/dark transition test at 2 dpf, 3 dpf and 4 dpf. Larvae at 2 dpf were dechorionated if they had yet to hatch. The trial design for this experiment consisted of a 10-minute acclimatisation period in darkness, followed by one minute of 100% light intensity and three minutes of complete darkness. This deviation from the usual 10-min intervals was chosen due to the early age of the larvae. The short light period would prevent detrimental exhaustion, especially the youngest larvae. The 84 larvae tested were as follows: at 2 dpf (n = 30), 3 dpf (n = 24) and 4 dpf (n = 30).

Age comparison of 4 dpf vs. 5 dpf: Data were gathered from a retrospective experiment that measured activity of larvae at 4 dpf and 5 dpf over the light/dark transition test. The trial design for this experiment consisted of a 20-minute acclimatisation period in darkness, followed by one minute of light at 100% intensity and three minutes of complete darkness. The sample size for each age group was 12. The variability in activity decreases with age, making a smaller sample size appropriate for older larvae (Farrell *et al.*, 2011).

3.2.1.2 Results: Age has a significant effect on movement capacity during early development

Qualitatively (Fig. 3.2A), larvae at 2 dpf exhibited very low activity. At 3 dpf, larvae had considerably higher activity, but showed a delayed and reduced hyperlocomotion response to a light/dark

transition. Larvae at 4 showed a more substantial response to the light/dark transition when compared to both earlier ages.

Quantitative data analysis confirms these observations. There was not a statistically significant difference between the distance between the distance moved during the one-minute light period at different ages (Fig 3.2 B). When considering activity during the dark period (minutes 11-14), there was a statistically significant difference between the activity during the dark period at different ages (ANOVA main effect of age, $p < 0.001$). The average distance moved during the dark period at 2 dpf was significantly lower than that at 3 dpf and at 4 dpf ($p < 0.001$) (Fig. 3.2C). There was a significant difference in the peak change in locomotion in response to the light/dark transition test for larvae of different ages (ANOVA main effect of age, $p < 0.001$). The peak change in locomotion at 2 dpf was significantly lower than the peak change in locomotion at 3 dpf ($p < 0.001$) or at 4 dpf ($p < 0.001$). There was no significant difference between the response at 3 dpf and the response at 4 dpf (Fig. 3.2D).

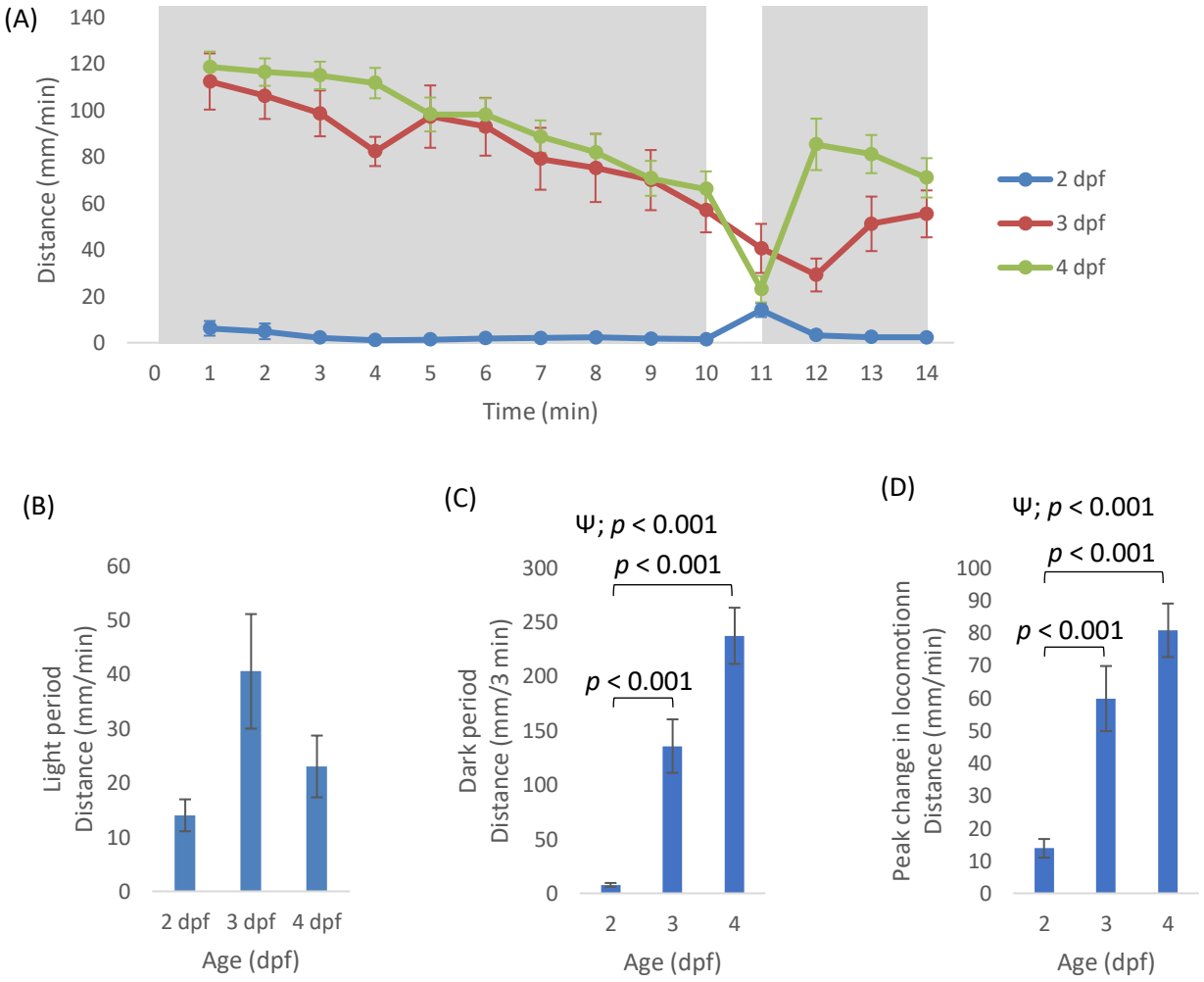


Figure 3.2. Effect of age on general activity and responsiveness in the light/dark transition test

(A) Line graph showing the activity of larvae of different ages (2 dpf, 3 dpf and 4 dpf; n = 24, 22 and 29, respectively)

Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs indicate activity of larvae of different ages (2 dpf, 3 dpf and 4 dpf) during **(B)** the light period and **(C)** during the dark period in the light/dark transition test, while **(D)** depicts the peak change in locomotion during the light/dark transition test for larvae of different ages (2 dpf, 3 dpf and 4 dpf; n = 24, 22 and 29, respectively). Statistical analysis: Independent-Samples Kruskal-Wallis Test with Bonferroni correction for multiple tests. Data are presented as means and SEM. Ψ represents a main ANOVA effect.

When comparing relatively older larvae (Fig. 3.3), larvae at 5 dpf appeared to have higher activity levels during both the light period (p = 0.008) and the dark period (p = 0.041)of the light/dark transition test (Fig. 3.3A). The 5 dpf larvae showed higher activity during the light period due to an increase in activity during the latter part of the light period (Fig. 3.3B). They also showed a significantly higher hyperlocomotion response in terms of total distance moved during the dark period (Fig. 3.3C). On the other hand, there was not a statistically significant difference between the peak change in locomotion at the two different ages (Fig. 3.3D).

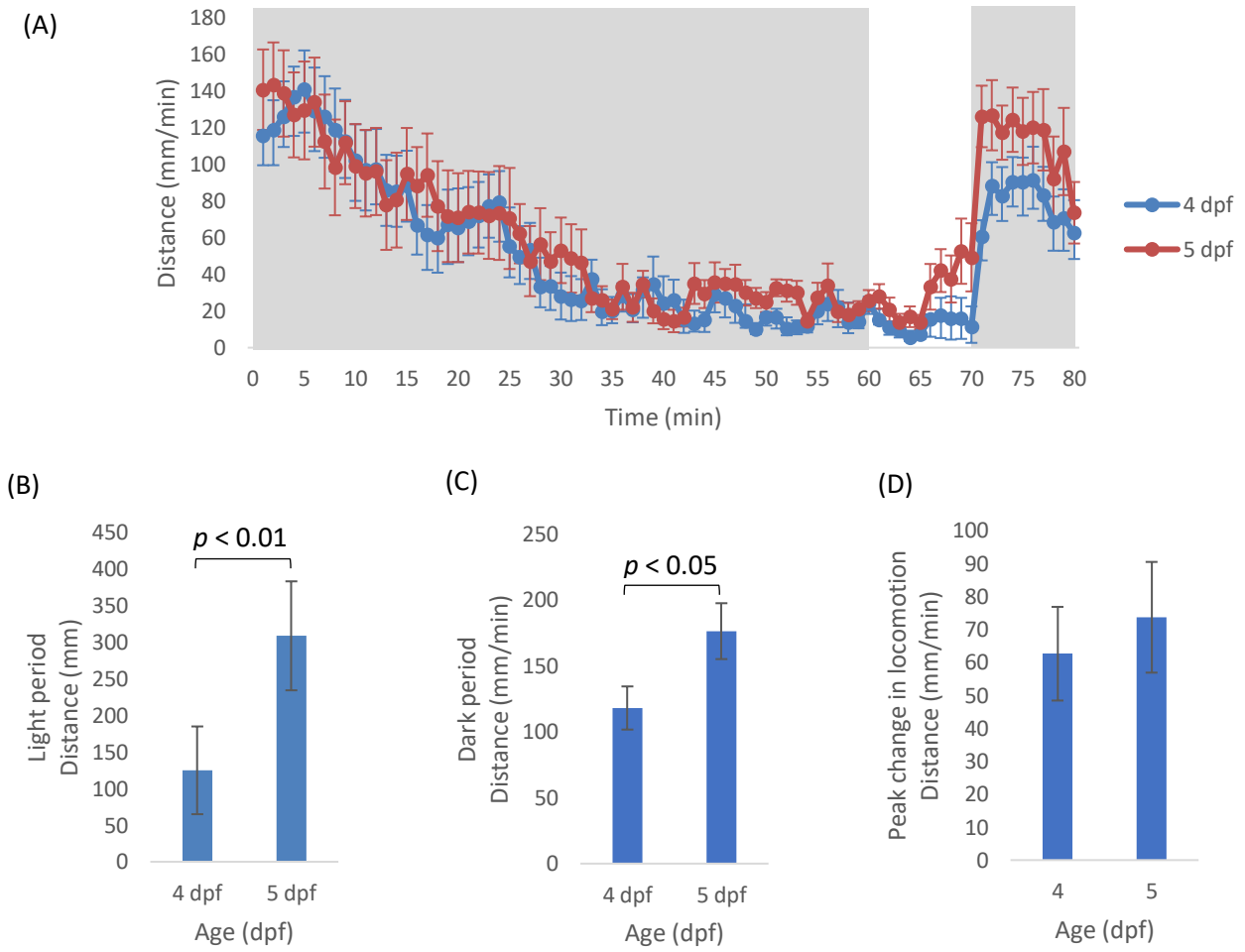


Figure 3.3. Comparison of 4dpf vs 5dpf zebrafish larvae in the LDTT

(A) Line graph showing the activity of larvae at different ages (4 dpf and 5 dpf; n = 12) over the course of the light/dark transition test. Data were acquired at a frame rate of 25 frames/sec, and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs show the activity of larvae during **(B)** the light period, **(C)** the dark period and **(D)** the peak response to bright light. Statistical analysis: Independent-Samples t-Test. Data are presented as means and SEM.

3.2.2 Experiment 2: Effect of lighting conditions during rearing

When pharmaceuticals whose labilities are increased by light exposure are investigated, it may necessitate the maintenance of larvae in the dark. However, disturbance in the natural diurnal light cycle has been associated with undesired outcomes (e.g. depression in humans) (Parry & Maurer, 2015; Walker *et al.*, 2020). It is therefore important to determine the significance of this potential confounder when assessing behavioural and neurocognitive parameters using zebrafish larvae.

3.2.2.1 Protocol used

The effect of darkness during early development was assessed by rearing zebrafish larvae either under standard light conditions or under consistent darkness until 4 dpf. Of the larvae, 12 were reared under standard conditions and 12 were wrapped in foil between refreshing to maintain them under dark conditions (the larvae were briefly exposed to light during the refreshing process). For the day prior to the light/dark transition test, all larvae were exposed to one day under a normal light/dark cycle (light period = 14 hours; dark period = 10 hours). The following day, they underwent a light/dark transition test. The trial design for this light/dark transition test consisted of a 10-minute acclimatisation period in darkness, followed by one minute of light at 100% intensity and three minutes of complete darkness.

3.2.2.2 Results: Lighting conditions during rearing did not show conclusive effects on development

Qualitatively, it appeared that larvae reared under standard lighting conditions had a larger response to the light/dark transition test than larvae reared under darkened conditions (Fig. 3.4A). In addition, at the start of the activity tracking, the darkness-reared larvae exhibited lower activity levels than those raised under standard lighting conditions. The effect sizes were large. Cohen's d was 0.825 for total distance moved and 0.836 for peak change in locomotion. Quantitatively however, these differences did not reach statistical significance (Fig. 3.4B, C).

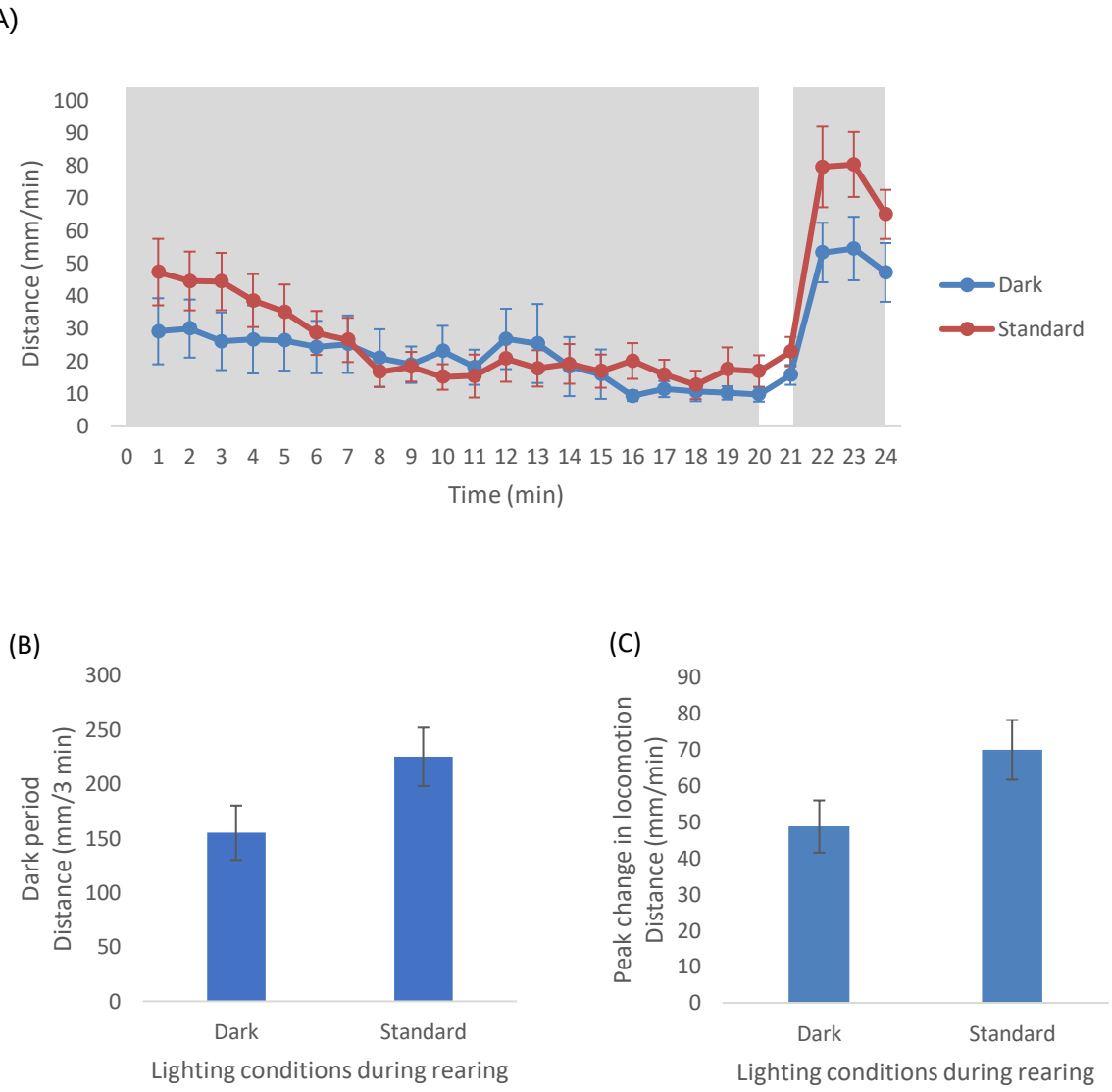


Figure 3.4. Effect of lighting conditions on LDTT outcomes

(A) Line graph showing the activity of larvae reared under different lighting conditions (darkness or normal lighting; n = 10 and n = 11) over the course of the light/dark transition test. Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs showing the activity of larvae during **(B)** the dark period and **(C)** the peak change in locomotion in the light/dark transition test. Statistical analysis: Independent-samples t-Test. Data are presented as means and SEM.

3.2.3 Experiment 3: Effect of capture order on activity levels

Before the light/dark transition test, the larvae must be pipetted from the Petri dish in which they have been reared to individual wells in a 96-well plate. If larvae that are captured first have a lower innate activity level, this could affect subsequent experiments, including the light/dark transition test. This experiment therefore investigated whether there was a difference in the light/dark transition response of the first larvae that were caught compared with that of the last larvae caught.

3.2.2.1 Protocol used

Larvae were assessed at <5 dpf (118 hpf). Rearing and husbandry took place until 4 dpf, using standard husbandry protocols. On the morning when the larvae reached 4 dpf, they were refreshed and placed in a single large Petri dish. At this point, 220 fish were placed into a single large volume container. Between 3:30 and 5:00, they were refreshed again and moved into 96-well plates. The larvae were left in the 96-well plates overnight to allow ample time for acclimatisation to their environment before the light/dark transition test was conducted. The next day, the performance of the first 20 larvae that were caught was compared with that of the last 20 larvae caught (the rest of the fish transferred were used in other experiments). The trial design for this experiment consisted of a 30-minute acclimatisation period in darkness, followed by 10 minutes of light at 100% intensity and 10 minutes of complete darkness. Our hypothesis was that the last larvae to be caught would be the strongest and most active larvae, therefore they would show a greater response to the light/dark transition tests.

3.2.2.2.Results: Capture order does not skew LDTT responsiveness

Both qualitatively and quantitatively, the first and the last larvae showed similar activity throughout the LDTT (Fig 3.5).

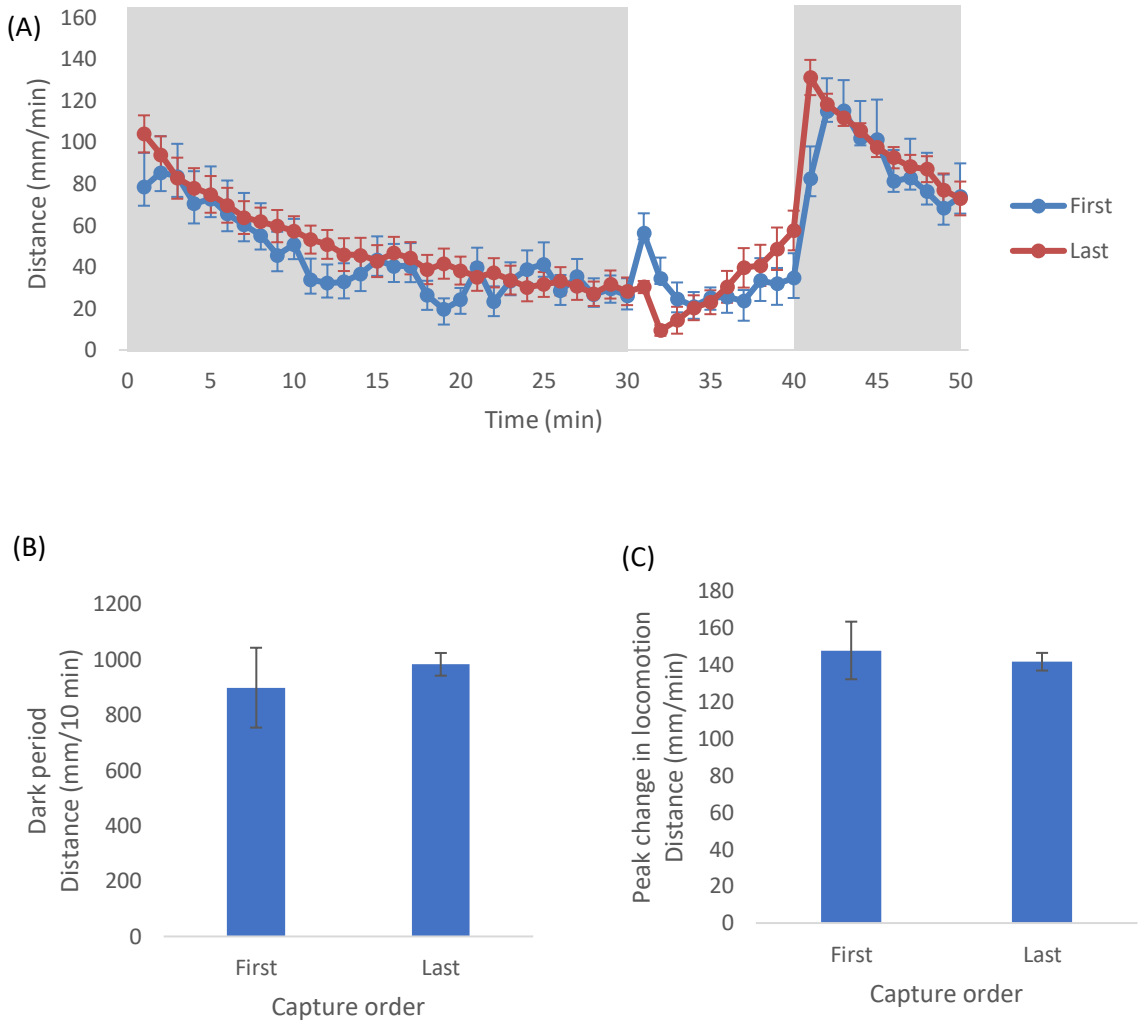


Figure 3.5. Effect of capture order on behavioural outcomes in the LDTT

(A) Line graph showing the activity of the first and the last larvae (n = 17 and n = 20) captured for transfer into 96-well plates, over the course of the light/dark transition test. Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs show the activity of the first and last larvae (n = 17 and n = 20) caught and placed in 96-well plates in the the dark period **(B),** while **(C)** shows peak change in locomotion. Statistical analysis: Independent-samples t-Test. Data are presented as means and SEM.

3.2.4 Experiment 4: Effect of time of day

3.2.4.1 Protocol used

Larvae at 5 dpf were subjected to a standard light/dark transition test starting at either 9:00, 10:00, 11:00, 12:00, 13:00, 14:00, 15:00, 16:00 or 17:00 (n = 44, 45, 40, 44, 45, 45, 44, 20 and 21, respectively) to cover the effect of being tested at different times throughout the workday. The larvae were placed in the 96-well plates for testing the previous day to allow for ample time for acclimation to the wells and recovery after handling before testing. The larvae were placed in the observation chamber five minutes before their trial was started. Each trial consisted of 30 minutes of acclimatisation in darkness, followed by 10 minutes of light at 100% intensity and 10 minutes of complete darkness.

3.2.4.2 Results: Time of day may be a confounder in the LDTT

Although the general activity pattern seemed similar at different times during the day (Fig. 3.6A), average activity levels varied visibly. Although no clear trend is seen over the course of a day, statistical analysis revealed an ANOVA main effect of time in both the distance moved during the dark period (p = 0.014) and the peak change in locomotion (p = 0.028). Post hoc analysis indicated that the hyperlocomotion response in the dark period (min 40-50) was significantly higher at 9:00 than at 10:00 (p = 0.032) (Fig. 3.6B), and peak change in locomotion was significantly higher at 9:00 than at 16:00 (p = 0.034) (Fig. 3.6C).

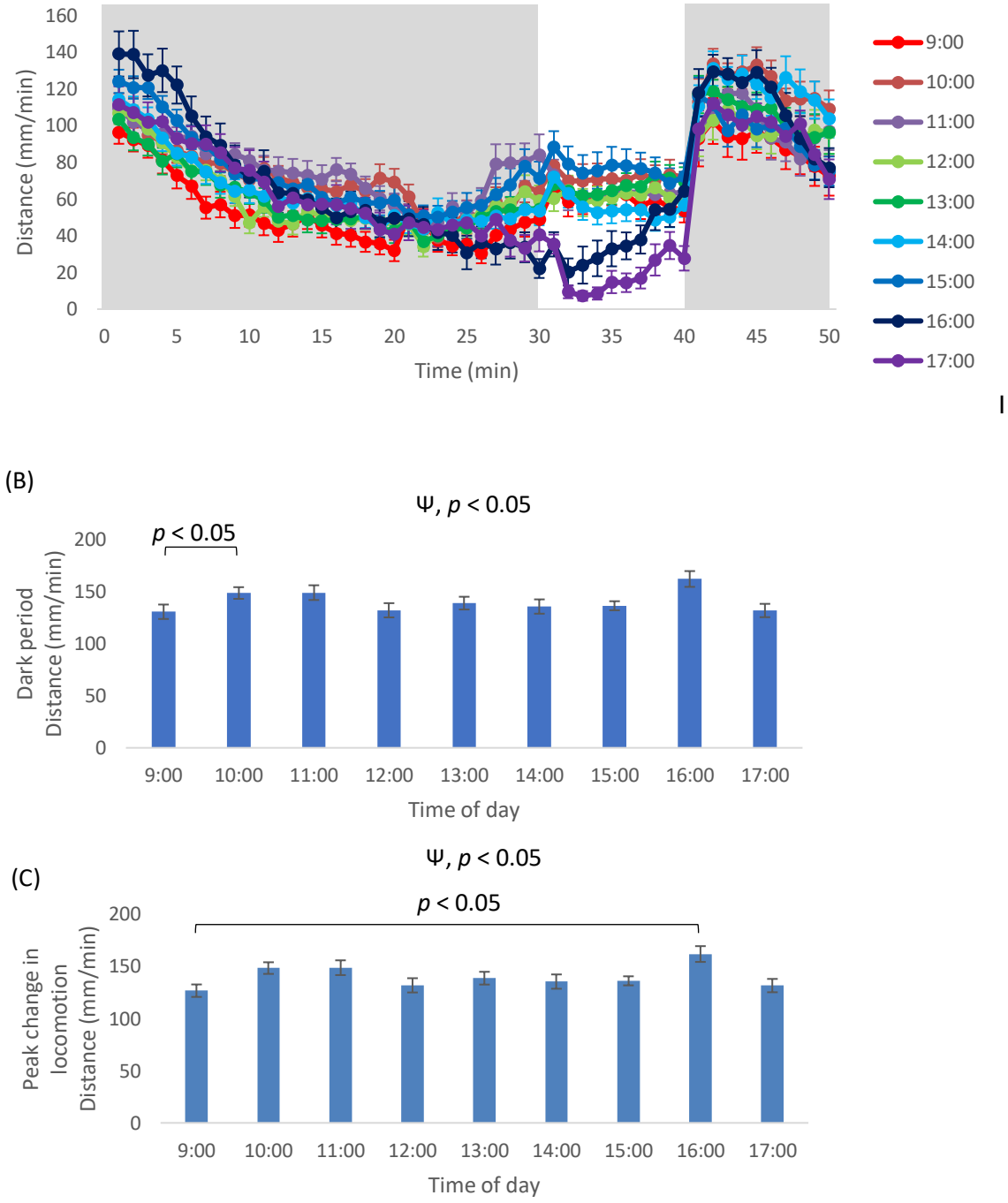


Figure 3.6 Time of day as potential confounder in the LDTT

(A) Line graph showing the activity of larvae at different times of day (9:00; 10:00; 11:00; 12:00; 13:00; 14:00; 15:00; 16:00; and 17:00, with n = 44, 45, 40, 44, 45, 45, 44, 20 and 21, respectively) over the course of the light/dark transition test. Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs show activity of larvae at different times of day (9:00; 10:00; 11:00; 12:00; 13:00; 14:00; 15:00; 16:00; and 17:00, with n = 44, 45, 40, 44, 45, 45, 44, 20 and 21, respectively). **(B)** Shows movement during the dark period and **(C)** the peak change in locomotion. Statistical analysis: Independent-samples Kruskal-Wallis test with Bonferroni correction for multiple tests. Data is presented as means and SEM. Ψ represents a main ANOVA effect of time.

3.2.5 Experiment 5: Effect of repeated cycles of light/dark within a single LDTT

3.2.5.1 Protocol used

Due to the expected small effect size for this measure, a relatively large number of larvae were employed in the assay. A total of 88 larvae were refreshed and transferred to a 96-well plate at 4 dpf. The larvae were left in the 96-well plate overnight to allow ample time for acclimatisation to their environment before the light/dark transition test. The next morning, the plate was placed in the observation chamber at 8:10. The trial was started at 8:20. The trial design for this experiment consisted of a 30-minute acclimatisation period in darkness, followed by 3 cycles, each consisting of 10 minutes of bright light exposure at 100% intensity followed by 10 minutes of complete darkness. The larvae underwent this light/dark transition test at 5 dpf.

3.2.5.2 Results: Repeated light/dark cycles do not affect response to bright light exposure

Qualitatively, there appeared to be a very slight decrease in the hyperlocomotion response in the dark period in subsequent cycles (Fig. 3.7A). However, statistical analysis revealed that repeated cycles did not have a statistically significant effect on activity during the dark period (Fig. 3.7B) or on the peak change in locomotion (Fig. 3.7C).

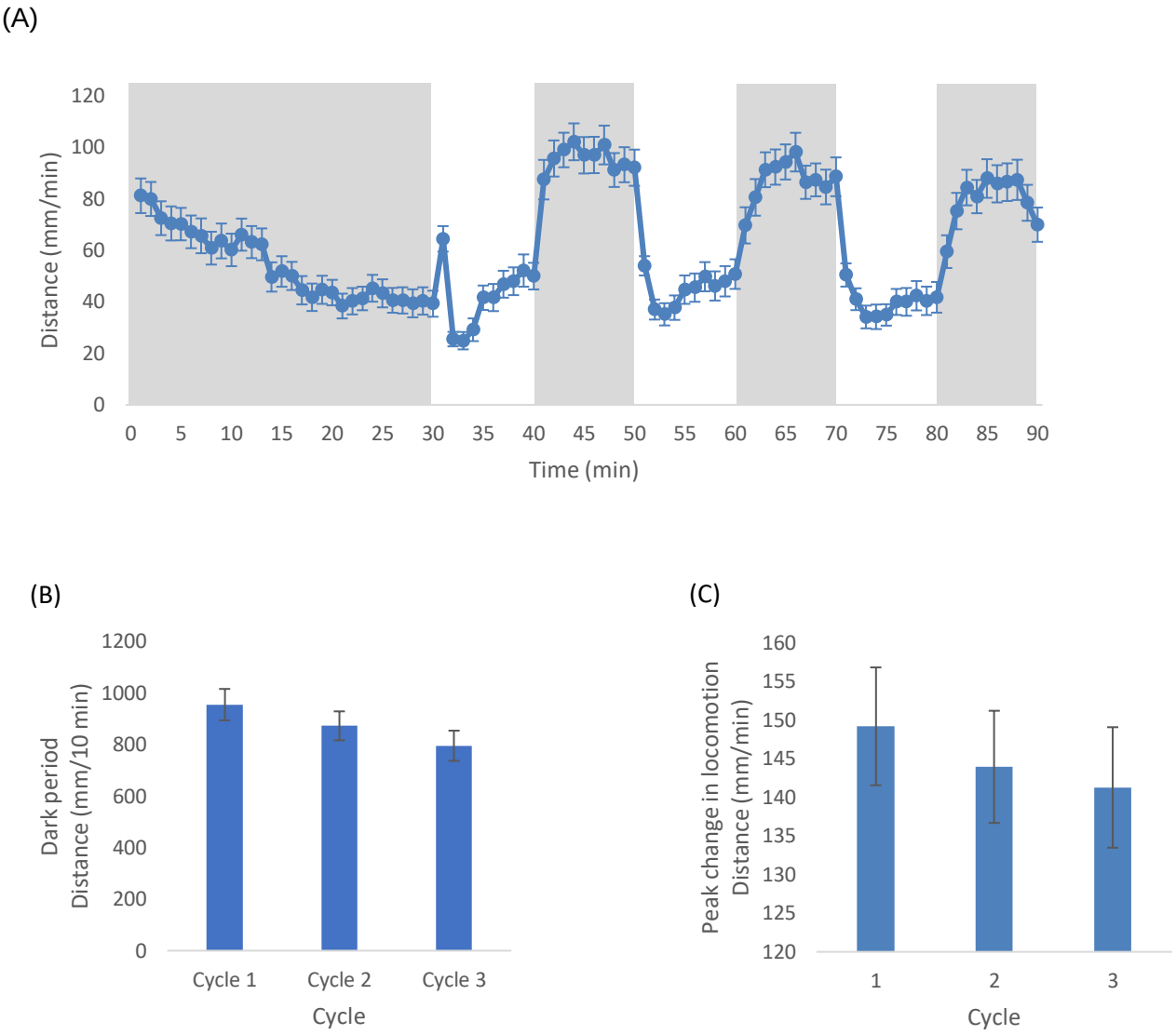


Figure 3.7. Effect of repeated light/dark cycles on responsiveness to bright light within a single LDTT
(A) Line graph showing the activity of larvae of different ages over three repeated cycles of light/dark transition during the light/dark transition test (n = 88). Bar graphs show the activity of larvae (B) during consecutive dark periods and (C) the peak change in locomotion after an increasing number of light/dark transition cycles. Data are presented as means and SEM.

3.2.6 Experiment 6: Effect of repeated light/dark transition tests

3.2.6.1 Protocol used

Larvae at 5 dpf were refreshed between 8:00 and 9:00 in the morning. The larvae were pipetted into the 96-well plate an hour before their EthoVision trial started. At each time point, 24 larvae (i.e. two rows on a 96-well plate) were added to the trial. A starting point of 9:00 was chosen to avoid handling before the normal lights-on period. The trial design for this experiment consisted of a 20-minute acclimatisation period in darkness, followed by 10 minutes of exposure to bright light (intensity at 100% capacity) followed by 10 minutes of complete darkness. The plates were stored in the incubator where they were maintained with the lights on between the repeated trials. The group that

was to undergo four exposures to a LDTT was exposed at 9:00, 11:00, 13:00 and 15:00. An additional 2 rows of larvae were added to the plate after each LDTT. This meant that the group that would undergo three exposures was exposed to a LDTT at 11:00, 13:00 and 15:00, while the group that would undergo two exposures was exposed at 13:00 and 15:00. Finally, the group that would undergo only one exposure started at 15:00. The trial run at 15:00 was analysed to investigate whether repeated exposures to LDTTs affected the larvae's responses to the LDTT.

3.2.6.2 Results: Repeated light/dark transition tests do not result in habituation to the LDTT

Despite their exposure to a different number of light/dark transition tests, the larvae seemed to exhibit similar hyperlocomotion throughout the final test (Fig. 3.8A). The number of light/dark transition test exposures did not have a statistically significant effect on their activity during the dark period (Fig. 3.8B) or on the peak change in locomotion during the light/dark transition (Fig. 3.8C).

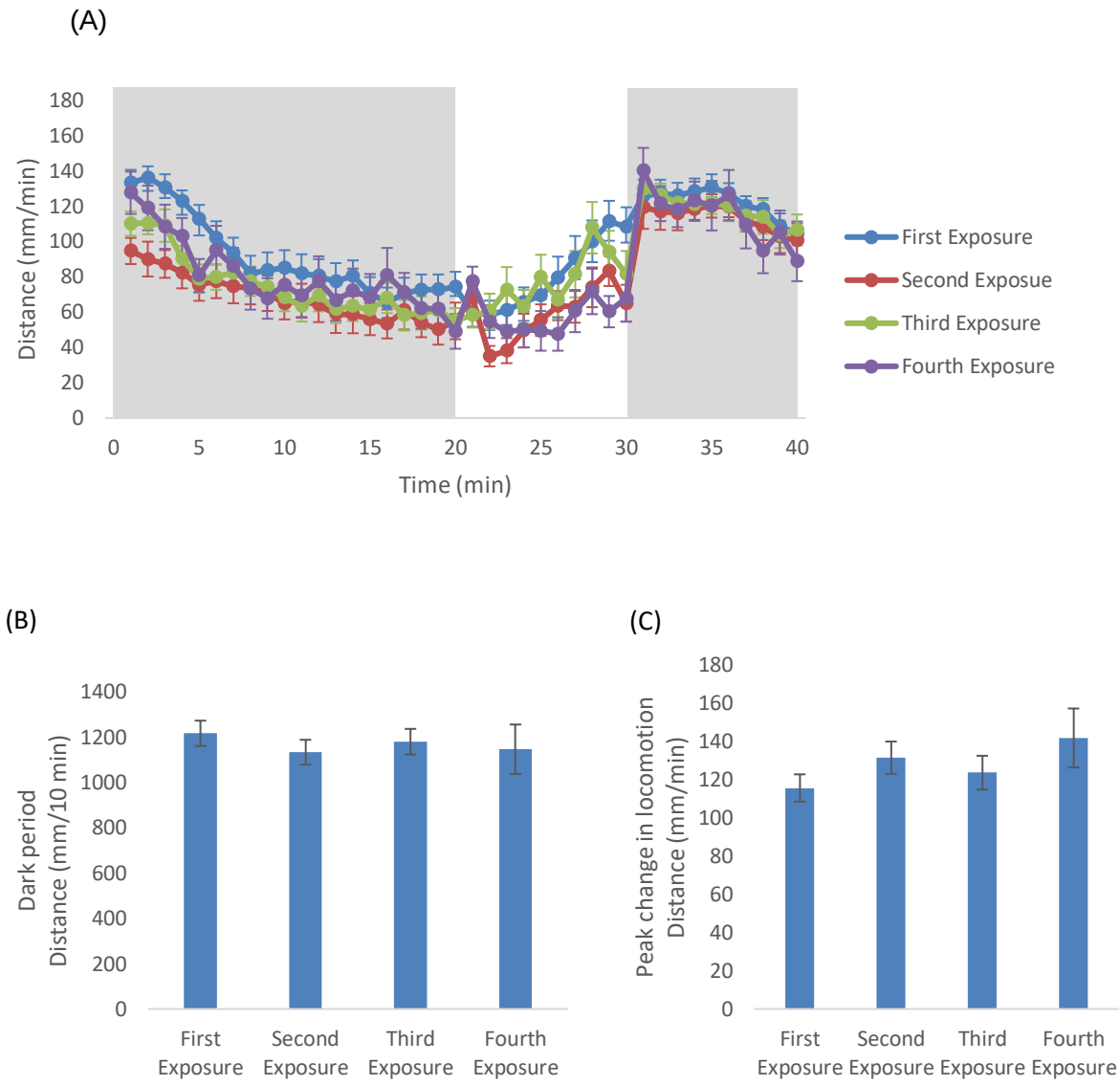


Figure 3.8. Repeated LDTT tests do not result in habituation

(A) Line graph showing activity after exposure to different numbers of light/dark transition tests (first exposure, second exposure, third exposure, fourth exposure; n = 23, 24, 23 and 24, respectively). Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs show the activity after different numbers of light/dark transition tests on activity during the dark period**(B)** and **(C)** the peak changes in locomotion Statistical analysis: Independent-samples Kruskal-Wallis test with Bonferroni correction for multiple tests. Data are presented as means and SEM.

3.2.7 Experiment 7: Effect of light period length on hyperlocomotion response as measured in the dark period

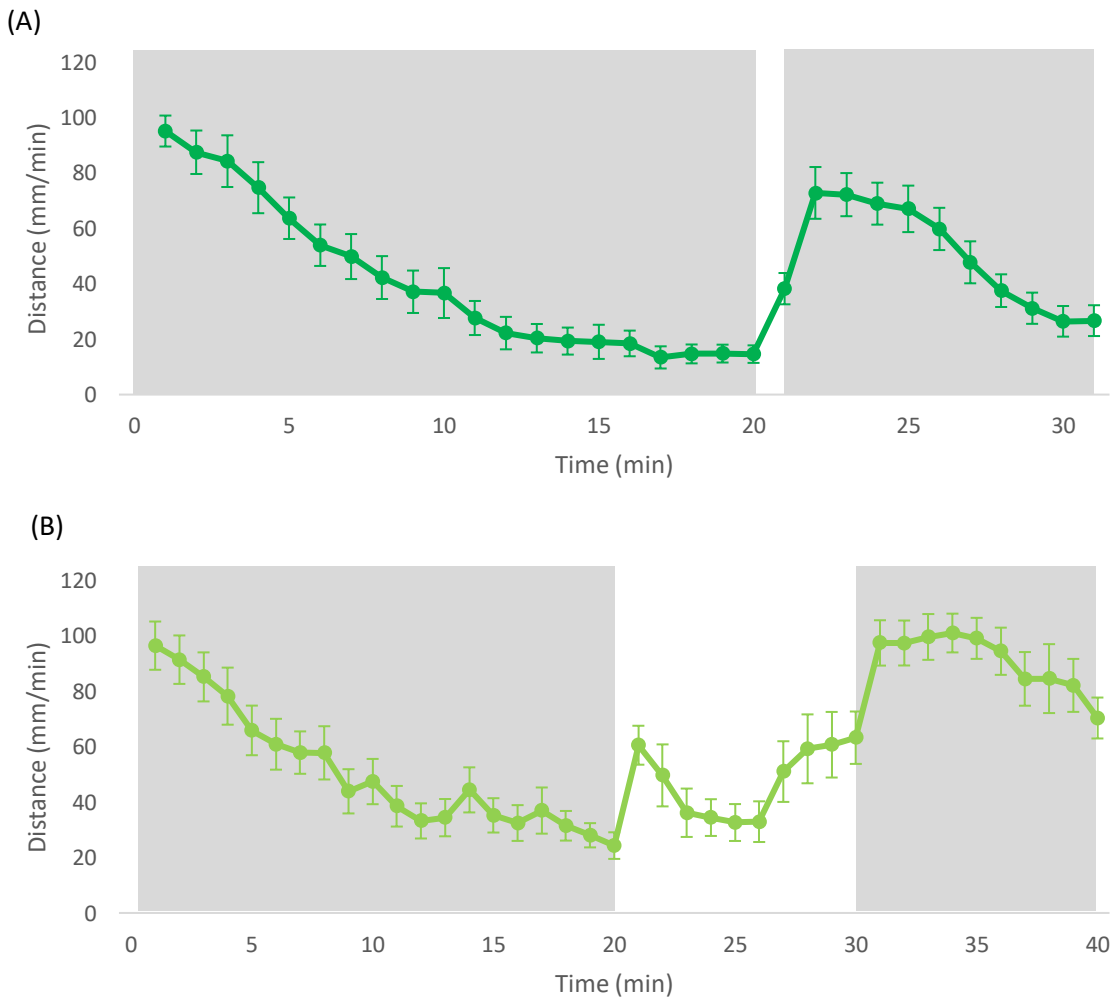
3.2.7.1 Protocols used

Larvae at 5 dpf were pipetted into the individual wells of the 96-well plate at least an hour before the start of the test after being refreshed in the morning. One group of larvae underwent a light/dark transition test with a 1-minute light period, and a second group of larvae underwent a light/dark

transition test with a 10-minute light period (n = 24 per group). The trial design for the group with a 1-minute light period consisted of a 20-minute acclimatisation period in darkness, followed by 1 minute of light intensity at 100% and 10 minutes of complete darkness. The trial design for the group with a 10-minute light period consisted of a 20-minute acclimatisation period in darkness, followed by 10 minutes of intensity at 100% and 10 minutes of complete darkness.

3.2.7.2 Results*:* The duration of the light period has a significant effect on response in the dark period

Light/dark transition elicits a hyperlocomotion response after both a 10 minute and 1 minute exposure to bright light (Fig. 3.9A,B). When overlaying the activity graphs to align with the start of the dark cycle, the magnitude of the hyperlocomotion response to a light/dark transition seemed more robust after the 10-minute light period than it was after the 1-minute light period (Fig. 3.9C). This was confirmed by statistical analysis of both total movement during the dark phase (Fig. 3.9D) and the peak response (Fig. 3.9E).



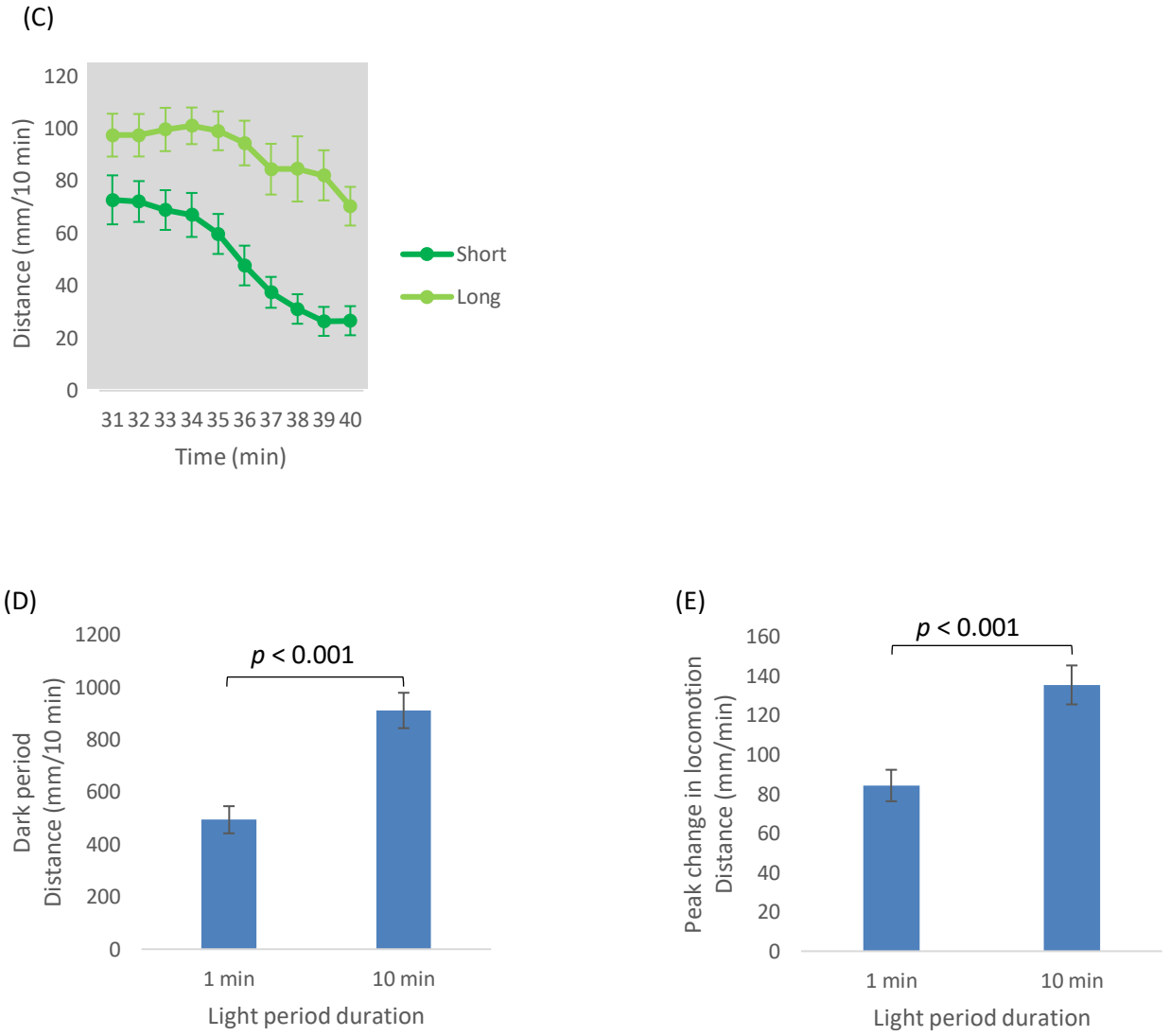


Figure 3.9. Effect of duration of bright light exposure on magnitude of subsequent hyperlocomotion response
Line graphs show the activity of larvae during different phases of a light/dark transition test with a one-minute light period **(A)** vs a ten-minute light period **(B)**, as well as an overlay of the hyperlocomotion in the dark phase **(C)** (n = 24 per group). Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs show the activity of larvae exposed to the different light period lengths during the dark period **(D)** and peak change in locomotion after different light period lengths **(E).** Statistical analysis: Independent-samples Mann-Whitney U test. Data are presented as means and SEM.

3.2.8 Experiment 8: Effect of light intensity of the bright light stimulus

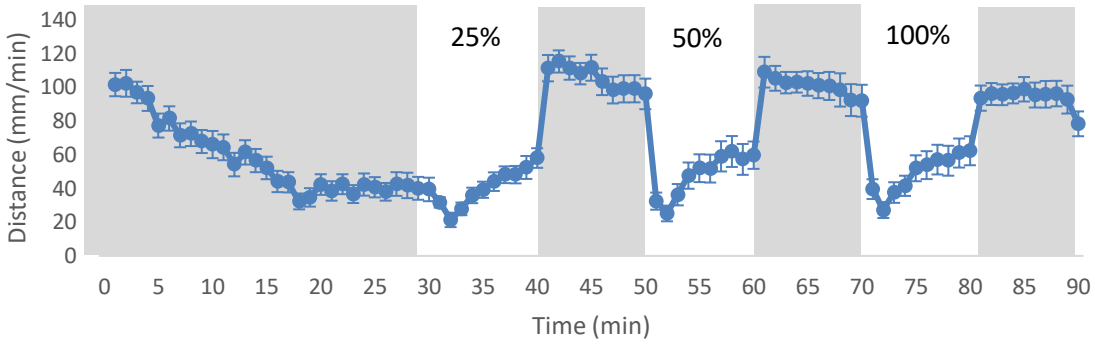
3.2.8.1 Protocols used

Two experiments were conducted to assess the significance of the magnitude of differences in light intensity between the bright light exposure and the darkness. In the first experiment, relatively large differences in light intensity were employed, while in the second, far more subtle differences between darkness and light were implemented. For both assays, larvae were tested at 5 dpf (n = 72 and 75, respectively). Standard rearing protocols were used for the larvae until an hour before testing. The larvae were refreshed on the morning of testing. They were pipetted into 96-well plates an hour prior to testing to give them time to acclimatise to their testing environment. The first trial was started at 10:00 and the second trial was started at 11:30. In order to avoid a confounding effect from the order in which the fish were exposed to light of different intensities, the results from a trial where larvae were exposed to light periods of increasing intensity and the results from a trial where larvae were exposed to light periods of decreasing intensity were combined for each experiment. Both trial designs consisted of a 30-minute acclimatisation period in darkness, followed by three cycles of 10 minutes of light followed by 10 minutes of complete darkness. For the experiment using large light intensity increments, the first trial employed increasing light intensities of 25%, 50% and 100% of system capacity for the consecutive light exposure cycles, while the second trial used decreasing light intensities at 100%, 50% and 25% of system capacity for the consecutive cycles. For the experiment with smaller increments, the same protocol was used, but consecutive light cycle intensities were set at 1%, 5% and 10% for the first trial, and at 10%, 5% and 1% for the second trial.

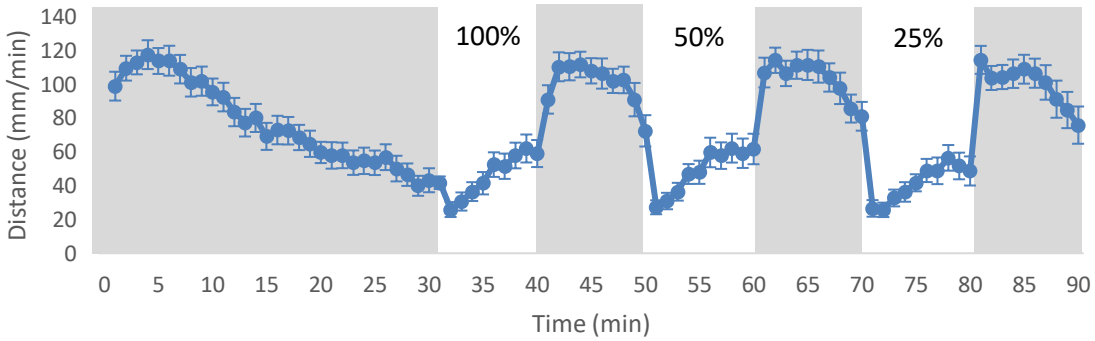
3.2.8.2 Results: Light to dark transition intensity did not have a significant effect on the subsequent hyperlocomotion response

Qualitatively, neither the magnitude of change in light intensity, nor the directionality of change during the light period caused any evident changes in the hyperlocomotion response after a light/dark transition (Fig. 3.10 A-D). Statistical analysis confirmed that neither total movement during the dark phase (Fig. 3.10 E,G) nor peak response (Fig. 3.10 F,H) were affected.

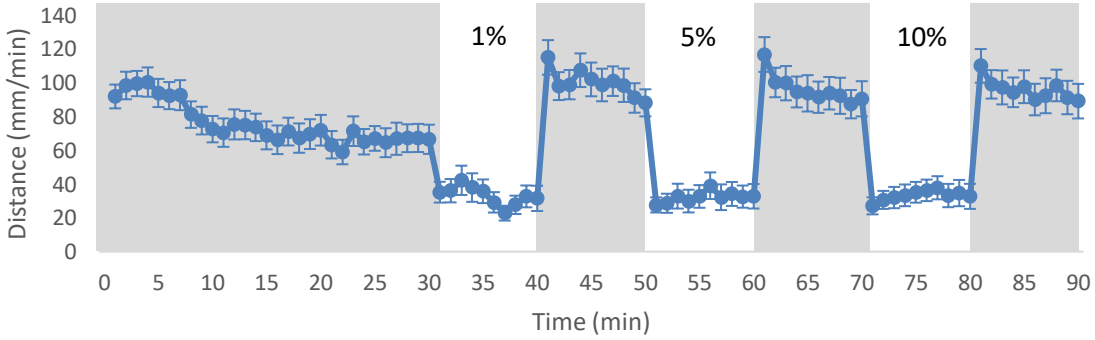
(A)



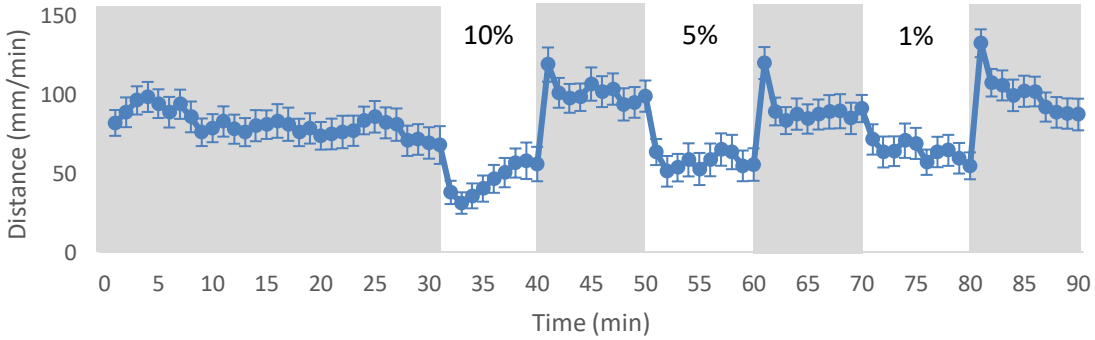
(B)



(C)



(D)



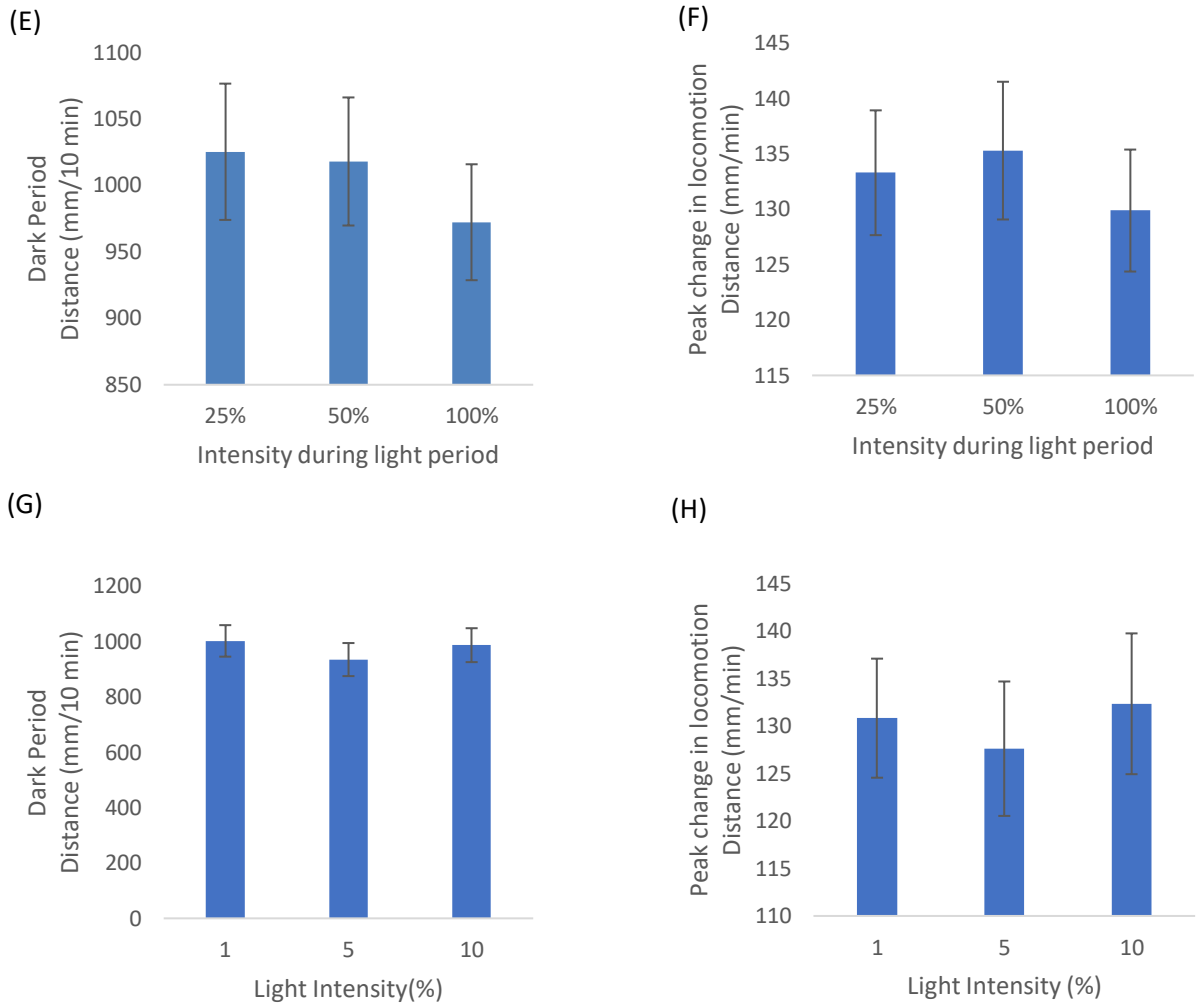


Figure 3.10. Bright light intensity did not affect the hyperlocomotion response in the subsequent dark phase Line graphs show activity levels in the LDTT with large incremental increases in white light intensity **(A),** large incremental decreases **(B),** small incremental increases **(C)** and small incremental decreases **(D).** (All groups n = 72). Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Quantitative data confirms no effect of either large or small incremental changes on hyperlocomotion during the dark phase **(E, G)** or on peak response **(F, H).**

3.2.9 Experiment 9: Effect of breeding stock differences

3.2.9.1 Protocol used

A standard LDTT protocol (30-minute acclimatisation period in darkness, followed by 10 minutes of light at 100% intensity and 10 minutes of complete darkness) was conducted at 5dpf on wild-type zebrafish larvae supplied by three different breeding stocks (A, B, and C; n = 75, n = 21 and n = 31), to evaluate the extent to which different genetic background may affect responsiveness in the LDTT.

3.2.9.2 Results: Genetic differences may be a confounder in the LDTT

When comparing normal activity between larvae produced by three genetically different lots of breeding pairs in the facility (Fig 3.11A), the hyperlocomotion response to the LDTT was greater in breeding stock B and C than in A. These observations were confirmed statistically for both the hyperlocomotion response and the peak response in the dark phase (Fig 3.11B, C). The total distance moved by stock A was lower than in stock B ($p = 0.002$) and stock C ($p = 0.006$). The peak change in locomotion is also lower in stock A than in stock B ($p = 0.011$) and stock C ($p < 0.001$). In addition, the peak change in locomotion was higher in stock C than in stock B.

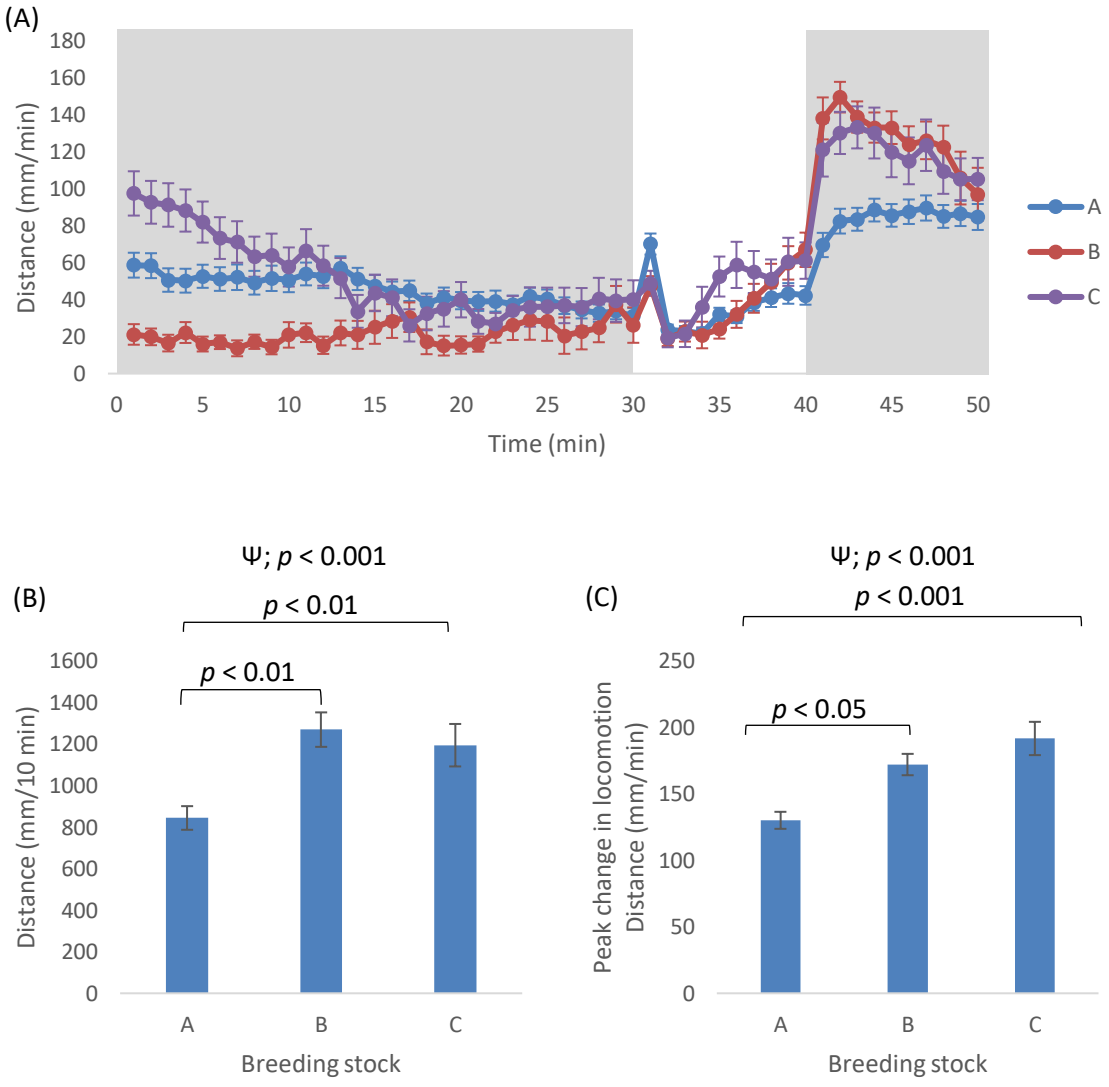


Figure 3.11. Genetic differences may be a confounder in the LDTT

(A) Line graph showing the activity of larvae from different breeding stocks (A, B, C) over the course of the light/dark transition test (n = 75, 21, 31 respectively). Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data are presented as means and SEM. Bar graphs show **(B)** hyperlocomotion response and **(C)** the peak change in locomotion during the light/dark transition test. Statistical analysis: Independent-samples Kruskal-Wallis test. Data were acquired at a frame rate of 25 frames/sec and binned into 1-minute time bins. Data is presented as means and SEM.

Chapter 4: Discussion

The main aim of this thesis was to investigate the effect of different protocol variations on the light/dark transition test in order to identify which variables must be controlled and accounted for to allow for comparison across studies. Identifying the effects of protocol variations also indicates which variables need to be standardised and which can vary according to logistics without significantly impacting the outcome of this widely-used test. The effects of age, lighting conditions during rearing, capture order, repeated light/dark transition cycles, repeated light/dark transition tests, duration of the light period, light intensity and breeding stock differences were all investigated.

Several of the factors investigated had a significant effect on the response to the light/dark transition test. These were age, time of day, light period duration and breeding stock. This means that these factors need to be controlled and accounted for if groups are to be compared. On the other hand, variations in lighting conditions during rearing, capture order, repeated cycles, repeated light/dark transition tests and light to dark transition intensity did not have significant effects on the total distance moved during the dark period. This has many practical implications that will simplify protocols and allow the number of larvae used to answer research questions to be reduced. In the next few sections, the data for each of these protocol parameter variations is contextualised with the relevant literature. For clarity, the topics for discussion have been organised in the order of experiments conducted.

Neither the acclimation period nor the light period are usually analysed during light/dark transition tests, therefore, in most experiments, the contextualisation has been limited to the dark period and the peak change in locomotion. Although light period activity was revisited to allow for comparison with Padilla *et.al* (2011), qualitatively, there were no consistent patterns in the light period for most conditions investigated in this study, so light period activity was only revisited for the age experiment. Although the peak change in locomotion is not one of the main parameters analysed in the light/dark transition test, this parameter has been included in evaluating the hyperlocomotion response to the light/dark transition test. It is recommended that the validity of the analysis of the light period be further investigated.

In the next few sections, the most important findings of this thesis are discussed in more detail.

4.1 Age has a critical threshold prior to which quantification of locomotion is not feasible

Before larvae are able to respond to the light/dark transition test, there needs to be sufficient development of the visual system, nervous system and musculoskeletal system to enable movement in response to stimuli. The first movement that zebrafish show is spontaneous coiling in the egg.

From 27 hpf, embryos from dechorionated eggs may be able to swim briefly in response to tactile stimulation (Drapeau *et al.,* 2002; Legradi *et al.,* 2015; Saint-Amant & Drapeau, 1998). Similarly, newly hatched larvae (2 dpf and 3 dpf) show spontaneous burst swimming as well as burst swimming in response to tactile stimulation (Brustein *et al.,* 2003; Drapeau *et al.,* 2002; Legradi *et al*., 2015; Saint-Amant & Drapeau, 1998). The burst swimming—as opposed to constant movement—suggests limited mobility in the larvae at this early stage of development. This interpretation is in line with current data, where the newly hatched and dechorionated larvae at 2 dpf showed extremely low activity levels during all stages of the light/dark transition test.

In terms of probable capability to respond to the light stimulus of the LDTT, zebrafish larvae exhibit evidence of sight very early. Already at 2 dpf, distinctions between light and dark changes over time are possible (Easter & Nicola, 1997). This means that they are able to detect changes in lighting conditions. However, current data indicate that larvae at 2 dpf show only a small increase in activity—a potential startle response—in the first minute after a transition from darkness to bright light, and do not show a hyperlocomotion response to a light/dark transition. At that stage many systems are still undergoing rapid development: these can affect perception, processing and the mechanical ability to respond.

The larvae at 2 dpf show extremely low activity during the acclimation period, a brief increase in activity after the dark/light transition and no hyperlocomotion response to the light/dark transition. Although distinctions between light and dark changes over time are possible, the ability to process light changes may not be optimally functional yet. In terms of potential neural mechanisms controlling movement, Mauthner neurons are known to control startle responses to noxious stimuli (Maximino *et al.*, 2010), and startle response to touch has been demonstrated to be present by 2 dpf (Drapeau et al., 2002; Legradi et al., 2015; Saint-Amant & Drapeau, 1998). The increase in activity after the dark/light transition may thus be due to a startle response. However, the hyperlocomotion resulting from startle only last a few seconds, while the hyperlocomotion seen in the dark phase is a more sustained increase in activity. The hyperlocomotion response is controlled at least in part by deep brain photoreceptors (Fernandes *et al*., 2012). Different neurons and receptors may be involved in the startle and hyperlocomotion responses, which may explain the presence of the one and not the other. The presence of a startle response after a dark/light transition but not a light/dark transition may be due to differences in detection. Further investigation would be of interest. It should be noted that the larvae at 2 dpf had been dechorionated, and therefore some differences between dechorionated larvae and larvae that have hatched may be present.

Activity levels (both at baseline and during all parts of the light/dark transition test) are expected to increase with age (Colwill & Creton, 2011; Padilla *et al.*, 2011; Thirumalai & Cline, 2008), as indeed can be seen in current data. Brief ‘beat and glide’ swimming first emerges in 3 dpf larvae, while 4 dpf and 5 dpf larvae show sustained ‘beat and glide’ swimming (Brustein *et al*., 2003; Buss &

Drapeau, 2001). Despite this, 3 dpf and 4 dpf larvae in this experiment showed similar levels of activity during the initial acclimation period. This similar level of activity could be explained by the larvae being inadvertently exposed to a light/dark transition test when they are transferred from the incubator to the activity chamber. In addition, it is very challenging to avoid movement of the media during the transfer process. Therefore, it is likely that what is being observed in the acclimation period represents the latter part of a light/dark transition test and recovery after media disturbance. This shows the importance of the acclimation period when baseline activity levels are being measured, as well as the recording of the timing between transfer to the DanioBox and the start of recording. If the exact timing of transfer to the observation chamber relative to the start of recording is noted, this phase could potentially serve as a valuable source of additional information.

The hyperlocomotion response to the LDTT appeared at 3 dpf. At 4 dpf, the larvae showed a qualitatively larger hyperlocomotion response than larvae at 3 dpf, however this did not reach statistical significance. It was clear, however, that a light/dark transition test response could be measured from 3 dpf.

At 4 dpf, zebrafish larvae show a robust response to light and dark conditions (Basnet *et al*., 2019), and at 5 dpf, the zebrafish visual system is considered fully functional (Legradi *et al.,* 2015); therefore, a strong response to the light/dark transition test was expected in the second experiment where the light/dark transition test response of larvae at 4 dpf and 5 dpf was compared. Unlike Padilla *et al.* (2011), we found a significant effect of age on the activity during the dark period. However, Padilla *et al.* (2011) found a significant age x time interaction. They used a 22-minute dark period, while we used a 10-minute dark period. It is possible that the significance of the differences in activity during the dark period were lost in Padilla ***et al.'s*** (2011) study due to the longer dark period used (Padilla *et al.*, 2011). This shows the importance of the choice of the length of the dark period if the study is to be sensitive to differences at the beginning of the dark period.

In our study, the peak change in locomotion was not significantly different between larvae at 4 dpf and 5 dpf. This may be due to differences in their recovery from the sudden dark/light transition during the light period. Initially, the light period was not analysed as it is not usually one of the parameters considered during the light/dark transition test. However, it was noted that the activity during the light/dark transition test was qualitatively similar to the responses previously observed at different ages (Padilla *et al.*, 2011). Statistical analysis confirmed that, as in Padilla *et al.* (2011), larvae at 5 dpf had significantly higher activity than the 4 dpf larvae. Padilla *et al.* (2011) speculated that this may be due to OFF-retinal ganglion cells being more developed than ON-retinal ganglion cells, which would result in a response to a light/dark transition before a response to a dark/light transition (Padilla *et al.*, 2011). In our opinion, the reasons may be more complicated. The Mauthner neurons are known to be important for startle responses after transitions in lighting conditions, however their activity does not explain the differences between the response during the light period

at 4 dpf and 5 dpf because the difference is caused by a gradual increase in activity in the 5 dpf larvae that resembles recovery from fear. It is likely that both stress processing and age are involved in the rate of recovery after a dark/light transition, and this is clearly affected by developmental changes that occur between 4 dpf and 5 dpf. This was revisited in our study, but it was not the original research question. Future research should investigate the role of anxiety-like behaviour on activity during the light period. This is a promising additional parameter to consider when screening for anxiety-modulating treatments.

Age had a significant effect on both activity and peak change in locomotion during the light/dark transition test, and therefore affected the hyperlocomotion response to the light/dark transition test. Larvae at 2 dpf did not show a large enough response to the light/dark transition test to be used for this assay. The hyperlocomotion response is present from 3 dpf, therefore it is possible for larvae to be used for the light/dark transition test from this age onwards. The response is, however, more variable. Moreover, with the complete lack of response to the light/dark transition at 2 dpf, and the potential for slower development when temperature is lower, it is recommended that using larvae at 3 dpf for the light/dark transition test should be avoided unless it is absolutely necessary to address a specific research question. The response to the light/dark transition continued to increase with age, and the larvae at 5 dpf had a larger hyperlocomotion response than the larvae at 4 dpf. Larvae at 4 dpf and at 5 dpf are both suitable for use in the light/dark transition test. This means that the light/dark transition test can be performed with ethics exemption, rather than requiring a full ethics application. Age must, however, be considered when comparing results across studies. Due to the large age-related differences during the light period, the use of larvae at 4 dpf will need to be revisited when activity during the light period is used as an important parameter.

Although from an ethical perspective, it is better to use younger larvae, there are many scientific benefits to using slightly older larvae. Firstly, older larvae show less variation in locomotor activity than younger larvae (4 dpf versus 7 dpf) (Ingebretson & Masino, 2013). Moreover, behaviour during the dark period of a light/dark transition test has particularly low intra-individual variability at 6 dpf and 7 dpf. This emergence of larvae-specific behaviour could be beneficial for pre- post-test designs (Fitzgerald *et al.*, 2019). In addition, older larvae have better-developed central nervous systems. The same factors that reduce the ethical concerns in younger larvae make them less suitable for modelling certain neuropsychiatric conditions. However, as shown in zebrafish research on the innate immune system, developmental stages can be utilised to investigate isolated systems and mechanisms. This means that it is very important to know how the age of the larvae influences their response to the light to dark transition test. The best age at which to perform the light/dark transition test will vary, depending on the specific application.

Two retrospective experiments were used to compare larvae from different age groups to reduce the number of larvae used and to ensure that maximum value was extracted. This meant that larvae at

3 dpf, 4 dpf and 5 dpf were not compared in the same experiment. While this may be considered a limitation, our findings were in line with existing literature. Nonetheless, future research can compare larvae at 3 dpf, 4 dpf and 5 dpf at the same time. This was a logistically challenging experiment to perform due to the size of the Zebrafish Research Unit at the time of this thesis, and has thus been left to future studies.

It should also be noted that specific treatments may have different effects on the light/dark transition test at different ages due to changes in the pharmacokinetics (absorption, distribution, metabolism and excretion) at different ages (Kristofco *et al.*, 2016; Souder & Gorelick, 2017). In addition, until the systems involved in the mechanism of action are sufficiently developed, potential therapeutic effects could be missed. Therefore, despite the ethical and cost benefits of using younger larvae, screening in younger larvae increases the chance of type II error. These factors should be considered when choosing which age of larvae to use when performing light/dark transition tests. Nonetheless, the cost, time and throughput benefits of screening using younger models (<5 dpf) mean that more compounds can be screened, providing an overall benefit in the number of potential treatments identified.

This study expands the resources available for investigating responses to the light/dark transition test by including younger ages than Padilla *et al.* (2011) did. In addition, the nuanced differences during the different phases show how important it is to investigate the effect of the test of interest at different ages.

4.2 Effect of lighting conditions during rearing inconclusive

When screening light-sensitive compounds, it may be necessary to rear larvae under dark conditions. However, lighting conditions during rearing have been shown to affect the visual behaviour of zebrafish larvae even in the absence of gross morphological changes (Saszik & Bilotta, 1999). In this study, we investigated the effect of rearing in the dark on response to the light/dark transition test, which to the best of our knowledge, has not been specifically investigated before. Qualitatively, there was a large difference between the hyperlocomotion response distance of larvae reared under different lighting conditions. Larvae that were raised under continuous darkness showed delayed development (Bilotta, 2000). This could explain the qualitatively lower hyperlocomotion response identified in this study, as we found that the hyperlocomotion response increased with age. A lack of significance for the total distance moved during the dark period was probably due to the small sample size (n = 10 dark, n = 11 light) (the data were very variable), as the effect size was large. In future, this experiment should be repeated with a larger sample size. The small sample size was used for ethical reasons due to the reduced survival observed when larvae are reared under constant darkness (Villamizar *et al.*, 2014). Although Villamizar *et al*, (2014) had shown that larvae transferred from constant darkness to normal lighting conditions at 5 dpf had recovered by 30 dpf (we transferred the larvae to normal lighting conditions at 4 dpf), caution was used, as this was the first experiment in this laboratory that reared larvae under conditions of

constant darkness, so small group sizes were preferred. In addition, larvae were exposed to 24 hours of normal lighting conditions prior to the light/dark transition test, which may have allowed the larvae reared under dark conditions some level of recovery. Therefore, despite the lack of a significant difference in this experiment, caution is advised when rearing larvae under dark conditions, particularly if the larvae are not exposed to standard lighting conditions for 24 hours prior to testing. More research is needed to determine the consequences of rearing larvae under dark conditions on their response to the light/dark transition test.

4.3 Capture Order does not skew activity levels

During the process of transferring zebrafish larvae from the Petri dishes, in which they are usually housed, to the 96-well plates in which behavioural testing was performed, it seemed that some larvae were more active (and therefore more challenging to capture) than others. As no literature was found that addressed this, an experiment was designed to investigate whether there were systematic differences between the first and the last larvae captured that could affect the results of the light/dark transition test. No significant differences were observed between the first larvae captured and the last larvae captured. This suggests that it would not be detrimental to capture the larvae one group or plate at a time. This has practical and welfare benefits, particularly when the larvae being used to answer a particular research question are spread across multiple plates. When the larvae for each plate can be captured consecutively, they spend less time outside of the incubator and therefore have a smaller chance of getting cold. The temperature and lighting conditions that they are exposed to will therefore be controlled for longer.

This finding suggests that there were no consistent differences between the larvae's activity levels, or between other traits that affected ease of capture, which significantly affected their response to the light/dark transition test. This test did not, however, test whether being recently disturbed by the process of capture and transfer would affect light/dark transition test response because the larvae had been allowed to recover overnight. This allowed for the identification of consistent differences, but it means that future research should investigate whether capture order significantly affects response to the light/dark transition test when capture and transfer to the 96-well plate occurs directly before the light/dark transition test. Moreover, larvae develop rapidly, and it is possible that the less-developed, slower larvae that were captured first caught up to the other larvae overnight. Particular caution should be exercised when using younger larvae. It should be noted that the person who transferred the larvae to the 96-well plate was very experienced. Someone who is still learning this technique is more likely to distress the larvae. The use of a longer period between the transfer to the 96-well plate and the start of the test could compensate for less efficient transfer during training. If capture order does have an effect, one larva should be captured for each group/plate before a second larva is captured for each group or plate.

4.4 Time of day has a significant effect on the response to the light/dark transition test

A number of studies have found that time of day affects baseline activity levels (MacPhail *et al.*, 2009; Vignet *et al.*, 2013). Although some studies have compared the response to the light/dark transition test at different times of day, they used only two timepoints (9 am and 2 pm) (Fitzgerald *et al.*, 2019). Fitzgerald *et al.* (2019) found no significant differences in the response to the light/dark transition test at different times of day, but this needs to be confirmed over a wider range of timepoints. Thus, we compared the light/dark transition test with tests conducted at a number of timepoints throughout the working day.

Unlike Fitzgerald *et al*. (2019), we found that time of day did have a statistically significant effect on the response to the light/dark transition test. The time of day that differed the most from other starting times was the test that started at 9:00. There was a significantly reduced response to the light dark/transition test at this time compared to the response to the test performed at 10:00 and the test performed at 4:00. Fitzgerald used a measure called the activity index and compared correlation levels. These differences in the analysis may have prevented the detection of differences caused by the time of day in that by Fitzgerald *et al* (2019).

Time of day response may also have been affected by differences in how busy the laboratory was over the course of the day. The laboratory was most busy early in the morning when the larvae in the incubator needed to be refreshed. Although as many larvae as possible were refreshed before the start of the EthoVision experiments, some larvae needed to be refreshed between 8:00 and 11:00. It is recommended that treatment groups should be evenly distributed across different testing times to account for the effect of time of day. Moreover, if facilities allow for this, it may be beneficial to use separate laboratories for behavioural testing and general zebrafish husbandry.

Despite the low level of variability this study and others found during the early afternoon (Macphail *et al.*, 2009; Vignet *et al.*, 2013), there may be disadvantages to using this time of day due to the low level of activity then. This low level of activity may decrease sensitivity in detecting decreases in activity that may be present after treatments with anxiolytic drugs. If the low level of activity is due to the larvae being more tired later in the day, this may also affect their responses to assays that stimulate activity. The other study that measured the effect of time of day on response to the light/dark transition test found greater variability in the afternoon (Fitzgerald *et al.*, 2019). This suggests that the morning may be a more suitable time to apply the light/dark transition assay.

4.5 Repeated light/dark cycles do not affect the response to the light/dark transition test

Repeated cycles did not affect response to the light/dark transition test. Repeated cycles with different conditions could be used to decrease the number of fish needed, because the same fish could be measured repeatedly when investigating the effect of a treatment on the light/dark transition

test response over time. This would be useful when one was not sure what the effect of a treatment would be, or how long it would take to have an effect. This could therefore reduce type II errors due to screening the compound of interest before or after it is effective. Being able to use repeated cycles would therefore save time and resources.

4.6 Repeated light/dark transition tests do not affect the response to subsequent light/dark transition tests

The most appropriate time between treatment and testing will depend on the pharmacokinetics and the pharmacodynamics of the compound being tested. It may be beneficial to perform light/dark transition tests at a range of intervals after testing to screen adequately for potential neuropsychiatric effects.

We found that repeated light/dark transition tests on the same day did not affect activity during the dark period or peak change in locomotion during the light/dark transition test. This is in line with the findings of Fitzgerald *et al*. (2019), who found low intra-individual variation across a particular day given repeated testing (at 9 am and 2 pm on the same day) (Fitzgerald et al., 2019). The lack of any significant effect from repeated testing in this study suggests that study designs with a pre-test and a post-test could be utilised. This would both reduce the number of larvae used in experiments and reduce the confounding effect of interindividual variation when comparing treatment groups. Due to the differences across age groups, it is less advisable to do a pre-test/post-test design that requires testing on subsequent days. If this is done, larvae should be separated so that the individual pre-test/post-test responses can be compared.

4.7 The duration of the light period has a significant effect on the response to the light/dark transition test

As observed by MacPhail *et al.* (2009), a longer light period results in a larger response to the light/dark transition (MacPhail *et al.*, 2009). This is consistent with the bright light being aversive, as well as with the change to darkness being more aversive when the larvae have had more time to adjust to the light condition. If a longer light period and therefore a greater hyperlocomotion response is present, it may be easier to detect a decrease in the hyperlocomotion response caused by an anxiolytic treatment. If a shorter light period is used, and there is therefore a smaller hyperlocomotion response, there will be greater sensitivity to an increase in activity caused by an anxiogenic treatment. This has potential to prevent ceiling effects. Moreover, it is possible that trait anxiety may be better measured using a shorter light stimulus, while acute anxiety may be better represented when longer light stimulus is used. Nonetheless, in order for the hyperlocomotion response of different treatments to be compared, the length of the light period must be standardised.

4.8 Light to dark transition intensity did not have a significant effect on the response to the light/dark transition test

No effects were seen when comparing a sequence of 100%, 50% and 25% light intensity or when comparing a sequence of 10%, 5% and 1% light intensity. However, previously, both the degree of hyperlocomotion and the amount of thigmotaxis response after a light/dark transition had been reported to be significantly reduced when there had been constant illumination followed by different decrements of light during the darker period (in other words the light period was constant but the dark period had different levels of illumination) (Schnörr *et al.*, 2012). This provides support for the decrease in illumination level being an aversive part of the light/dark transition test.

Although very high and very low light intensities were investigated, the highest and the lowest light intensities were not directly compared. Future studies could be designed to compare these conditions directly, however current data from this study indicates that studies within the high light intensity range can be compared with each other and studies within the lower intensity range can be compared with each other.

4.9 Breeding stocks

Zebrafish research in South Africa is still in its infancy, and it is not yet possible to access the specific, well-characterised strains that are used internationally. All the breeding stocks from the Zebrafish Research Unit at Stellenbosch University, Tygerberg campus, Clinical Pharmacology, would be considered wild-type zebrafish and were sourced from pet shops. Nonetheless, it is valuable to conduct some basic characterisation of the breeding stocks being used in the experiments as this may be relevant to the research topics of interest because there is evidence of strain-specific effects on anxiety (Robison & Rowland, 2005; Wagle *et a*l., 2017) and on the light/dark transition test (Van Den Bos *et al.*, 2017). In this study, the light/dark transition test responses for the breeding stocks provided by the Zebrafish Research Unit at Stellenbosch University, Tygerberg campus, Clinical Pharmacology, were investigated.

The older C breeding stock was the F2 generation of stock obtained from a pet shop in 2021. The younger A and B breeding stocks were obtained from a pet shop during 2022. Although the parents of stock C were used to produce the larvae used in some of the experiments, that stock had been retired, due to age, by the time the different breeding stocks were being compared. Only those breeding stocks that were being actively used between July and October 2022 (A, B and C) were compared.

The offspring of breeding stock A showed the lowest hyperlocomotion response to the light/dark transition test. There could be genetic and epigenetic differences that affected their response to the light/dark transition test, and therefore reduced the magnitude of their response to the stimulus of a light/dark transition.

The offspring from breeding stock C showed the highest magnitude of response to the light/dark transition test, and therefore showed the highest anxiety-like behaviour. This suggests a greater sensitivity to stressors such as a light/dark transition. Once again, this could have been caused by genetic and/or epigenetic differences between stock C and the other stocks. Interestingly, the C breeding stock showed increased bottom-dwelling, which is associated with anxiety-like behaviour (Egan *et al*., 2009) (this was a qualitative observation during husbandry that was not a part of the original study design). Studies have found that older zebrafish do tend to show more anxiety-like behaviour in terms of bottom dwelling and thigmotaxis (Kacprzak *et al.*, 2017). Qualitatively, the oldest breeding stock (stock C) used in our study showed more anxiety-like behaviour in terms of bottom dwelling, startle sensitivity and latency to feed. Epigenetic modification may have increased the anxiety-like behaviour in their offspring. A potential explanation could be that their higher anxiety-like behaviour of breeding stock C was related to their more advanced age, and that epigenetic changes were passed on to their offspring that increased their anxiety-like behaviour. It may therefore be valuable to investigate whether the age of the parent fish has a significant effect on the response of offspring in the light/dark transition test, but that was beyond the scope and resources available for this project.

Zebrafish are shoaling fish which often spawn in groups. Using small groups of fish maximises the yield because if there are too many males, competition can interfere with breeding efficiency (Tsang *et al.*, 2017). Every time that a particular breeding stock is put up for breeding, different combinations of individuals may participate, which increases the variation between offspring from different breeding events from the same breeding stock. In addition, each fish can produce offspring with multiple partners. Pair breeding can be used, but this reduces egg yields. Very inbred stocks may result in some effects being missed and others being overestimated. Sometimes laboratory strains are outbred with wild type fish for toxicology studies to increase variation—this will often decrease effect size but the sample will more closely resemble a normal population (Brown *et al.*, 2012). Nonetheless, the large effect of different breeding stocks as observed in our study implies that breeding stock should be considered, and that the genetic and epigenetic factors that differ between breeding stocks can have a significant effect on response to the light/dark transition test.

The breeding stock can affect the light/dark transition test response. This could act as a confounder, and unequal contribution to groups tested by different breeding stocks could increase type I error. Going forward, the breeding stock used for each experiment should be noted. If multiple breeding stocks are used for the same experiment, there should be a similar number of larvae from each breeding stock in each group. Experiments could be conducted/repeated in all available stocks, as validation that results are repeatable in different populations

4.10 Limitations and future work

Despite the contributions of this study, there is much future work. The effect of lighting conditions during rearing, where results were inconclusive, should be replicated in a larger sample size. It would also be useful to repeat the time-of-day investigation with repeated measurement of the same larvae now that subsequent experiments indicated that repeated exposures do not affect the response to the light/dark transition test. The experiment in this study used a new group of larvae at every time point. Breeding stock among wild type fish significantly, suggesting that it would be good to repeat this with common research strains with a well-characterized genetic background. The investigation of protocol variations should be replicated with larger sample sizes and in different genetic backgrounds.

In addition, this study did not directly investigate the biochemical parameters of the anxiety-related response in the light/dark transition test. Future work can investigate how these protocol variations affect neurotransmitters and cortisol levels for example. This would also improve the ability to interpret and explain the results of this test. The effect of these protocol changes on neurotransmitters and whole-body cortisol should be investigated, and when available the use of transgenics with fluorescent indicators should be leveraged.

4.11 Concluding Remarks

This study showed that age, time of day, light period and breeding stock all have a significant effect on the response to the light/dark transition test and should be controlled and standardised wherever possible. Caution needs to be employed when comparing studies reported in the literature when these factors differ between studies. On the other hand, capture order, repeated cycles, repeated light/dark transition tests and light/dark transition intensity can vary according to logistical requirements without significantly impacting the results of the light/dark transition test. This opens up possibilities for study designs with repeated tests. In addition, studies where these factors vary can be compared. Although large qualitative differences in the light/dark transition test were visible after rearing larvae under different lighting conditions, there was high variability, and these differences did not reach statistical significance. Further investigation is necessary to elucidate whether it is important to consider lighting conditions during rearing when the light/dark transition test is to be used. In addition, future research can look into the effect of combinations of significant protocol variations to investigate potential additive and interaction effects. Overall, this study has made a valuable contribution to the design of light/dark transition test protocols, and it will facilitate comparison of the many studies using this test that are reported in the literature.

The light/dark transition test is an important screening tool for neuroactive compounds, including those that modulate anxiety. An increase in the hyperlocomotion response after a treatment can indicate a potential anxiogenic treatment, while a decrease in the hyperlocomotion response can indicate a potential anxiolytic treatment. As the light/dark transition test is so widely used, the potential anxiolytic and anxiogenic effects of many treatments can be identified from the existing

literature. However, it is essential that the effects of different protocol variations in the light/dark transition test are well understood so that this literature can be effectively evaluated.

The light/dark transition test is best used as an initial screening tool. One of the light/dark transition test's strengths is its sensitivity to a wide array of neuroactive and toxic compounds, which makes it useful for tests for toxicology and neuroactivity. This means that it is important to confirm potential anxiolytic or anxiogenic effects with more specific anxiety related tests (the light/dark preference test and thigmotaxis measurement are two good options that can be measured in zebrafish larvae). Nonetheless, the light/dark transition test is an important tool in identifying potential treatments for anxiety disorders.

The initial context that prompted the investigation of the effects of protocol variations on the light/dark transition test was the need to support screening for potential anxiolytic treatments. Nonetheless, the findings of this study are useful for all applications of the light/dark transition test. These findings inform both protocol design and the comparability of existing literature, and will help to ensure that maximum value is extracted from the zebrafish larvae used in this screening test. It therefore has the potential to play a broader role in the support of toxicology and pharmacology in addition to meeting its initial aim of supporting the search for new anxiolytic treatments.

Chapter 5: Reference List

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Appendices

Appendix A

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Protocol Approval

Date: 04 May 2021

PI Name: Prof C Smith

Protocol #: ACU-2021-21677

Title: Use of zebrafish larval model of IBS for drug screening

Dear C Smith ,

Your response to modifications, was reviewed by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report (due 30 days prior to expiry date), up to a maximum of three years.

Approval Date: 04 May 2021 - 03 May 2022

Animal Species: Zebrafish

Animal Numbers: 10080

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 21677 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU Secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

Appendix B

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approved with Stipulations

14 April 2020

PI: Prof Carine Smith

REC: ACU Reference #: ACU-2019-11820

Title: Use of Zebrafish larvae (<5dpf) for drug screening

Dear Prof Carine Smith

Your Notification ACU-2019-11820 was reviewed on 25 March 2020 y the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is valid for a period of five years. A new application must be submitted when the source of the material changes.

Stipulations for clarification:

1. How will it be ensured that the larvae do not come in direct contact with the ice?

2. What is the treatment that will be done to/on the larvae?

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your REC: ACU reference number: # ACU-2019-11820 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: ACU policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Animal Care and Use)