Anxiety as a personality trait in the zebrafish, *Danio rerio*

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ABSTRACT

In recent years, there has been an increasing interest in animal personality traits. The term personality trait refers to individual behavioural variations that are consistent within individuals and over time. Recently, scientists have shown an increased interest in anxiety-like behaviour, but this has been limited to modelling stress responses that are essential in the study of drugs and diseases. However, up to now, there has been no detailed examination of anxiety, and little is known about whether it is a personality trait, is heritable or has any fitness effects. This thesis investigated the individual behavioural differences in anxiety in the zebrafish, *Danio rerio*, using three different tests (novel tank diving test, open field test and light/dark test) in order to determine whether it could be regarded as a personality trait. It also determined whether anxiety was heritable, differentially expressed and had any fitness consequences. The repeatability and consistency of anxiety-like behaviour measured in the three tests was assessed in male and female zebrafish and Principal Component Analysis (PCA) was used to determine the association between these measures and, based on these results, individuals were ranked according to their level of anxiety. RNA was extracted from the brains of six least anxious and six most anxious males and females to identify any variation in gene expression. Four different groups of least and most anxious males and females were established and randomly crossed to determine their reproductive success and therefore the fitness consequences of this trait. The heritability of anxiety was determined by crossing males with known levels of anxiousness with females and assessing the behaviour of the offspring using the novel tank diving test. Furthermore, least and most anxious individuals were exposed to chemical alarm cues as a stressor to measure their stress responses. We found that anxiety was inconsistent between different behavioural measures in the novel tank diving test, open field test, and light/dark test, but this inconsistency was highly repeatable within males and females. From the PCA, the

results revealed that most individual variation in anxiety was explained by the novel tank diving test and the open field test. We also found that the novel tanks diving test was not correlated with the open field test in measuring anxiety while the open field test and light/dark test were negatively correlated. We identified some genes that were expressed differently depending on level of anxiety and sex, indicating that anxiety is genetically controlled. We also found that anxiety was heritable ($h^2 = 0.03 - 0.18$). Moreover, we found that level of anxiety had no effect on the number of eggs laid and fertilised, suggesting that this trait is not linked to this aspect of fitness. Finally, the results demonstrated that individuals did not display any difference in behaviour before and after exposure to conspecific alarm cues, except that they increased the amount of time they spent in the upper half of the novel tank diving test after the treatment. The most anxious males and females responded to the alarm cues in unexpected ways, and they reduced their anxiety levels. Taken together, these results suggest that anxiety is a personality trait that is repeatable, heritable, and is controlled by differential gene expression in zebrafish. Furthermore, anxiety has no direct effect on zebrafish fitness as measured by number of eggs laid and fertilised and stress responses.

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PRESENTATIONS AND CONFERENCES

ASAB (Association for the Study of Animal Behaviour) Easter Conference, 04-06 April, 2018 at the University of Plymouth, UK.

Posters

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Other conferences attended

EpiStressNet Research Network Meeting held at Halifax Hall, the University of Sheffield, 22-23 February 2018.

Epigenetics symposium held at the University of Sheffield, 25 May 2018.

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Chapter I

General introduction

1.1 Animal personality behavioural traits

Behaviour is the way an animal responds to their environment. Animals can behave in different ways to enhance their survival, and this can give an animal characteristics that distinguish them from others (Manning & Dawkins, 1998). Personality traits can have an impact on animal behaviour, such as mating strategy, dispersal, and territory defence (Seyfarth et al., 2012). We can say that a personality trait is possessed by an individual animal only if they display behavioural variation that distinguishes them from others (individual differences), and this difference is consistent over time (temporal stability) and between contexts (contextual consistency) (Kaiser & Müller, 2021). Thus, a personality trait refers to a behaviour that differs between individuals in a population and is consistent over time and among contexts (Amin et al., 2016; Ariyomo et al., 2013; Bevan et al., 2018; Castanheira et al., 2013; Groothuis & Maestripieri, 2013; Neave et al., 2020; Thomson et al., 2020; Urbánková et al., 2020; Wolf & Weissing, 2010). Although research on behavioural consistency has been mostly restricted to the adulthood stage (Alfonso et al., 2019; Alfonso et al., 2020; Ariyomo & Watt, 2013a), some research has focused on behaviour across time from larval to juvenile stages (Alfonso et al., 2020).

Individuals within populations vary in their personality traits and this variation is consistent over time. For example, boldness, a measure of an individual's risk-taking and exploration of their environment (Ariyomo & Watt, 2012; Ariyomo et al., 2013b), and aggression, the agnostic response of an individual to its conspecifics (Réale et al., 2007), are two personality traits that have been studied in different groups of animals such as fish

(Ariyomo & Watt, 2012), birds (Barnett et al., 2012), lizards (McEvoy et al., 2015) and rodents (Yuen et al., 2015). Individuals have been found to be bold, shy, aggressive or non-aggressive. Moreover, these personality traits are sometimes correlated, which is referred to as a behavioural syndrome. Behavioural syndromes have been investigated in many studies (Bell, 2005; Dingemanse et al., 2007; Dochtermann & Jenkins, 2007; Johnson & Sih, 2005). In some birds, fish and rodents, more aggressive individuals are bolder in novel environments (see references in Dingemanse & Réale, 2005).

Research on personality has increased since the success achieved by Wilson in the field of behavioural ecology when he published his paper (1994) about animals' personality traits (shyness and boldness), which included humans (Réale et al., 2010). Traits such as novel environment exploration/boldness, sociability (an individual's responses to visible or non-visible conspecifics), activity (an individual's movements in a familiar situation; Réale et al., 2007), and aggressiveness have been used to illustrate personality differences in animals (Conrad et al., 2011; McEvoy et al., 2015; Réale et al., 2007) and they have been found to be repeatable (Schuster et al., 2017).

1.2 The maintenance of personality traits

Personality traits vary genetically between individuals. Although the advantageous traits are selected, personality genetic variation is maintained in a population (Verweij et al., 2013). Three possible reasons can explain this maintenance (Penke et al., 2007; Verweij et al., 2013). The first possibility is selective neutrality which describes a situation when genetic variation in personality traits is free to frequently drift because it is not affected by selection or fitness (Verweij et al., 2013). In other words, selective neutrality occurs when there is no selection for mutation (Penke et al., 2007). The second possibility is mutation selection balance (Kirzhner

et al., 2003; Penke et al., 2007; Verweij et al., 2013) when genetic variation is reduced because deviations from an optimal level of personality traits are selected against leading to some but not all of the alleles being eliminated that do not match this optimum (Penke et al., 2007; Verweij et al., 2013). The third possibility is balancing selection which describes a situation when genetic variation is maintained by selection itself (Penke et al., 2007; Verweij et al., 2013).

1.3 Fitness consequences of personality traits

Variation in personality traits can affect fitness. This variation can lead individuals to be different in their activity, use of habitat, diet, and capture of prey, which in turn affects their fitness by reinforcing the productivity and density of the population (Wolf & Weissing, 2012). Variation in behavioural traits leads an individual to be challenged in an environment that is different in regards to intruders, competitors, sociability, parasites, and resource abundance, which in turn has an impact on fecundity and mortality (Smith & Blumstein, 2008; Wolf & Weissing, 2012). For instance, in general, individuals with a high level of aggressiveness usually exhibit a higher reproductive success from competition with conspecifics for mates while being aggressive in a risky context is costly and resulting in a decreased chance of survival (Smith & Blumstein, 2008). Male western bluebirds (*Sialia mexicana*) with a high level of aggressiveness make territories in higher-quality habitats with more competition than less aggressive individuals do (Wolf & Weissing, 2012). Aggressiveness and boldness have been associated with foraging, reproduction, survival (Oswald et al., 2012; Ariyomo & Watt, 2013b; Ariyomo et al., 2013), and dispersal (Hirsch et al., 2016). Moreover, boldness has a positive effect on fitness (reproductive success) in adult largemouth bass, *Micropterus salmoides* (Ballew et al., 2017), and zebrafish (Ariyomo & Watt, 2012). Furthermore, in a meta-analysis study, reproductive success was found to be positively associated with boldness

(Smith & Blumstein, 2008). Personality traits may affect the selection and evolution of other traits such as morphological ones. For instance, the caudal region in bolder zebrafish was shown to be greater than shyer individuals after several generations (Kern et al., 2016). Personality traits may also negatively affect lifespan and reproductive success. For example, it has been shown that hyper-aggressive behaviour significantly reduces mating success in a water strider, *Aquarius remiges* (Sih & Watters 2005), and boldness has been found to have a negative impact on juvenile largemouth bass survival (Ballew et al., 2017).

Variation in behaviours is linked with variation in life history and an individual's mortality and fertility, which in turn affects their fitness (Wolf & Weissing, 2012). An individual's size, energy, age, and environmental and bodily conditions are examples of these states (Wolf & Weissing, 2010). According to the life history theory, consistent variations in the behaviour of risk-taking in individuals are predicted to affect their differences in future fitness, where risk-averse behaviour should be displayed by those with high expectations of future fitness to survive and achieve them while risk-prone behaviour should be displayed by those with low expectations because they do not have much to lose (Wolf et al., 2007).

1.4 A trait's plasticity and consistency at the same time

Animals with consistent personality traits are not able to change their behaviour to match environmental fluctuations. In other words, individuals can express their personality traits in the presence or absence of stimuli. For instance, in the situation of predation, bold individuals behave in the same way in the presence or absence of the threat (Bell et al., 2013). However, consistency of trait differences does not require that individuals in the same population have the same reaction toward a situation. Instead, they are different in the expression of these differences and this is stable throughout time and among contexts (Bell et

al., 2013; Biro & Stamps, 2008; Dingemanse & Réale, 2005; Wolf & Weissing, 2010). For example, in responding to the risk of predation, both juvenile and adult rainbow trout (*Oncorhynchus*) who were bold consistently exhibited lower responses compared to those who were shy in the same population (Biro & Stamps, 2008).

Therefore, many researchers believe that personality traits are not plastic nor influenced by environmental factors (Dingemanse et al., 2010; Roche et al., 2016). However, an individual's personality traits may vary slightly with environmental conditions, which is known as phenotypic plasticity, and can vary among individuals (Cornwell et al., 2018; Dingemanse & Wolf 2010; Nussey et al., 2007). Behavioural plasticity is described as a difference in behavioural scores caused by external (contextual plasticity), internal (intraindividual variability such as the random difference in the brain's activity) stimuli difference or learning (developmental plasticity) (Stamps & Biro, 2016). Plasticity can also explain the changes in behaviours over time and between ages (temporal plasticity) (Stamps & Biro, 2016). For instance, it has been shown that zebrafish (*Danio rerio*) differ in aggression and foraging depending on the levels of water flow (Bhat, 2015; Suriyampola et al., 2017) and vegetation (Bhat, 2015). Other examples in different species have shown that personality traits can show some plasticity under different social situations or environmental conditions (Biro et al., 2010; Dosmann & Mateo, 2014; Galhardo et al. 2012). Plasticity in personality traits is induced by factors such as an individual's prior experience (Brown et al., 2005; Frost et al., 2006). For example, Frost et al. (2006) found that the plasticity of rainbow trout (*Oncorhynchus mykiss*) in regards to boldness was affected by prior experience of fights or watching individuals' responses that varied from boldness to novelty. Bold individuals who watched shy fish or those who did not win a fight became shy, while shy individuals who watched bold activities did not exhibit any behaviour change. The losers and winners who were shy both

became bolder by reducing their latency to contact a novel object. Moreover, an individual can show flexibility in their behaviour because of their age and size, which leads them to respond differently to the environment depending on their development stage (Brown et al., 2006). Sex can affect personality traits because of the different life history strategies displayed by males and females (Brown et al., 2006). Plasticity can also be induced by transferring an individual from their natural habitat to the laboratory environment (Brown et al., 2005).

However, plasticity in personality traits is very limited and varies between individuals (Bell, 2007 & Dubois, 2019; Dingemanse & Réale, 2005). Perhaps this is because of the difficulty in undergoing a personality transformation by individuals (Bell, 2007). For instance, the transformation of an individual to be aggressive is costly because it requires time, energy, for neural machinery rewiring and physiology building needed for metabolism. Therefore, individuals prefer to stick to an intermediate strategy (Bell, 2007). Furthermore, uncertain information related to the environment makes an individual behave consistently to eschew the probability of making a mistake (Bell, 2007; Wolf & Weissing, 2010). Another possibility of the limitation in personality plasticity is that individuals' ability in detecting environmental changes varies (Dubois, 2019). For example, shy individuals in the same population are thought to be more plastic than bold, aggressive and explorative individuals (Dubois, 2019). According to the speed-accuracy trade-off hypothesis, bold individuals' abilities to detect environmental changes are less than shy individuals because of their speedy and inaccurate exploring (Dubois, 2019). Individuals' conditions and energetic needs could be a reason for the variation in personality plasticity, as they affect how optimal plasticity is displayed (Dubois, 2019). For individuals, being plastic or not can be costly or beneficial (Dewitt, et al., 1998; Dubois, 2019). For example, being non-plastic can be costly for individuals which demand higher energy with

a higher rate of metabolism because they regularly need to adjust their behaviour to meet the needs of their resources compared to those which demand the opposite (Dubois, 2019).

1.5 Measuring personality traits

Although there are several methods available for measuring personality traits, considering their reliability is important. The measurement of personality traits depends on the traits to be tested. Emergence tests, assays that measure an individual's willingness to emerge from a holding area into a novel environment, have been commonly used to measure boldness in many studies (Beckmann & Biro, 2013; Binder et al., 2016; Brown, 2004; Budaev, 1997; Fraser et al., 2001; Reddon, 2009; Toms et al., 2010). However, when this test was repeated, it was found to yield inconsistent results (Beckman & Biro, 2013). In one study that tested the accuracy of three different tests (emergence, open field and novel object), the open field test was found to be the most accurate (Burns, 2008). Therefore, it is often used to measure boldness, for example, in fish such as zebrafish (Ariyomo & Watt, 2012, 2013a, 2015; Ariyomo et al., 2013), guppies, *Poecilia reticulata* (Ariyomo & Watt, 2013b), reptiles, such as the Australian social skink, *Egernia whitii* (McEvoy et al., 2015), and mammals such as Merriam's kangaroo rat, *Dipodomys merriami* (Hurtado & Mabry, 2017). However, other tests have been used to measure boldness such as exposure to predators' odour (e.g. Merriam's kangaroo rat*;* Hurtado & Mabry, 2017*)*.

Tests must measure the target trait to prevent the assessment of other behaviours. For example, the assessment of an individual's fear of a novel object as a target behaviour is carried out in a familiar environment because a novel one may induce exploration behaviour (Réale et al., 2007). Using more than one measure to quantify a trait assesses the strength of the genetic correlation between them (Réale et al., 2007). For example, the measure of early exploratory

behaviour has been tested using an open field test or a novel environment test (Dingemanse et al., 2002; Krause & Naguib, 2011; Naguib et al., 2011) and the arena construction test (an arena with connected rooms used as a complex novel environment) in wild great tits, *Parus major* (Arvidsson et al., 2017). Counting the number of flights between artificial trees has been commonly used for measuring the exploratory behaviour of birds such as wild great tits (Arvidsson et al., 2017; Dingemanse & Goede, 2004; Dingemanse & Réale, 2005).

The validity of using the mirror test, an assay that records an individual's response to its mirror image (e.g. Ariyomo & Watt, 2012), to assess aggression behaviour has been investigated in species of sympatric cichlids of Lake Tanganyika, *T. vittatus, L. elongates and N. pulcher,* (Balzarini et al., 2014). Furthermore, researchers have tried to compare and test the accuracy of many aggression measures using tests such as a live conspecific test (an assay that records the aggressive activities between two fish), a flat and inclined mirror test (an individual is exposed to a mirror flat against the tank or at an angle), a clay model stimulus test (an assay in which a clay model that is matched to a fish's colour and size is used as a stimulus), and a video recording test (an assay in which a fish is exposed to a recorded film of aggressive behaviours conducted by a random fish in front of a mirror) (Way et al., 2015). These tests have elicited repeatable aggression responses in zebrafish, but their rates were different depending on the test that was used. High expression rates of aggressive responses were associated with live conspecific and flat mirror tests in zebrafish (Way et al., 2015). The mirror test has become the most popular technique for measuring aggressive behaviour in fish such as zebrafish (Ariyomo et al., 2013; Ariyomo & Watt, 2013a, 2015). Considering the weak ability of many animals to recognize their mirror image (Balzarini et al., 2014; see references in Cattelan et al., 2017), the mirror test has also been reliably used to measure sociability behaviour in many species (e.g. guppy, *Poecilia reticulata,* Cattelan et al., 2017; mosquitofish,

Gambusia holbrooki, De Santi et al., 2001; zebrafish, Moretz et al., 2007). However, other tests, such as using an aquarium that is divided vertically into two sections (Cote et al., 2010), divided into three sections (Ward et al., 2004), or set inside another aquarium (Brown & Irving, 2013) to measure the responses of target fish to their visible single or group conspecifics, have also been used to assess sociability.

1.6 Factors affecting personality traits

Behavioural flexibility is constrained by genetic and physiological mechanisms, and this is reflected by an individual's traits (Gosling, 2008). In addition, their early social and physical environment can contribute to such variation (Trillmich & Hudson, 2011). Other factors, such as interspecific interactions such as predation and ecological effects in the early stage of an individual's life may contribute to their personality traits (Trillmich & Hudson, 2011). Research on the influences of the environment and genes on personality traits has been demonstrated before; for example, variation in innovativeness in great tits (Quinn et al., 2016), and in emotionality and learning and memory in horses, *Equus caballus* (Martine et al., 2004). In addition, in fruit flies (*Drosophila* sp.), foraging behaviour is controlled by individual gene differences and environmental factors, such as food, that reflect behavioural plasticity (Mather & Logue, 2013), and in mangrove killifish (*Kryptolebias marmoratus*), level of exploration, boldness, and aggressive behaviour are affected by predation risk, and boldness has a genotype-environmental interaction (Edenbrow & Croft, 2012).

1.6.1 Gene expression

An animal's phenotype can be affected by variations in its genes. For example, an investigation conducted on the fruit fly genome demonstrated that variation in a fly's behaviour was associated with variations in its genes (Groothuis & Maestripieri, 2013). Edwards et al. (2006) found that variation in aggression in a fruit fly (*Drosophila melanogaster*) was affected

by differences in the level of gene expression, and they discovered about 15 novel genes influencing this trait. Genes associated with personality traits have been demonstrated in many studies. For example, the gene DRD4 is associated with exploratory behaviour in great tits (Verhulst et al., 2016) and wariness and local site selection in black swans, *Cygnus atratus* (Van Dongen et al., 2015), OXTR polymorphisms are linked to roughness as a personality trait, irregularity of surface in cats, *Felis catus* (Arahori et al., 2016; Chumley et al., 2023), the SERT genotype is associated with traits correlated with harm avoidance in blackbirds, *Turdus merula* (Mueller et al., 2013), the Avpr1a gene is linked to sociality in chimpanzees (Staes et al., 2015), and boldness is associated with genes of the stress axis in zebrafish (Oswald et al., 2012).

1.6.2 Heritability of personality traits

The plasticity of personality traits becomes limited if they are heritable and under genetic control. Heritability describes the proportion of variance in phenotypes resulting from genetic factors, and can be assessed by determining the degree of that variance (Falconer & Mackay, 1996; Wu et al., 2017). Additive genetic variance expresses the heritability of phenotypic traits (Falconer & Mackay, 1996). When a trait is under directional or disruptive selection, it leads to reduced additive genetic variation (Oers & Sinn, 2013). The genetic control and heritability of personality traits have been studied in many animals under laboratory conditions (e.g. great tits, Drent et al., 2002; Sinn et al., 2006; Van Oers et al., 2004; mice, Miczek et al., 2001) and natural conditions (e.g. German Angus and Simmental cattle, Gauly et al., 2001). Moreover, some personality traits are heritable such as boldness and aggressiveness in zebrafish (h^2 = 0.76 and h^2 = 0.36, respectively; Ariyomo et al., 2013) exploratory behaviour in great tits (h^2 = 0.10–0.78; Drent et al., 2002), boldness in dumpling squids, *Euprymna* tasmanica (h^2 = 0.2-0.8; Sinn et al., 2006), and temperament in German Angus and Simmental cattle (h^2 = 0.0-0.61 and h^2 = 0.0-0.59; Gauly et al., 2001).

1.6.3 Maternal and environmental effects

The offspring's phenotype may be affected by other factors such as parental or maternal effects. Egg provisioning and parental care are usually exhibited by mothers; thus, parental influences are often due to maternal effects (Groothuis & Maestripieri, 2013). Similarities in phenotypes between offspring and their parents can include personalities along with physiological and behavioural factors (Groothuis & Maestripieri, 2013). Ariyomo et al. (2013) demonstrated evidence of maternal effects in zebrafish. Offspring may be affected by the environmental conditions that their mothers experienced during their lives to increase their fitness. For example, it has been shown that female three-spined sticklebacks (*Gasterosteus aculeatus*) and crickets (*Gryllus pennsylvanicus*) can transfer their experience with predators to theiroffspring (Giesing et al., 2010; Storm & Lima, 2010).

1.7 Effect of stressors on individuals with different personality traits

Stress is a state that describes the responses of the hypothalamic-pituitary-adrenal (HPA) axis to a stressor, which is a factor that causes such a state (Cockrem, 2013). In fish, the HPA equivalent is the hypothalamic-pituitary-interrenal (HPI) axis, which once stimulated leads to the secretion of cortisol (Cockrem, 2013). Avoidance of stressors can cause animals to exhibit anxiety-like behaviours (Bai et al., 2016). Stress can negatively affect organisms' immune systems (Barcellos et al., 2007; Schreck et al., 2001) and growth (Hyun et al., 1998) and therefore influence fitness. Cortisol is a physiological hormone that helps to restore the stressed body to its normal state (homeostasis) (Alderman & Bernier, 2009; Bonga, 1997) and its presence can be used to estimate the stress response (e.g., in jundia*, Rhamdia quelen, Quoy & Gaimard*, Barcellos, 2004; dogs, Barker et al., 2010; and zebrafish, Alderman & Bernier, 2009; Bai et al., 2016; Egan et al., 2009; Kitson et al 2022).

Exposure to alarm cues can evoke stress responses and has been used for modelling anxiety in animals (Maximino et al., 2012). For instance, in zebrafish, erratic movements were exhibited, shoal cohesion was higher and exploration was reduced following exposure to alarm cues, which have been used as an indicator of anxiety (Blaser et al., 2010; Cachat et al., 2010). Alarm cues can be secreted from damaged skin through predatory aggression (Ferrari et al., 2007; Nieuwegiessen et al., 2008). For some fish, exposure to conspecific alarm cues elicits stress responses that illustrate conspecifics are injured (Barcellos et al., 2007). Stress responses can be elicited when individuals see, smell, or hear conspecific cues (Barcellos et al., 2007). For example, zebrafish larvae displayed reduced activity following exposure to conspecific alarm cue (Lucon-Xiccato, et al., 2020). Redfin darters (*Etheostoma whipplei*) have demonstrated low activity (low movements) during conspecific alarm cues exposure (Commens-Carson et al., 2007). When alarm cues were paired with heterospecific cues, rainbow darters (*E. caeruleum*) showed a decreased number of moves (Abudayah & Mathis, 2016). Individuals vary in their response to stressors with some of them being vulnerable to stress and others exhibiting weak or almost no response (Cockrem, 2013). Bell et al. (2007) indicated that sticklebacks (*Gasterosteus aculeatus*) exhibited consistent stress responses following exposure to different stressors (conspecific and heterospecific cues). Furthermore, these responses may be affected by individuals' personality traits (Ferreire et al., 2020). For example, birds were found to exhibit stress responses that were attributed to personality (reactive versus proactive) when exposed to different stressors, with the reactive group exhibiting higher responses than the proactive group (Cockrem, 2007). Although the responses of animals with different personality traits to stressors—such as conspecific cues—have been investigated before, none have attempted to examine the effect of stressors on zebrafish with consistent variations in anxiety.

1.8 Anxiety-like behaviour and how to measure it

Describing anxiety is challenging because it could imply a lot of concepts and components such as a state or trait and (Endler & Kocovski, 2001; Reiss, 1997; Spielberger & Rickman, 1988). Anxiety can be regarded as a non-permanent emotional state elicited by a real or potential threat, environment or stimuli at a specific time that affects an individual's physiology and behaviour and leads to the elicitation of neuronal, endocrinal, and defensive responses (Endler & Kocovski, 2001; Kalueff et as., 2013; Spielberger & Rickman, 1988; Steimer, 2011). Individuals can differ in their anxiety-like behaviour and how they respond to specific situations, and this is usually consistent over time (Endler & Kocovski, 2001; Reiss, 1997; see references in Steimer, 2011). As anxiety is unconscious, it can be revealed in behaviours and other signs such as an increase in vigilance and urine discharge (Reiss, 1997; Ohl et al., 2008). Anxiety-like behaviour can involve thigmotaxis, geotaxis, scototaxis, zig-zagging movement, body colour alteration, freezing and a reduction in exploratory behaviour (Kalueff et as., 2013). Activity and exploration are the most studied animal personality traits that are related to anxiety (see references in Pawlak et al., 2008).

Anxiety is distinguished from shyness, which is when an individual avoids social interactions and risk-taking behaviour, decreases activity and increases the latency to respond to novelty or stimuli (Kalueff et as., 2013; Pilkonis, 1977; Thörnqvist et al., 2019).
Shyness can be caused by novelty, others' presence and actions (Russell et al., 1986).

1.8.1 Measurement of anxiety

It has been mentioned in this thesis that the novel tank diving test, open field, and light/dark tests have been used for measuring different behaviours. For example, in zebrafish, boldness and shyness can be measured using the open field test because it measures an

individual's willingness to explore and take risks in a new environment (Ariyomo et al., 2013; Ariyomo & Watt, 2012, 2013a, 2015). Risk-taking behaviour has been measured using the novel tank diving test in zebrafish (Thörnqvist et al., 2019). Validating anxiety-like behaviour is difficult because this trait may overlap with the behavioural measures for other traits (Maximino et al., 2012). Consequently, using more than one assay to quantify anxiety and determining the association between them in measuring this trait is needed (Maximino et al., 2012). In this thesis, the novel tank diving, the open field, and the light/dark tests were used to assess the association between them in measuring the anxiety-like behaviour in the zebrafish.

In zebrafish, the most established tests that measure anxiety-like behaviour are the novel tank diving and light/dark tests (Jesuthasan, 2012; Kysil et al., 2017; Stewart et al., 2011). These two tests measure an individual's natural responses to new environments when they dive to the base of the tank (geotaxis), freeze or decrease their exploration of it for defence (Cachat et al., 2010; Stewart et al., 2011; Cachat et al., 2010). In the novel tank diving test, a fish is transferred to a tank divided into equal horizontal portions. The fish will instinctively swim to the bottom of the tank and reduce its exploratory behaviours, and depending on the responses to the novel environment, anxiety can be assessed (Cachat et al., 2010; Levin et al., 2007). Many studies on zebrafish have demonstrated that preference for the bottom of a tank is associated with a threat response; the time spent at the bottom, the number of entries into this area, and the distance that they moved within the area have been used to measure anxiety-like behaviour (Fontana et al., 2022; Kalueff et al., 2013; Sackerman et al., 2010). Other indexes such as erratic movement, immobility, latency to enter the upper half of a tank, number of entries and time spent there has been used as an indicative of anxiety (Fontana et al., 2022; Hamilton et al., 2017; Haghani et al., 2019). In the light/dark preference test (scototaxis, Kysil et al., 2017), a fish is transferred to a half-white and half-black tank and the amount of time it

spends in the dark half of the tank is recorded. An increased time spent in the dark side is taken as a reflection of higher anxiety (Jesuthasan, 2012; see references in Mathur & Guo, 2011; Magno et al., 2015; Maximino et al., 2010; Serra et al., 1999).

However, other tests can measure anxiety in zebrafish, such as the open field test in which anxious responses can be measured by exploratory behaviour and thigmotaxis, a centrophobic or horizontal behaviour, which concerns an individual's position in the centre versus the edge of a tank (Godwin et al., 2012; Maximino et al., 2010; Stewart et al., 2011; Stewart et al., 2012; Schnörr et al., 2012; Singer et al., 2016; Scatterty et al., 2023). Less movement and more thigmotaxis in the open field test suggest high levels of anxiety (Kalueff et al., 2013; Lucon-Xiccato et al., 2020). The open field test is also used to measure boldness and shyness in zebrafish because it measures an individual's willingness to explore and take risks in a new environment (Ariyomo et al., 2013; Ariyomo & Watt, 2012, 2013a, 2015). In the open field test, a fish is transferred to a novel tank, and its behaviour is recorded such as motionlessness, behaviour inhibitions (freezing) and velocity of swimming; increased freezing, reduced exploration, increased time spent at the bottom or thigmotaxis, increased vigilance, and decreased swimming speed reflect anxious responses (Blaser et al., 2010; Egan et al., 2009; Maximino et al., 2010; Singer et al., 2016). Shoaling behaviour has been used to measure anxiety-like behaviour in fish according to whether they increase their time with or swim away from a group, but this is following exposure to a stimulus (see references in Stewart et al., 2012).

Previous research on anxiety-like behaviours in zebrafish has focused on aspects such as drug impact (Johnson & Hamilton, 2017; Mitchell & Moon, 2016; Singer et al., 2016; Spielberger & Rickman, 1988), stress responses (Egan et al., 2009; Levin et al., 2007),

validation indexes of anxiety (Blaser et al., 2010; Maximino et al., 2012) mental disorder (Spielberger & Rickman, 1988) or modelling anxiety for genetic, molecular, biology, and pharmacological investigations (Stewart et al., 2012; Spielberger & Rickman, 1988). However, there have been no previous investigations on consistent individual variation in anxiety in zebrafish.

1.9 Zebrafish as a model species

Zebrafish have been used as a model species for several years for research on biomedicine (Parichy, 2015), genetics (Eliceiri, et al., 2010; Howe et al., 2013; Nowik et al., 2015; Parichy, 2015), cell biology (Eliceiri et al., 2010), human disease (Howe et al., 2013; Nowik et al., 2015; Weyan & Shavit, 2014), embryology, neurobiology, microbiology and immunology (Nowik et al., 2015) and hematology (Weyan & Shavit, 2014), as well as that related to drug development (Eliceiri et al., 2010; Nowik et al., 2015). This model species has many features that make it useful for research (Weyan & Shavit, 2014); zebrafish are easy to breed and raise in the laboratory, are small (approximately 2.5 cm), have a short generation period (Nowik et al., 2015), and have a fast reproductive rate (Nowik et al., 2015; Parichy, 2015). Moreover, their embryonic life stages are easy to follow and study (Parichy, 2015; Weyan & Shavit, 2014). Since zebrafish fertilization is external (Nowik et al., 2015; Parichy, 2015; Weyan & Shavit, 2014) and the embryos are transparent, researchers can manipulate their development at any stage and study various aspects (Nowik et al., 2015; Parichy, 2015). In addition, it is possible to get tissue and blood samples from them for genetic and hormone analysis, such as by fin clipping (Nowik et al., 2015; Zahangir et al., 2015).

Zebrafish are found in the streams and rivers of India (Graham et al., 2018; Spence et al., 2008). They are also observed in Bangladesh and Nepal rivers and streams (Sundin et al.,

2019). In the wild, zebrafish are found in clear and slow-flowing rivers with muddy or rocky bases, and feed on small animals or plants, and are consumed by other fish and birds (Parichy, 2015; Spence et al., 2008). The number of individuals varies within groups depending on the water flow of the river; for example, in slow-flowing water, the number has been ranged from 4–12, and in running water, this number reaches to 300 fish (Graham et al., 2018; Suriyampola et al., 2016).

As previously mentioned, a large and growing body of studies on a variety of different animal species has demonstrated that individuals show consistent differences in behavioural traits. However, there have been insufficient investigations of anxiety as a personality trait. The research described in this thesis has focused on studying anxiety as a personality trait in zebrafish. The overall aim of this research is to determine whether there are consistent individual differences in anxiety that are under genetic control that affect aspects of fitness.

Objectives

The objectives of this project are the following:

1- Measure whether zebrafish display consistent and repeatable anxious behaviour using repeated different tests: the novel tank diving, open field, and light/dark tests.
2- Estimate whether anxiety is affected by specific genes by extracting RNA from the brains of males and females with known anxiety levels
3- Evaluate the reproductive success of pairs with known levels of anxiety to determine the fitness consequences of anxiety
4- Compare offspring's behaviour with that of the parents following crosses conducted between males with known anxiety and females to determine the heritability of anxiety.

5- Determine the individuals with different levels of anxiety responses to stressors

The project's aims are as follows:

1. Measure anxiety-like behaviour in individual zebrafish and test the consistency and repeatability of it
2. Determine whether specific genes are associated with anxiety-like behaviour in both males and females
3. Determine whether there are any fitness benefits associated with levels of anxiety
4. Determine whether anxiety is heritable
5. Determine whether individuals respond to stress differently depending on their level of anxiety

This thesis consists of seven chapters and each chapter was written following a scientific paper structure. Chapter I is a general introduction to the themes covered in this thesis. Chapter II investigates whether zebrafish's differences in anxiety are consistent and repeatable within individuals and between contexts. Chapter III determines whether variation in anxiety level is associated with variation in specific gene expression. Chapter IV establishes whether anxiety has an impact on fitness (reproductive success). Chapter V investigates whether phenotypic differences in anxiety are attributed to additive genetic variance. Chapter VI examines the behavioural responses of individuals with known levels of anxiety following exposure to alarm cues using the novel tank diving test. Chapter VII generally discusses all the experiments conducted and summarizes all of the results.

1.10 References

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Chapter II

Assessing anxiety using different behavioural tests in zebrafish, *Danio rerio*

2.1 Abstract

The term "personality" has been used to describe human and non-human animals' behavioural variations that are consistent over time and among contexts. Many researchers have investigated anxiety as a response to stressor exposure; however, previous research has not investigated anxiety as a personality trait. This study measured variation in anxiety in zebrafish (*Danio rerio*) and the consistency and repeatability of this behaviour using robust tests to determine whether it was a personality trait. The study also determined whether the tests used to measure anxiety were correlated and therefore reflected the same outcome of individual variations. The behaviour of adult male and female zebrafish was assessed using three different tests (novel tank diving, open-field, light/dark), which were repeated on the same individuals. A generalised linear mixed model (glmer) was used to determine whether behaviours measured in the three tests varied between sex (males and females), between repeated tests (test 1 and test 2) and if there was an interaction between sex and tests. The model was fitted with number of tests and sex as fixed effects and individual identity as a random effect to determine their effects on behavioural responses including latency to enter the upper half of the tank, number of entries to the upper half of the tank, and time spent in the upper half during the novel tank diving test, and number of lines crossed, and time spent in the centre during the open field test, and time spent in the light half during the light/dark test. We found that sex has a significant main effect on the latency to enter the upper half during the novel diving test, lines crossed, time spent during the open field test, and time spent in the light half during the light/dark test. Moreover, test had a significant effect on the latency to enter the upper half, the number of entries to the upper half of the tank and time spent in the upper half of tank during the novel

diving test, time spent in the centre during the open field test, and time spent in the light half during the light/dark test. Principal Components Analysis (PCA) was used to estimate the variation explained by the measurements analysed from each of the tests. Results revealed that measures of anxiety were repeatable in all three tests. PCA indicated that most of the individual total variation in anxiety was associated with the novel tank diving test, which explained 59 % of the total variance for females and 65% for males, followed by the open field test and the light/dark test which explained 35 % of the total variance for males and just the open field test which explained 41 % of the total variance for females. The repeatability and the differences in anxiety levels in zebrafish make it likely that this trait is heritable.

2.2 Introduction

Animal personalities refer to individual differences in behaviour that are consistent in different contexts and through time (Silva et al., 2014; Thomson et al., 2020; Van Dongen et al., 2010). In the field of animal personality research, characterising behavioural responses requires accurate measurements. One problem that animal personality researchers face is determining which personality traits are being measured in a selected test. For example, one test such as the open field test, whereby animals are exposed to a novel or unfamiliar environment (Careau et al., 2009), may measure more than one trait. For example, this test can be used to measure both boldness (Ariyomo & Watt, 2012; Ariyomo et al., 2013; Axling, 2022; Dahlbom et al., 2011; Fu et al. 2021; Nordberg et al., 2021; reviewed in Perals et al., 2017) and exploration (Baker et al., 2018; Careau et al., 2009; Dingemanse et al., 2002; Perals et al., 2017). Furthermore, exploration can also be measured using the free-choice exploration test, whereby an additional space is provided to an animal in its home or familiar environment and its latency and inspections numbers are recorded (Graham et al., 2018). The other problem is that a given trait may be referred to differently in different studies, such that boldness,

exploration and anxiety may be used to describe distance displayed in an open field test or the same name may be used for different traits, and so boldness has been used to describe response to predation risk (Frost et al., 2013), a novel object, and a novel environment (Ariyomo & Watt, 2012; see references in Roche et al., 2016).

Anxiety-like behaviour can be assessed using novelty suppressed feeding (inhibition of feeding behaviour by exposure to a novel stimulus), open field test, light/dark test, elevated plus maze test (an apparatus that is raised off the ground and includes four arms shaped like a cross with a central area and two of the arms have a wall and are open at the top), and stress-induced hyperthermia (reviewed in Belovicova et al., 2017). As aforementioned, some of these tests have been used to measure different traits because it lets researchers assess several behaviours. For example, in the open field test, willingness to explore a new environment could measure exploration, how much time an animal is active could measure activity, time spent to emerge from a shelter into a novel tank, time spent near to a novel object and movement rate could measure boldness, how much time spent in different area of a tank including thigmotaxis (wall hugging) could measure anxiety, so one testing method could provide data on more than one personality trait (Ariyomo & Watt, 2012; Ariyomo et al., 2013; Bechmann & Biro, 2013; Maximino et al., 2010a; Perals et al., 2017; Roy & Bhat, 2018).

Using multiple tests (called the triangulation approach) for measuring a one trait helps to determine the robustness and validity of the outcomes (Fontana et al., 2022). Anxiety can be distinguishable from other traits using same tests. Top avoidance in the novel tank diving test, thigmotaxis (wall hugging) in the open field test and scototaxis (light avoidance) in the can be used as indicative of anxiety in zebrafish (Richendrfer et al., 2012). Anxiety is induced by novelty, and so using anxiolytic drugs that inhibit anxiety in zebrafish, leads to a decrease in

top avoidance, thigmotaxis and light avoidance in these tests, hence the validity of using these tests in measuring anxiety in zebrafish (Richendrfer et al., 2012).

Repeatability can be estimated by assessing and analysing an individual's behaviour multiple times to establish differences in variation within and among individuals. Usually, repeatability is measured by analysing an individual's behaviour in the same context but at a dissimilar time (Stamps & Groothuis, 2010). Individual differences in behavioural traits have been extensively investigated, and some of them have been shown to be repeatable such as boldness and aggressiveness (Ariyomo et al., 2017; Roy & Bhat, 2018) and exploratory behaviour and activity (Baker et al., 2018; Thomsom et al., 2020) in the zebrafish and five personality traits, including exploratory tendencies, risk-taking behaviour, activity, neophobia, and obstinacy (an individual's struggling intensity after being caught) in female zebra finches, *Taeniopygia guttata* (David et al., 2011). However, no studies have investigated the consistent variation in anxiety in zebrafish among different contexts.

In general, animals stressed by being handled or being in a new apparatus can change their behaviour, which can be attributed to their innate responses (see references in Roche et al., 2016). Recording an animal's innate responses to novel stimuli is the common way to measure anxiety (Lucon-Xiccato et al., 2020a). Anxiety is a reaction that reflects an individual's defence when faced with threats (Marks & Nesse, 1994). Several methods have been developed to assess zebrafish's behavioural activities. The novel tank diving and open field tests are believed to be the most robust tests to assess anxious responses in zebrafish (see references in Lucon-Xiccato et al., 2020; Maximino et al., 2011). In the novel tank diving test, anxiety can be measured according to an individual's innate reaction to initially swim to the bottom and reduce their activities in a novel environment (Audira et al., 2018; Cachat et al.,

2010). Although fish have an innate preference for the lower part of a novel tank when transferred to one, they will gradually swim to the upper areas and explore the new environment (Blaser & Rosemberg, 2012; Kysil et al., 2017; Levin et al., 2007). The tanks used in the novel tank diving test are horizontally divided into two (Cachat et al., 2011) or three sections (Levin et al., 2007; Mezzomo et al., 2016; Mocelin et al., 2015; Pilehvar et al., 2019). In the open field test, anxiety can be measured according to a fish's preference for the outer or central area of the open field tank (Lucon-Xiccato et al., 2020; reviewed in Maximino et al., 2010a). A preference for the outer edges over its inner ones is referred to as thigmotaxis or "wall hugging" or "boundary hugging", and is an indication of anxiety because it rises as the animal becomes more anxious (Anderson et al., 2006; Dahlén et al., 2019; Schnörr et al., 2012; Seibenhener & Wooten, 2015). The open field test consists of a circle, square, or rectangular enclosure with surrounding edges to avoid an escape (Gould et al., 2009). The light/dark or scototaxis test has also been validated and has been used for assessing anxiety-like behaviour in zebrafish (Dahlén et al., 2019). This test, which measures zebrafish's preference for light or dark sections of the tank, has shown that adults prefer the dark sections and larvae usually prefer the light sections, and this is thought to be an adaptation to predator avoidance (Dahlén et al., 2019). Gaining consistent results using multiple tests can establish the validity of an assay (Maximino et al., 2012). Although anxious responses' ability to help in drug improvement, neurobiological and psychopathological investigations (Harro, 2018) has been extensively reported (Cachat et al., 2010; Dahlén et al., 2019; Lucon-Xiccato et al., 2020; reviewed in Maximino et al., 2010a; 2010b), the consistent variation in anxious responses between contexts and the consideration of this behaviour as a personality trait has not been yet investigated.

The aim of this study was to assess individual differences in anxiety and determine whether this behaviour can be considered a personality trait in zebrafish. We assessed variation

in the level of anxiety within and between zebrafish using three different tests: novel tank diving, open field, and light/dark. Many researchers have found that when multiple tests are used to measure one trait, the behavioural responses may not be correlated. For instance, responses to a novel food test and a threatening novel stimulus test used to measure boldness were not correlated (Carter et al., 2013). However, Burnes (2008) used open-field, emerge, and novel-object tests to assess boldness in guppies (*Poecilia reticulata*), and he found that emerge and open-field tests were correlated with each other but not with the novel-object test, which suggested that the latter was not a good measure of boldness in guppies. To avoid the problem of using multiple tests to measure one trait, a data reduction method such as principal component analysis (PCA) can be used to investigate whether these measurements are loaded with others into the same component and if there is a correlated between tests, and if they measure the same trait (Carter et al., 2013). PCA converts a large number of original variables that are possibly correlated into equal or a smaller number of linearly uncorrelated variables named principal components (Saccenti & Camacho, 2015). We investigated whether variation in the behaviours measured were consistent and repeatable by repeating the whole procedure, as some researchers have shown that conducting two tests in a short period of time is sufficient to validate the repeatability of the behaviour (Thomson, 2020). Repeatability might reduce as the time between tests increases due to some genetic and environmental changes that might affect a target trait (Bell et al., 2009). From this perspective, the three tests were repeated at two-week intervals. The association between the three tests in assessing anxiety was investigated using PCA, as well as any sex-dependent differences in anxiety.

2.3 Methodology

2.3.1 Study animals

Adult zebrafish (age 12-20 months) were reared in an aquarium maintained in the Department of Animal and Plant Sciences at the University of Sheffield. Before the tests were carried out, fish were sexed, and males and females were kept individually in 10 l holding tanks (30 x 15cm, 24.5 cm high; Fig. 2.1) in a recirculatory system at 26–27 °C and on a 12:12 h light: dark cycle. Fish were fed twice a day with brine shrimp (*Artemia sp*) and commercial dry food. The length of each fish, from the tip of its snout to the caudal fin, was measured using a ruler by gently netting the fish while in the tank and holding it in position. Mass was also taken by weighing each fish in a known weight of water. Next, 60 males (standard length $\pm$ SE 3.06 $\pm$ 0.11 cm, standard mass $\pm$ SE 1.20 $\pm$ 0.15 g) and 60 females (standard length $\pm$ SE 3.68 $\pm$ 0.07 cm, standard mass $\pm$ SE 1.65 $\pm$ 0.10 g) were transferred to separate labelled tanks on the recirculatory system. Immediately prior to behavioural testing, each fish was transferred from its individual tank to the test laboratory in a small tank (18.5 x 11.5 cm, 12 cm high; Fig. 2.2) containing 700 ml of heated dechlorinated water and held in this for 300 s for acclimatisation. The acclimatisation period is the time before a trial during which an animal recovers from the stress of being in a new environment (O'Neill et al., 2018). There is no standard acclimatisation time before experiments, as researchers have not investigated it in much detail (Makaras et al., 2021; Thompson et al., 2012). However, it can vary across species. For example, zebrafish have been acclimated for 60 s (Ariyomo et al., 2013; Ariyomo & Watt, 2012), 15 s (Thörnqvist et al., 2019) and 3 d (Jolles et al., 2015) before an experiment. In contrast, oysters, *S. glomerata* have been acclimated for 2, 7, 10 and 14 d before an experimanet (see references in Thompson et al., 2012). Moreover, Makaras et al. (2021) compared the optimal acclimation period before an experiment of different species of fish that included rainbow trout, *Oncorhynchus mykiss*, Atlantic salmon, *Salmo salar*, three-spined stickleback, *Gasterosteus aculeatus* and European perch, *Perca fluviatilis.* They found that the minimum time for acclimation is 1 h for the three-

spined stickleback and 2 h for the rainbow trout and Atlantic salmon. In these experiments, 300 s was chosen because the fish appeared to have adjusted to the new environment in this time.

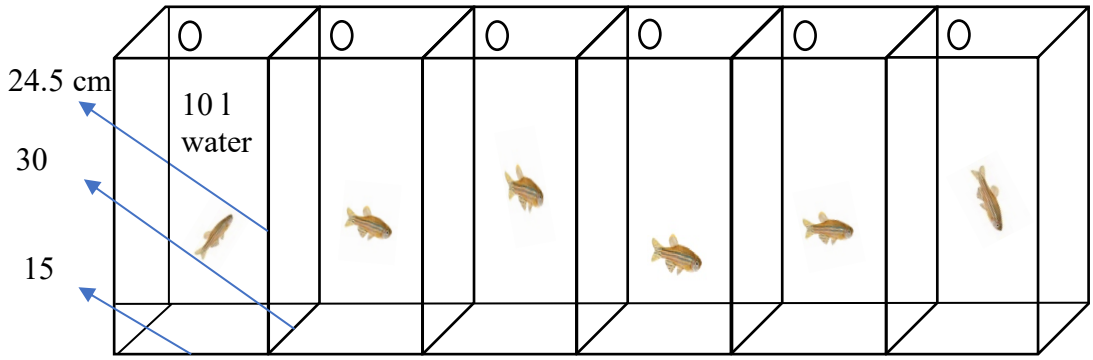

Figure 2.1. Separate tanks for housing zebrafish prior to behavioural testing.

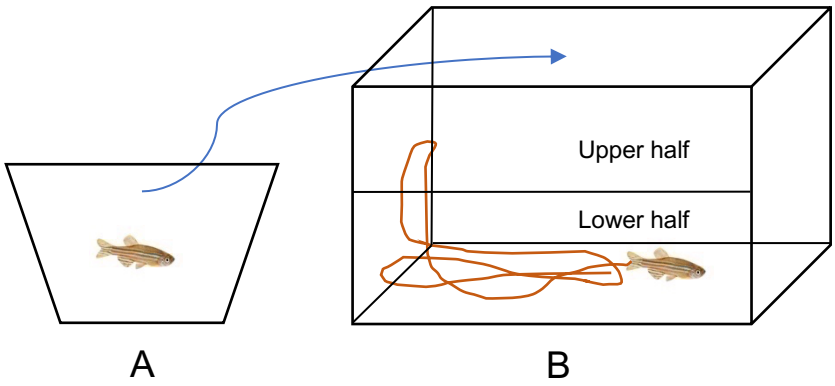

Figure 2.2. Small pre-test tank (A) and horizontally marked novel tank (B) used for assessing anxiety in zebrafish.

2.3.2 Assessing of anxiety

2.3.2.1 Novel tank diving test (NTDT)

Each fish was transferred from the small tank to a novel tank (25 x 15 cm, 15 cm high; Fig. 2.2). The novel tank was marked horizontally into two equal sections and contained 4 L of dechlorinated water heated to 27°C. The tank was placed on a flat, translucent platform raised by 10 cm by four supports and illuminated from below by two 10-W LED tube lights. A digital camera (Panasonic HC-V160) was placed in front of the tank for recording the behavioural tests (Fig. 2.3).

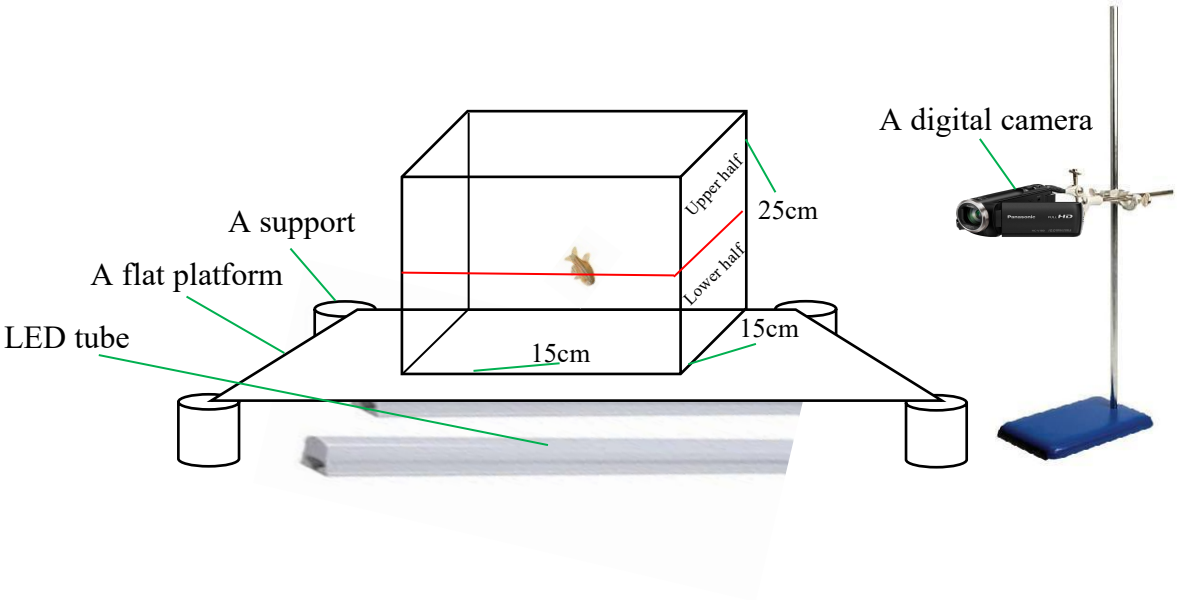

Figure 2.3. Experimental setup for NTDT.

Each fish was removed from the transfer tank and placed in the diving tank and after 60 s its behaviour was recorded for 300 s using the digital camera. Each fish was returned to its labelled individual tank in the aquarium after testing. Water in the novel tank and the small tank was replaced after each trial. Behaviours were analysed from the videos, and these included latency to enter the upper half of the tank, number of entries to the upper half of the

tank and time spent in the upper half of the tank. Fish that had a lower latency to enter the upper half of the tank, had more entries into the upper half of the tank and spent more time in the upper half of the tank were regarded as least anxious, while fish that had a higher latency, had fewer entries and spent less time in the upper half of the tank were regarded as most anxious.

2.3.2.1.1 Consistency and repeatability of anxiety

The whole procedure was repeated immediately after the first test has done to test the repeatability and consistency of these behaviours in the same fish.

2.3.2.2. Open field test (OFT)

Fish (60 females and 59 males) tested previously for anxiety using the NTDT were tested again using the OFT approximately 1 week later. At the beginning of each trial, each fish was transferred from its individual tank to the test laboratory in a small tank (18.5 x 11.5 x 12 cm; Fig. 2.4) filled with 700 ml of heated dechlorinated water where it was held for 300 s for acclimatisation. After this period, the fish was immediately transferred from the small tank to an open field tank (48 x 23 cm, 26 cm high; Fig. 2.4) and left for 60 s for acclimatisation. This tank was marked on its base into 24 rectangles (8 x 6 cm) and contained 3 L of dechlorinated water heated to 27°C. The tank was lit from below using a 40-W LED panel (600 x 600 mm) (Element Lighting, Colchester, UK). A digital camera (Panasonic HC-V160) was held over the top of the tank using a stand and clamp for behavioural recording for 300 s (Fig. 2.5). Movement of fish into the eight rectangles in the centre of the tank with the dimensions of 32 x 12 cm was considered to reflect a lower level of anxiety (Fig. 2.5).

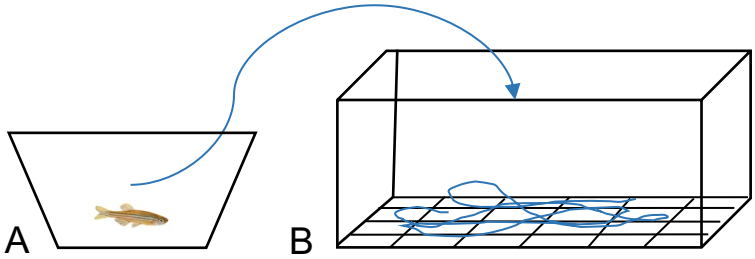

Figure 2.4. Small pre-test tank (A) and open field tank (B) used for assessing anxiety in zebrafish.

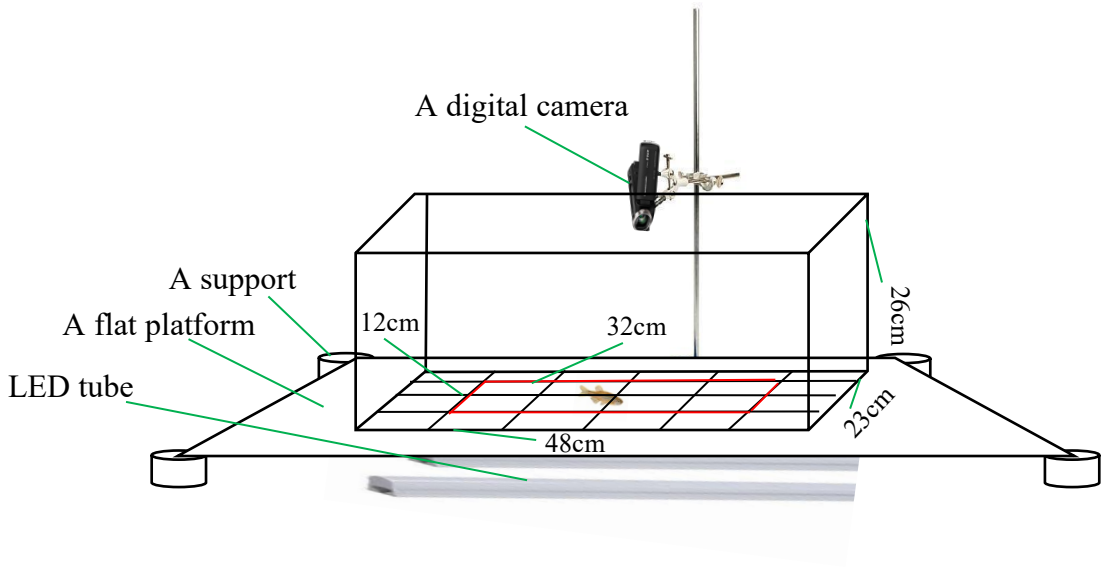

Figure 2.5. Experimental setup for OFT.

The same procedure as for the NTDT was followed (see Section 2.2.2.1), except that the behaviours recorded included the number of lines crossed by each fish and the time spent in the eight rectangles described above. Individuals that crossed more lines and spent more time in the rectangles in the centre of the tank were considered least anxious, while those that crossed fewer lines and spent less time in those rectangles were considered most anxious.

2.3.2.2.1 Consistency and repeatability of anxiety

The whole procedure was repeated immediately after the first test has done to test the repeatability of these behaviours and consistency in the same fish.

2.3.2.3 Light/dark test (L/DT)

Fish (60 females and 59 males) tested for anxiety using the NTDT and OFT were tested again using the (L/DT) about 1 week later. A similar procedure to the other two tests was followed (see Section 2.2.2.2), but a different tank was used. At the beginning of a trial, each fish was moved from its holding tank to the test laboratory in a small tank (18.5 x 11.5 x 12 cm; Fig. 2.6) containing 700 ml of heated dechlorinated water where it was held for the 300 s acclimatisation period. Fish were immediately transferred from the small tank to a light/dark tank (21 x 13 cm, 14 cm high) and held for 60 s acclimatisation. The L/D tank was vertically divided into light and dark sections using an outer covering of white and black paper and contained 3 L of dechlorinated water heated to 27°C (Fig. 2.6). The tank was lit from below using a 40-W LED panel (600 x 600 mm) (Element Lighting, Colchester, UK), and a stand and clamp were used to hold a digital camera (Panasonic HC-V160) over the top of the tank for behavioural recording (Fig. 2.7).

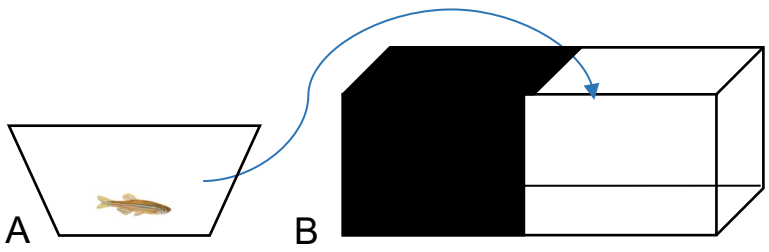

Figure 2.6. Small pre-test tank (A) and light/dark tank (B) used for assessing anxiety in zebrafish.

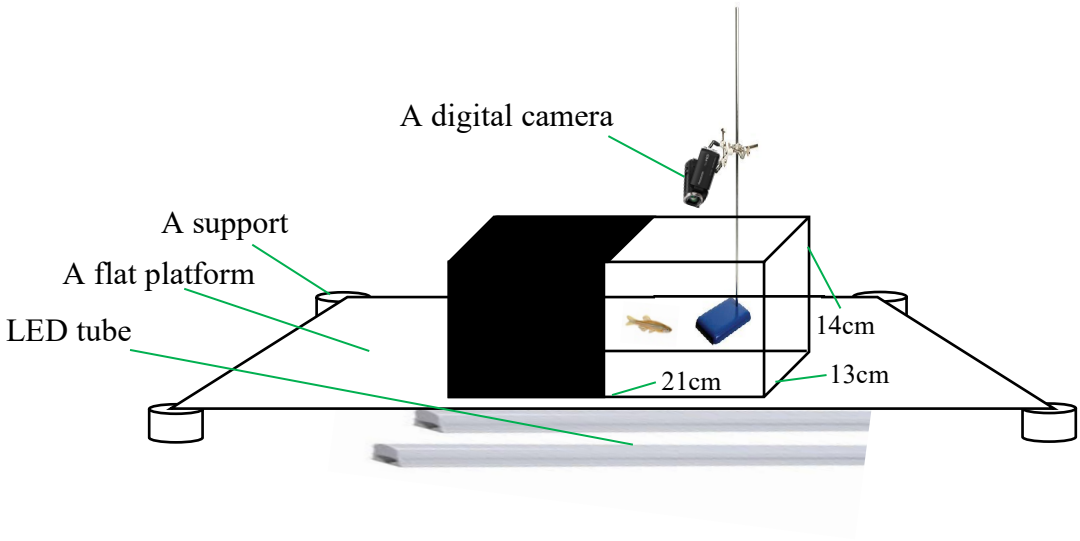

Figure 2.7. Experimental setup for L/DT.

Time spent in the light section was recorded. Fish that spent more time in the light section were deemed to be less anxious because of their instinctive preference for dark places for hiding purposes (Serra et al., 1999).

2.3.2.3.1 Consistency and repeatability of anxiety

Fish were tested again after the first one has done to test the repeatability and consistency of these behaviours.

2.3.3. Statistical analysis

2.3.3.1. Behavioural tests

For the NTDT, video recordings were processed manually, and data were statistically analysed using R software (version 1.1.383, R Core Team, 2017). For the OFT and L/DT, video recordings were processed both using the Viewpoint software program (http://www.viewpoint.fr/en/home) and manually due to some difficulties in fish movement

tracking using the program. The data were statistically analysed using R software (R Core Team, 2017).

A generalised linear mixed model was fitted to the data using the *glmer* function from the lme4 package in R (version 1.1.383; R Core Team, 2017). This model was fitted with the three variables measured in the NTDT (latency to enter the upper half of the tank, number of entries to the upper half of the tank, time spent in the upper half of the tank), the two variables measured in the OFT (number of lines crossed and time spent in the centre of the tank) and the one variable measured in the L/DT (time spent in the light half of the tank) as the response variables; sex (male and female) and test (Tests 1 and 2) as fixed effects variables; and fish identity as a random effects variable. The purpose was to determine whether the fixed effects variables significantly affected the response and whether there was an interaction between sex and test.

2.3.3.2 Repeatability

The repeatability within individuals of the different behavioural measures recorded for each of the three tests was estimated using the *rptr* function from the rptR package in R (version 1.1.383; R Core Team, 2017). Repeatability is represented by the intra-class correlation coefficient (ICC), and it is described as the proportion of the total variance attributed to within-individual variance (Nakagawa & Schielzeth, 2010). The rptR model was fitted with the datatype = "Poisson" to estimate the repeatability while accounting for the Poisson-distributed count data. Fish identity was included as a random effect variable, and fish sex (male and female) was included as a fixed effect variable to account for pseudoreplication and sex-specific effects. Likelihood ratio tests (LRTs) were used to determine whether the estimates of

model-based repeatability were statistically significant (see references in Nakagawa & Schielzeth, 2010).

Parametric bootstrapping (n=1000) was used to estimate 95% confidence intervals (CI) (Chaverri & Gillam, 2015; Nakagawa & Schielzeth, 2010; Whittaker et al., 2021). Repeatability of behaviours was considered depending on the lower 95% confidence interval when it exceeds zero (Whittaker et al., 2021). In two meta-analyses, it has been demonstrated that the average repeatability estimate of behavioural traits is between 0.4 and 0.48 with using two tests at short time intervals (Wuerz & Kruger, 2015). Repeatability estimates were deemed low when it was lower than 0.2, moderate when it ranged from 0.2 to 0.4 and strong when it was above 0.4 (Whittaker et al., 2021). Other researchers deemed repeatability slight when it is lower than 0.2, low when it is around 0.2 and 0.4, moderate when it is around 0.4-0.7, high when it is around 0.7 and 0.9 and very high when it is around 0.9 (Wuerz & Kruger, 2015). Moreover, when repeatability is close to one, it is considered high while when it is significantly less than one, it is considered low (Dohm, 2002).

Repeatability analysis was done on both the original and log-transformed data that depended on using the log-link Poisson distribution, which resulted in outputs of the original-scale approximation and link-scale approximation (transformed data) (Chaverri & Gillam, 2015). However, in the results, only the repeatability measure on the original scale is reported.

2.3.3.3 Principal component analysis (PCA)

Once all the tests were completed and the repeatability of the measures was calculated, a PCA was used to decrease the six behavioural measures recorded using the NTDT, OFT and L/DT to fewer dimensions to be able to assess the correlation between the behaviours in

estimating individual variation in anxiety. Based on the PCA results, individual variation in anxiety was ordered depending on the scores. Two separate PCAs were carried out for males and females in the case of sex-specific differences. The PCAs were fitted using the *Principal* function from the *psych* package (Revelle, 2017) in R (version 1.1.383; R Core Team, 2017) with *varimax* rotation. According to parallel statistical analysis using the *paran* function of the *paran* package (Dinno, 2012), the variation in these measures was explained by two principal components, which accounted for 65–67% of the total variation. The threshold for traits loading onto the components was >0.6, with all traits loading onto a component except one for females. The overall Kaiser-Meyer-Olkin value (a measure of correlation matrix sampling adequacy (Budaev, 2010) was 0.59 for females and 0.67 for males, and each trait correlated > 0.5 with at least one other trait and no traits correlated > 0.9. Although all components were associated with anxiety, each fish was just ranked according to its loading scores onto Component 1 that was associated with the NTDT, as this component represented most of the variation in anxiety within individuals (59% and 65% for females and males, respectively). While the second component was associated with the open field test that explained the remaining variation (41 % and 35 % for females and males respectively). Ranking was conducted using the *order* function in R, with males and females ranked separately (see Supplementary Tables 2.1 and 2.2). As Component 1 was explained by traits associated with anxiousness, this ranking allowed fish to be grouped into least anxious, most anxious and intermediately anxious. Only the six least and six most anxious fish of each sex were used in further work (Chapter III) to prevent overlap in the scores.

2.4 Results

2.4.1 Behavioural tests

2.4.1.1 NTDT

2.4.1.1.1 Latency to enter upper half of tank

There was a significant difference between the sexes, with males having a lower latency to enter the upper half of the NTDT compared to females (estimate = -0.734, SE = 0.29, $z = -2.54$, $p < 0.05$; Fig. 2.8A). There was also a significant difference between test 1 and test 2, in which test 2 fish had lower latency to enter the upper half of the tank compared to test 1 fish (estimate = -0.51, SE = 0.02, $z = -27.490$, $p < 0.001$; Fig. 2.8A). There was a significant interaction between sex and test in the latency to enter the upper half of the NTDT, which differed between test 1 and test 2 and also depended on sex, with females having a lower latency in the test 2 (estimate = 0.424, SE = 0.03, $z = 15.332$, $p < 0.001$; Fig. 2.9A).

2.4.1.1.2 Number of entries to upper half of tank

There was no significant difference between males and females in the number of entries (estimate = 0.293, SE = 0.220, $z = 1.330$, $p = 0.183$; Fig. 2.8B). There was a significantly higher number of entries during test 2 than test 1 (estimate = 0.177, SE = 0.045, $z = 3.962$, $p < 0.001$; Fig. 2.8B). There was also a significant interaction between sex and test, which significantly affected the number of entries to the upper half of the NTDT with females displayed higher number of entries in test 2 (estimate = -0.161, SE = 0.063, $z = -2.57$, $p < 0.05$; Fig. 2.9B).

2.4.1.1.3 Time spent in upper half of tank

There was no significant difference between males and females in the time spent in the upper half of the NTDT (estimate = 0.43, SE = 0.284, $z = 1.514$, $p = 0.13$; Fig. 2.8C). The time spent was significantly higher during test 2 than test 1 (estimate = 0.270, SE = 0.03, z =

10.29, $p < 0.001$; Fig. 2.8C). There was a significant interaction between sex and test, which significantly affected the time spent in the upper half of the NTDT with females spent much time in the upper half of the tank in the test 2 (estimate = -0.402, SE = 0.04, z = -10.820, $p < 0.001$; Fig. 2.9C).

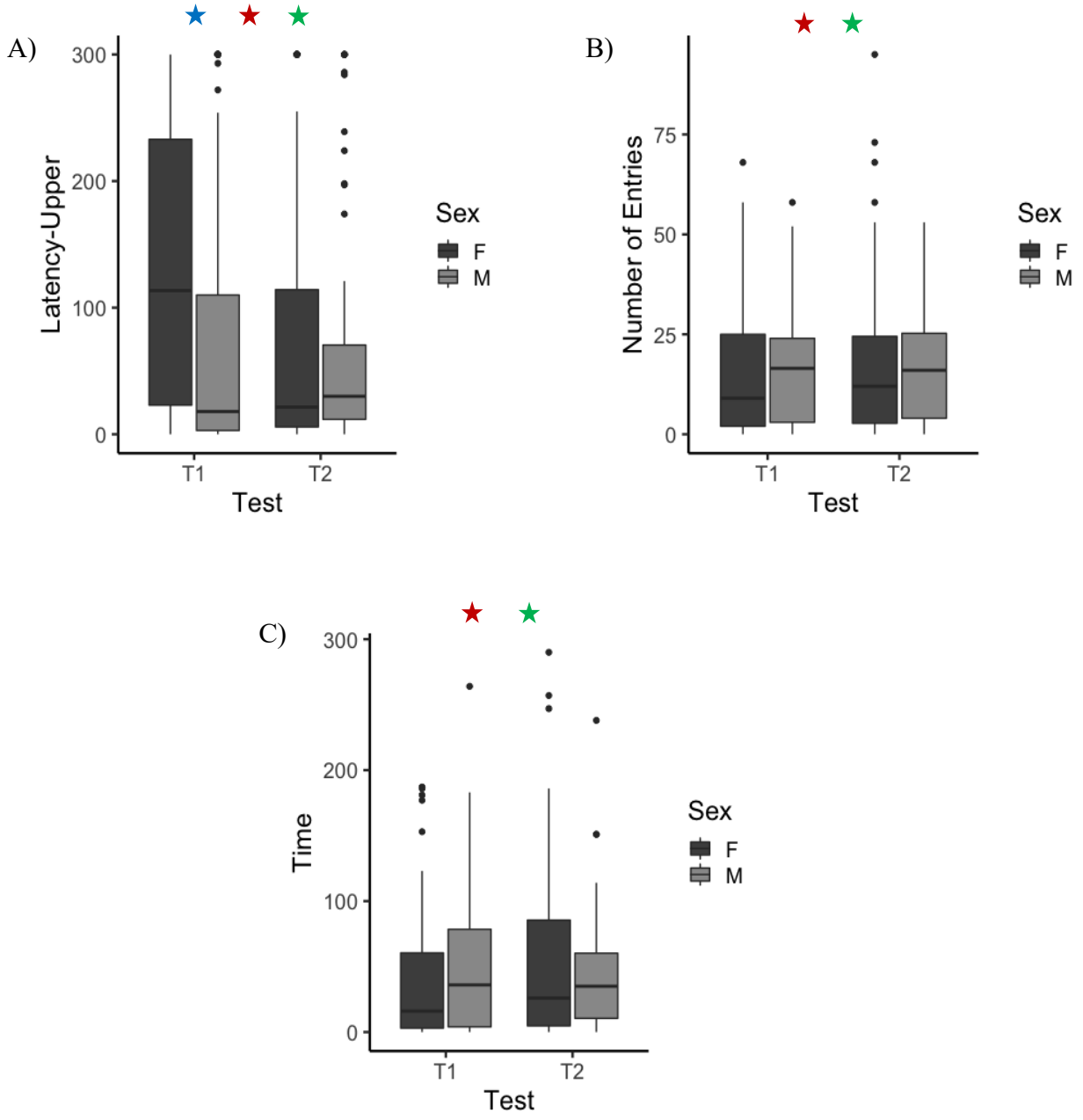

Figure 2.8. Medians (horizontal lines within boxes), interquartile ranges (second quartile refers to box under median, and third quartile refers to box above median) and outliers (circles) for (A) latency to enter upper half of tank, (B) number of entries to upper half of tank and (C) time spent in upper half of tank by female and male zebrafish for first and second NTDTs. Stars above the plot indicate significant differences: blue star, between males and females; red star between the first test and second test; green star interaction between sex and test.

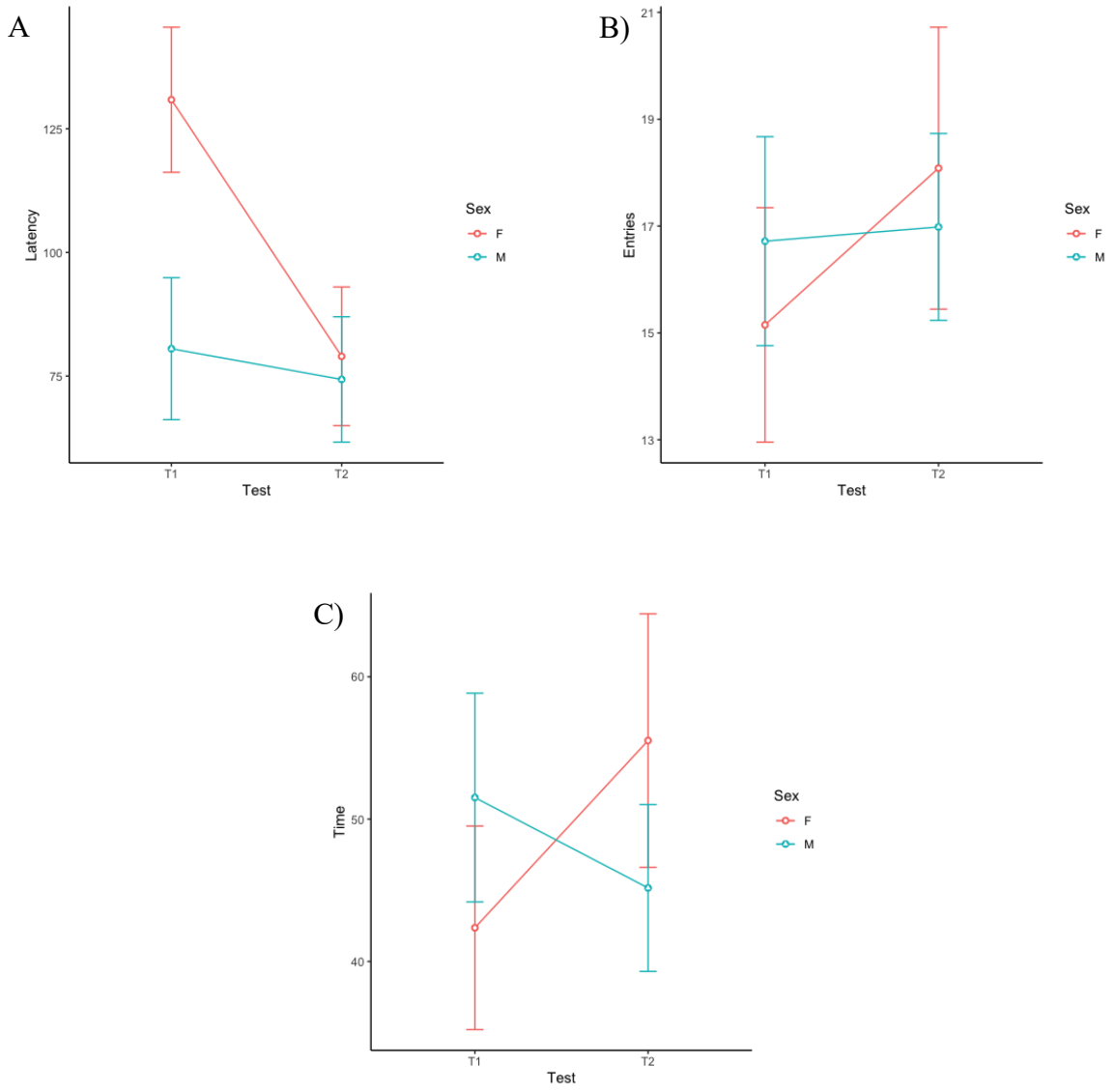

Figure 2.9. The effect of interaction between sex (males versus female) zebrafish and tests (test 1 versus test2) on (A) latency to enter the upper half, (B) number of entries and (C) time spent in the upper half of the novel tank diving test.

2.4.1.2 OFT

2.4.1.2.1 Number of lines crossed

There was a significant difference between males and females, with males crossing a significantly greater number of lines than females (estimate = 0.14, SE = 0.011, z = 12.61, p < 0.001; Fig. 2.10A). However, there was no significant difference between test 1 and test 2 (estimate = -0.014, SE = 0.011, z = -1.21, p = 0.24; Fig. 2.10A). There was an interaction between sex and test, which significantly affected the number of lines crossed (estimate = 0.12, SE = 0.02, z = 7.62, p < 0.001; Fig. 2.11A), with males crossing more and females crossing less lines in test 2 than test 1.

2.4.1.2.2 Time spent in centre

There was a significant difference between males and females, with males spending less time in the centre than females, which significantly affected the response (estimate = -0.05, SE = 0.021, z = -2.28, p < 0.05; Fig. 2.10B). There was also a significant difference between test 1 and test 2, with individuals spending less time in the centre in test 2 than test 1 (estimate = -0.1, SE = 0.02, z = -2.67, p < 0.01; Fig. 2.10B). There was no significant interaction between sex and test (estimate = 0.012, SE = 0.03, z = 41, p = 0.6855; Fig. 2.11B).

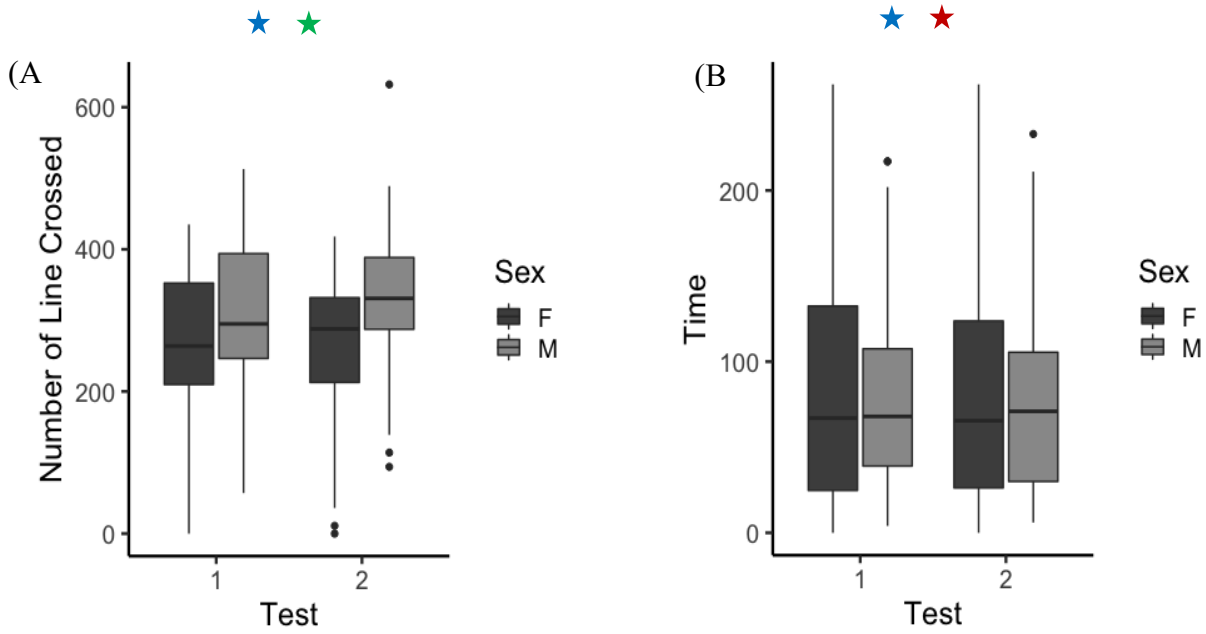

Figure 2.10. Medians (horizontal lines within boxes), interquartile ranges (second quartile refers to box under median and third quartile refers to box above median) and outliers (circles) for (A) number of lines crossed and (B) time spent in centre of tank by female and male zebrafish for first and second OFTs. Stars above the plot indicate significant differences: blue star, between males and females; red star between the first test and second test; green star interaction between sex and test.

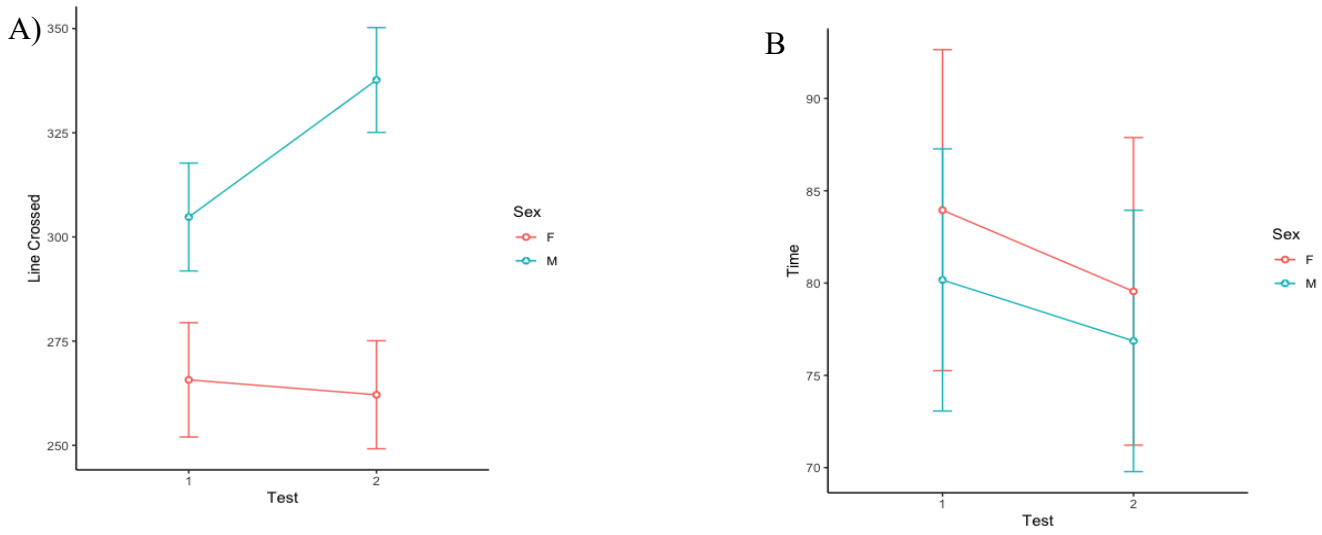

Figure 2.11. The effect of interaction between sex (males versus female) zebrafish and tests (test 1 versus test 2) on (A) number of lines crossed and (B) time spent in the centre in the open field test.

2.4.1.3 L/DT

2.4.1.3.1 Time spent in light

Males spent significantly more time in the light half of the tank compared to females (estimate = 0.561, SE = 0.233, z =2.41, p = 0.02; Fig. 2.12). There was a significant difference between test 1 and test 2, with individuals spending less time in the light half of the tank in test 2 than test 1 (estimate = -0.394, SE = 0.03, z = -15.697, p < 0.001; Fig. 2.12). There was a significant interaction between sex and test, which significantly affected the time spent in the light half of the tank during the light/dark test with both males and females spent less time in the light half of the light/dark test in the test 2 (estimate = 0.331, SE = 0.032, z =10.28, p < 0.001; Fig. 2.13).

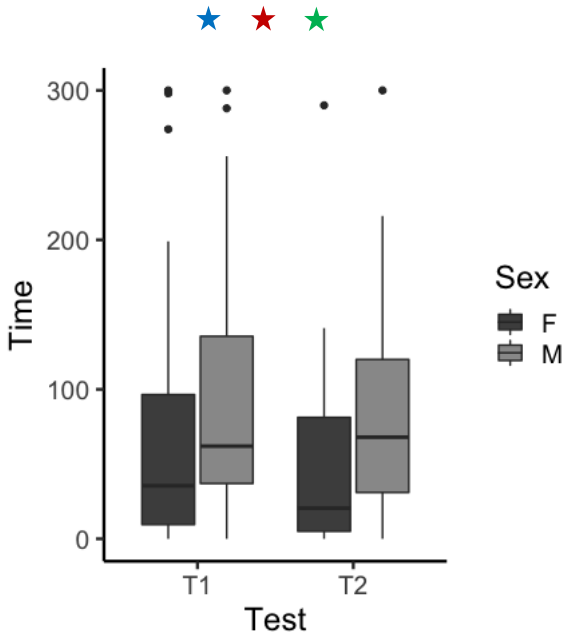

Figure 2.12. Medians (horizontal lines within boxes), interquartile ranges (second quartile refers to box under median and third quartile refers to box above median) and outliers (circles) for time spent in light half of tank by female and male zebrafish for first and second L/DTs. Stars above the plot indicate significant differences: blue star, between males and females; red star between the first test and second test; green star interaction between sex and test.

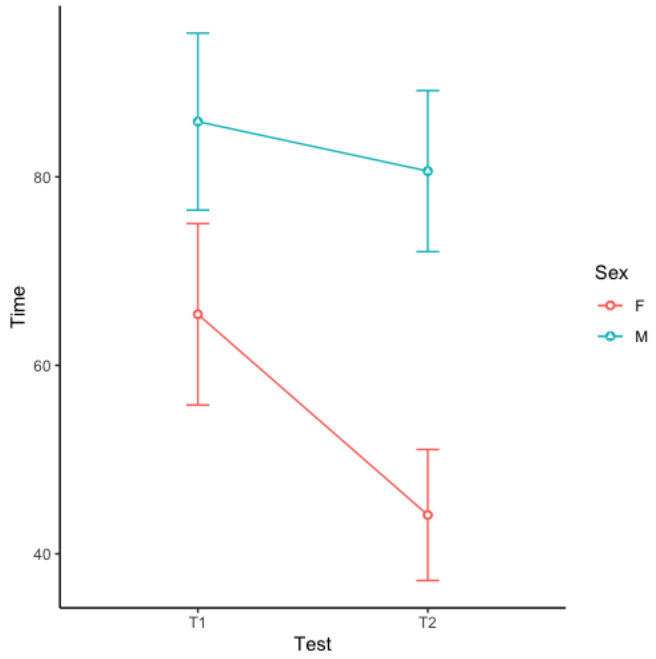

Figure.2.13. The effect of interaction between sex (males versus female) zebrafish and tests (test 1 versus test 2) on time spent in the light half of the light/dark test.

2.4.2 Repeatability

2.4.2.1 NTDT

Table 2.1 shows the results of the repeatability analysis. The behavioural traits in the NTDT were significantly repeatable, and they ranged from 0.09 to 0.36 for original-scale approximation.

2.4.2.2 OFT

The behavioural traits in the OFT were significantly repeatable, and they ranged from 0.46 to 0.68 for original-scale approximation (Table 2.1).

2.4.2.3 L/DT

The behavioural trait in the L/DT was significantly repeatable, and it was 0.31 for original-scale approximation (Table 2.1).

Table 2.1. Estimated repeatability of behaviours with 95 % confidence intervals in male and female zebrafish in NTDT, OFT and L/DT using glmm method and log link.

Behaviour recorded in NTDT, OFT and L/DT for male and female zebrafish	**Original-scale approximation of R (CI) ± SE df P value**
Latency period to move to upper half of tank (NTDT)	0.09 (0.04, 0.17) ± 0.03 1 P < 0.001
Number of entries to upper half of tank (NTDT)	0.36 (0.24, 0.50) ± 0.06 1 P < 0.001
Time spent in upper half of tank (NTDT)	0.22 (0.14, 0.35) ± 0.05 1 P < 0.001
Number of lines crossed (OFT)	0.68 (0.58, 0.76) ± 0.04 1 P < 0.001
Time spent in centre (OFT)	0.46 (0.33, 0.57) ± 0.06 1 P < 0.001
Time spent in light half (L/DT)	0.31 (0.20, 0.42) ± 0.06 1 P < 0.001

2.4.3 PCA

For females, two components explained 65% of the total variance; the first component explained 38%, and the second explained 27%. The traits loaded onto the two components could all be interpreted as measures of anxiety, with those loading onto Component 1 measured in the NTDT and those loading onto Component 2 measured during both the OFT and L/DT. Three behavioural measures loaded onto the first component, with latency to enter the upper half negatively loaded (-0.88) and the number of entries and the time spent in the upper half of the tank in the NTDT positively loaded (0.86 and 0.87, respectively; see Fig. 2.14A, X axis; Tables 2.2 and 2.3). Two traits, the number of lines crossed and the time spent in the centre of the tank in the OFT, loaded positively onto the second component (0.89 and 0.85, respectively; Fig. 2.14A, Y axis; Tables, 2.2 and 2.3). The final behavioural measure of time spent in the light half in the L/DT loaded most strongly onto Component 2, but this did not reach the loading threshold of 0.6 (0.31).

For males, two components explained 67% of the total variance, with the first component explaining 44% and the second explaining 24%. The same three traits loaded onto the first component in males as in females. Latency to enter the upper half of the tank was negatively loaded (-0.83), and the number of entries and time spent in the upper half of the tank in the NTDT were positively loaded (0.93 and 0.89, respectively; Fig. 2.14B, X axis; Tables 2.4 and 2.5). For the second component, the number of lines crossed (0.72) and the time spent in the centre of the tank in the OFT (0.66) loaded positively, while the time spent in the light during the L/DT loaded negatively (-0.62) (see Fig. 2.14B, Y axis; Tables 2.4 and 2.5).

Based on the results, each fish was assigned a loading score for the first component. The traits loading onto the first component were generally associated with anxiousness. Thus, fish with high loading scores for the first component were deemed less anxious, while those

with lower loading scores on the first component were deemed more anxious (see Supplementary Tables 2.1 and 2.2).

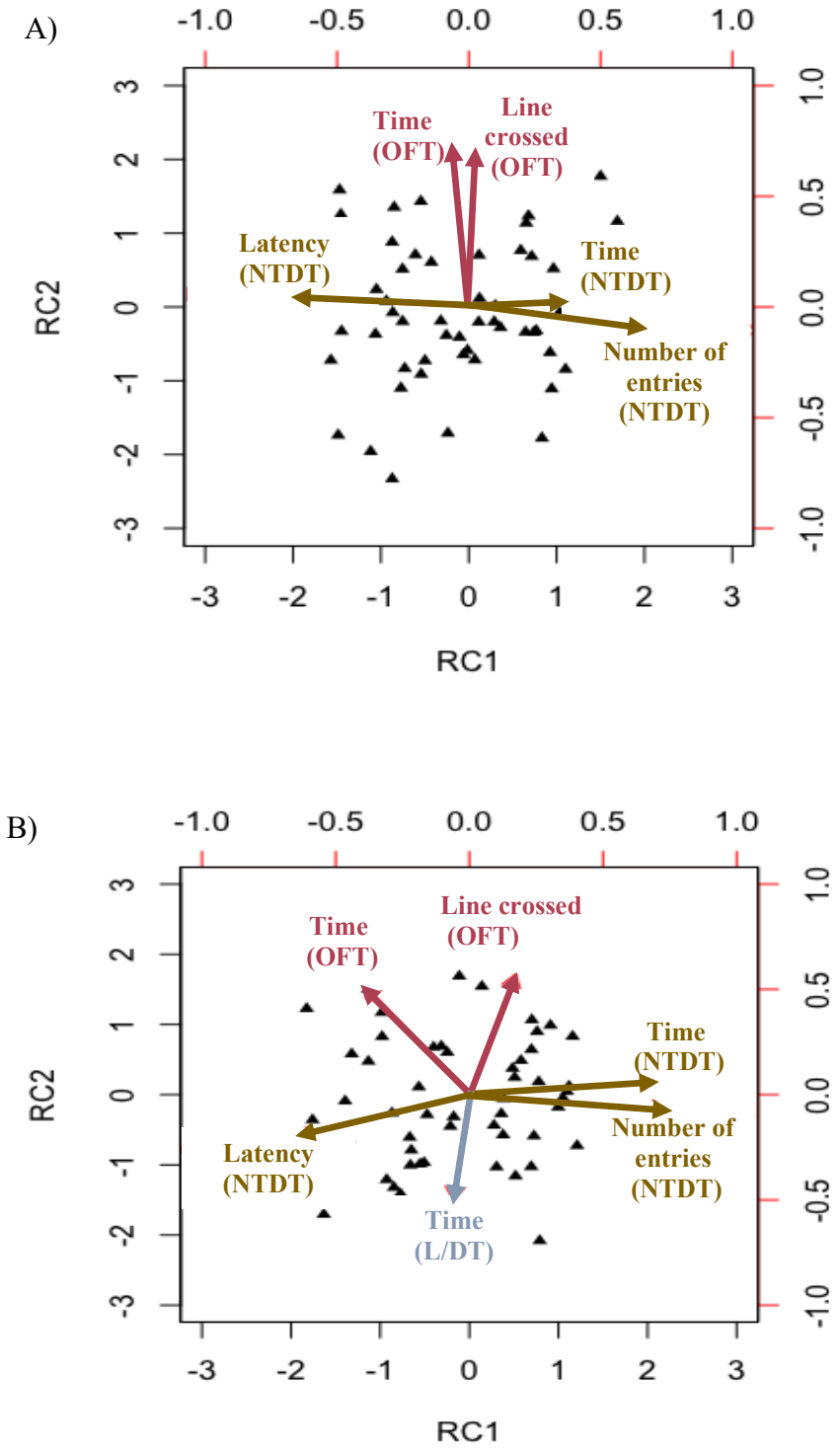

Figure 2.14. Bioplot of the behavioural measures of anxiety from NTDT, OFT and L/DT in (A) female and (B) male zebrafish with respect to the principle components.

Table 2.2. Principal component loading matrix of anxiety assessed in NTDT, OFT and L/DT loaded onto each component for female zebrafish. The traits loaded onto each component are presented in bold (>0.6).

Test	Behaviours	RC1	RC2
NTDT	latency to upper	**-0.88**	0.05
NTDT	number of entries	**0.86**	-0.02
NTDT	time spent in upper	**0.87**	0.08
OFT	number of lines crossed	-0.01	**0.89**
OFT	time spent in centre	-0.12	**0.85**
L/DT	time spent in light	0.04	0.31

Table 2.3. Total variation in anxiety explained by principal components of female zebrafish.

	RC1	RC2
The sum of square loadings	2.3	1.62
Proportion of variance explained	0.38	0.27
Cumulative of variance explained	0.38	0.65
Proportion Explained	0.59	0.41
Cumulative Proportion	0.59	1

Table 2.4. Principal component loading matrix of anxiety assessed in NTDT, OFT and L/DT loaded onto each component for male zebrafish. The traits loaded onto each component are presented in bold (>0.6).

Tests	Behaviours	RC1	RC2
NTDT	latency to upper	**-0.83**	-0.26
NTDT	number of entries	**0.93**	-0.09
NTDT	time spent in upper	**0.89**	0.08
OFT	number of lines crossed	0.22	**0.72**
OFT	time spent in centre	-0.49	**0.66**
L/DT	time spent in light	-0.08	**-0.62**

Table 2.5. Total variation in anxiety explained by principal components of male zebrafish.

	RC1	RC2
The sum of square loadings	2.62	1.41
Proportion of variance explained	0.44	0.24
Cumulative of variance explained	0.44	0.67
Proportion Explained	0.65	0.35
Cumulative Proportion	0.65	1

2.5 Discussion

The main aims of this experiment were to assess individual variation in the level of anxiety-like behaviour and to determine whether this variation was consistent and repeatable between three different tests: the novel tank diving test, the open field test and the light/dark test. Furthermore, this experiment aimed to determine whether there was a significant effect of sex and test on anxiety-like behaviours measured using the novel tank diving test, open field test and light/dark test. It also investigated whether there was an interaction effect between sex and test on anxiety-like behaviour.

Overall, females and males differed significantly in the behaviours measured. These included latency to enter the upper half of the tank in the novel tank diving test, which was significantly lower in males compared to females, as well as number of lines crossed in the open field test and time spent in the light area in the light/dark test, which were significantly higher in males compared to females, suggesting that females were more anxious than males. These findings were consistent with those reported by Genario et al. (2020), who found that females exhibited more anxiety-like behaviours than males. Furthermore, Fontana et al. (2019) demonstrated that females spent more time in the dark area in the light/dark test than males and spent more time in the bottom of the tank in the novel tank diving test compared to males. This suggested that females exhibit higher levels of anxiety-like responses than males and that this trait is sex dependent in zebrafish. The significant differences found between the sexes in this chapter might be due to variations in gonadal hormones, as is found in mammals and birds (Stamps & Groothuis, 2010), or central nervous system gene expression (Genario et al., 2020). Furthermore, in general, the differences shown between males and females in anxiety-like behaviours and movement levels may be explained by other factors such as exploratory

motivation, aggression, territoriality or reproductive behaviour (reviewed in Fontana et al., 2019; Tran & Gerlai, 2013).

This experiment showed that the first and second tests differed significantly in the behaviours measured. Individuals exhibited significantly lower latency to enter the upper half of the tank, showed a significantly greater number of entries to the upper half of the tank and spent significantly more time in the upper half of the tank in the second novel tank diving test compared to the first, suggesting that individuals became less anxious. Moreover, individuals spent significantly less time in the centre of the tank in the second open field test compared to the first. In addition, they spent significantly less time in the light half of the tank in the second light/dark test compared to the first, suggesting that individuals became more anxious. The inconsistency in behaviours may be partly explained by the number of trials, that is, a lower number of trials might decrease behavioural consistency due to sensitisation (an increase in reaction to stimuli, Blumstein, 2016) to the tests (Bell et al., 2009; Martin & Reale, 2008) or variation in acclimation (Biro, 2012). Individuals in this experiment were transferred from their home tank and put into a novel situation that might have been stressful, and measurement was only repeated two times, so sensitisation to novelty might have increased and acclimation before the tests might not have been achieved. This could explain why an individual's responses to the novel situation differed between the first and second tests. A study conducted on the boldness and activity of Ward's damselfish, *Pomacentrus wardi*, found that behaviours were not consistent using rapid tests and limited numbers of experiments, less than four times (Biro, 2012).

Although individuals showed inconsistent behaviours and responded differently in the second test compared to the first test, they responded in the same way, hence the significant repeatability. Having individual differences in behaviours and repeatable results suggesting

that anxiety is a personality trait. One explanation for the repeatability of individual variation reported in this study is that the conditions were stable, which prevented the fish from being subjected to environmental factors such as predation, starvation or group interactions and competition (Stamps & Groothuis, 2010). Therefore, it has been suggested that behaviour tested under such laboratory conditions is more repeatable (Bell et al., 2009). However, Bell et al. (2009) found that estimates of behaviour repeatability were lower in laboratory trials compared to the field trials. Another possible explanation is that these tests were conducted sequentially over a short period of time, with no more than two weeks between the first and second tests for each fish. It has been demonstrated that there is a significant correlation between behaviours measured by two tests no more than 30 days apart (reviewed in Spoolder et al., 1996). A long time between observations leads to ecological and physiological changes such as sexual maturity (Bell et al., 2009), and this may be due to age-dependent genes that affect individual traits and therefore their consistency and repeatability when the time between measurements is increased (reviewed in Bell et al., 2009). Additionally, Spoolder (1996) suggested that the consistency of personality traits decreases as an animal ages because an individual's reactions may become unstable according to the situation. Furthermore, animal behavioural variation has been shown to be more consistent in mature stages compared to earlier stages (Budaev et al., 1999). However, Verbeel et al. (1994) showed that the consistency of individual differences is not restricted to mature stages and can be shown in early stages of life too, such as in the exploratory behaviour of male great tits, *Parus major*. All individuals involved in the experiments described in this chapter were sexually mature.

The PCA of both male and female behaviour revealed that the most variation between individuals was clustered and presented in the first component, and these behaviours were measured using the novel tank diving test. The remaining variation was explained by

behaviours clustered and presented in the second component, and these were measured using the open field and light/dark tests in males and just the open field test in females. These results are in line with those of previous reports demonstrating that similar measurements could be used as indicators of anxiety. In previous studies in zebrafish, anxiety has been detected in the novel tank diving test as a decrease in movement leading to a higher latency to move to the upper half of the tank, a lower number of entries to the upper half of the tank and more erratic swimming and freezing (see references in Egan et al., 2009; Levin et al., 2007; Stewart et al., 2012). The preference for bottom dwelling is thought to be due to the fish avoiding the shallow/top section of the water (Maximino et al., 2012). The open field test has been used to assess an animal's willingness to explore novel environments (Dingemanse et al., 2002; Kalueff et al., 2006; Perals et al., 2017), but more recently, it has been developed to assess boldness, fear and anxiety via the phenomena of thigmotaxis (Lamprea et al., 2008; Perals et al., 2017). In the light/dark test, anxiety can be expressed as a light section avoidance (Fontana et al. 2022).

The results from this chapter indicated that there was not a correlation between the novel tank diving and open field tests but there was a correlation between the open field and light/dark tests in measuring anxiety in zebrafish. The results revealed that the first component loaded behaviours measured in the novel tank diving that were linearly uncorrelated to behaviours measured in the open field test and light/dark test loaded on the second component. The novel tank diving test and open field test are orthogonal in the analysis showing that there is no correlation between them. In males, the second component positively loaded behaviours measured in the open field test and these were related to low anxiousness, a greater number of entries and more time spent in the centre of the open field test. Moreover, this component negatively loaded behaviour measured in the light/dark tests and it related to high anxiousness,

less time spent in the light half of the light/dark test, suggesting that these tests have different endpoints in measuring anxiety in zebrafish. However, in females, behaviour measured in the light/dark test loaded in the second component with a small value (0.31) that did not reach the loading threshold of 0.6. These findings suggest that the open field and light/dark tests were negatively correlated in measuring anxiety in zebrafish. According to Budaev (2010), variables that are clustered and loaded onto a component usually correlate with each other and explain a significant amount of variance, and such variables are considered to share the same behavioural mechanism. For instance, a correlation of risk-taking measures usually refers to the trait of boldness (see references in Budaev, 2010).

Maximino et al. (2012) conducted a comparative review of the novel tank diving and light/dark tests in measuring anxiety that revealed insufficient findings to support a correlation between these tests, which led to the question of whether the novel tank diving and light/dark tests measure the same behaviours. For example, exposing zebrafish to a stressor such as fluoxetine using novel tank diving test and light/dark test lead to different outcome between the two tests (Maximino et al. 2012). Moreover, the current chapter raises the possibility that the reason behind the negative correlation between the open field test and the light/dark test is differences in genes and protein expression, which leads to variations in fish behaviours in those tests (Blaser & Rosemberg, 2012). The preference for the dark section in the light/dark test may be affected by stimuli, such as the way the tank is covered and the illumination rate, because some tanks have light/dark sections and others are black and transparent, which can lead to inconsistency in behavioural measures (see references in Maximino et al., 2012). For example, Stephenson et al. (2011) showed that in zebrafish, the preference for the light or dark sections, which is used as an indicator of anxiety, is affected by the levels of ambient light and odour stimulation as well. However, in this chapter, the way the light/dark test was divided and

set up was consistent between the first and second tests. Thus, this is an unlikely explanation of the negative correlation between the light/dark test and the other two tests.

In conclusion, this study investigated whether anxiety was a personality trait in zebrafish that was consistent and repeatable. The results showed that there was variation in anxiety-like behaviours between individuals and that this variation was repeatable within them. They also indicated that some anxiety behaviours in zebrafish are sex dependent due to behavioural differences recorded in the novel tank diving test, open field and light/dark tests. The PCA results revealed that the novel tank diving test, open field test, and light/dark test all contributed in explaining the variation in anxiety between the individuals, but the open field test was not correlated to the novel tank test and was negatively correlated to the light/dark test. Overall, based on the repeatability of the anxiety-like behaviours, this study suggests that anxiety could be heritable and that there may be specific genes that underlie these consistent differences. The following chapters will focus on investigating these questions.

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Chapter III

Gene expression varies with level of anxiety and sex in the zebrafish, *Danio rerio*

3.1 Abstract

Individual variations in behaviour across time and contexts are often referred to as personality traits. These traits are known to be under genetic control, but the underlying mechanisms are not well known. In Chapter II, anxiety was found to be repeatable in zebrafish, suggesting that this trait is genetically controlled. In this chapter, gene expression of zebrafish with different levels of anxiety was investigated, as well as potential candidate genes associated with this trait. Total RNA was extracted from the brains of the least anxious and most anxious male and female zebrafish that varied in their level of anxiety. Sequenced reads were mapped to the zebrafish reference genome and expressed genes identified. Several genes were differentially expressed depending on zebrafish anxiety level and sex. In the comparison of least anxious males and females, we identified four genes that were differentially expressed, while between most anxious males and females, we identified three genes that were differentially expressed. We identified one gene that was significantly expressed between least anxious and most anxious females and one gene between least anxious and most anxious males as well. When we compared the expression of genes among all groups and levels, we found just one gene that was different between them. This study suggests that individual behavioural differences in anxiety might be controlled by differences in gene expression.

3.2 Introduction

Data from Chapter II indicated that there were variations in anxiety within individuals and that some fish were consistently more anxious than others. Moreover, repeatability

estimates of behaviours that were indicative of anxiety varied from 9-68 %. High repeatability values indicate that a trait is genetically controlled, shows the effect of natural selection on a trait changing through time and shows individual consistency (Dohm, (2015). In eukaryotes, the expression of phenotypic variation is attributed to genetic differences that cause gene transcription alterations (Haas et al., 2018). Gene sequence transcription into a protein that results from the translation of an mRNA message and this is referred to as the gene expression (Bell & Aubin-Horth, 2010). Candidate genes are those that are likely contenders for involvement in a specific phenotype (Van Oers et al., 2005). The regulation of phenotypic differences by genes has received considerable attention (Staes et al., 2015). However, estimating the genetic variation underlying the consistency of individual differences in behavioural traits needs more investigation, and in non-human animals, data collected about both personality and genetics are limited (Millot et al., 2014; Staes et al., 2015). For instance, differences in aggressiveness levels are associated with variations in gene expression in sticklebacks (*Gasterosteidae*) (reviewed in Bengston et al., 2018). Moreover, Fidler et al. (2018), demonstrated that variation in personality traits (novelty seeking) was attributed to Drd4 gene polymorphisms in great tits (*Parus major*). A few studies have reported variations in gene expression and specific candidate genes associated with personality. For example, there is an association between variation in sociability in chimpanzees (*Pan troglodytes*) and the receptor genes *Avpr1a* and *OXTR* (Staes et al., 2015), and there is variation in gene expression of early- and late-emerging individuals of the Atlantic salmon (*Salmo salar*) (Thörnqvist et al., 2015).

Traditionally, rodents have been used for modelling anxiety (Adamec & Shallow, 1993; Kalueff, 1999; Ohl, 2003; Stewart et al., 2012). Furthermore, gene expression and candidate genes that are associated with anxiety have been investigated in mice, such as the *Cathepsin B*, *Ctsb* gene (Czibere et al., 2011). Many studies suggest that the environmental influences that

cause anxiety-like behaviour in rodents are the same as those in zebrafish and that both display genomic responses (Stewart et al., 2012). Zebrafish are an increasingly important model species in studying behavioural genetics and, by knowing more about their natural behaviour, we can understand more about how their behaviour is associated with the expression of specific genes (Cachat et al., 2010; Gerlai et al., 2000; Spence et al., 2008; Spence & Smith, 2005). Variations in gene expression in zebrafish with different personality traits have been investigated previously (Oswald et al., 2012; Rey et al., 2013). For example, differences in the expression of genes that control stress hormone production and glucocorticoid receptors have been found between bold and shy male and female zebrafish (Oswald et al., 2012). Furthermore, the *SIRPB1* gene has been associated with personality traits in humans using zebrafish (Laplana et al., 2014), and gene expressions differed between males and females of different ages (Arslan-Ergul & Adams, 2014). However, variations in the expression of genes that could be associated with anxiety have not yet been investigated.

The sequencing of an individual's whole genome depending on a reference genome can play a pivotal role in uncovering the genetic factors underlining behavioural variation by identifying differentially expressed genes upstream (Bengston et al., 2018).

Based on the previous chapter's findings (Chapter II) that individuals vary in anxiety and that some are consistently more anxious than others, by using the whole zebrafish genome as a reference, I predicted that genes associated with this behaviour would be expressed differently in the brains of zebrafish of different sexes and with different levels of anxiety.

3.3 Methods

3.3.1 Study animals

Adult zebrafish (60 females and 59 males), which were used in Chapter II, were investigated further in this chapter. The fish were housed in aquaria in the Department of

Animal and Plant Sciences at the University of Sheffield. Fish were held separately in 10 l tanks (30 x 15 cm,

24.5 cm high) on a recirculatory system. Conditions were as described previously in Chapter II (see: 2.3 Methodology, 2.3.1). To briefly summarise, the temperature was kept constant between 26–27°C and a 12:12 h light:dark cycle was used. Fish were kept visually separate from each other and fed commercial dry food and brine shrimp (*Artemia* sp) twice a day.

3.3.2 Experimental procedure

3.3.2.1 Pre-brain extraction procedure

Based on the behavioural tests and PCA analysis results described in Chapter II (see: 2.3 Methodology, 2.4.3), fish were ranked from the highest scores (low level of anxiety) to the lowest scores (high level of anxiety Appendix, Table 2.1, 2.2). A t test was used to compare the mean scores of the top least and the bottom most anxious fish.

3.3.2.2 Brain dissection procedure

The brain tissue of least anxious and most anxious male and female zebrafish (a total of 60 females (30 least anxious and 30 most anxious) and 59 males (30 least anxious and 29 most anxious)) was dissected. Each fish was transferred from its tank in the aquarium to the laboratory in a small tank (18.5 x 11.5 cm, 12 cm high) containing 800 ml of heated dechlorinated water. The bench surface was sterilised using 70% ethanol and the decontamination solution RNaseZap to destroy any RNase and prevent the extracted RNA from being degraded or contaminated. Prior to the fish dissection, the fish was immersed in a fresh aqueous solution of anaesthetic (tricaine methanesulfonate, MS222), which is the most established method for anaesthesia of adult zebrafish (Collymore *et al*,. 2014), and left for 15–20 min. After death, the fish was transferred to a sterile petri dish (75 x 15 mm) in preparation

for dissection. The brain was dissected using scissors and a fine scalpel that was sterilised by flaming with 70% ethanol. The scalpel and scissors were inserted into the mouth following the cut of the head from the dorsal side and all the way down to the brain. The whole brain was taken and submerged in 1.5 ml RNAlater solution stored in a 1.5 ml microfuge tube. The tube was moved to a container containing ice and then to a fridge at 4 °C overnight, to allow the brain tissue to be penetrated by the RNAlater solution. The following day, the brains were transferred to a freezer at -80°C until RNA extraction.

3.3.2.3 RNA extraction procedure

The top six (least anxious) and the bottom six (out of 30 most anxious) fish of each sex were used for RNA extraction and sequencing (24 samples in total). Several methods have been used to extract and isolate total RNA from tissues such as the magnetic bead method (He et al., 2017), Ribozol RNA extraction reagent, the acid guanidinium thiocyanate phenol chloroform extraction method (Braakman et al., 2015; Chomcznski et al., 2006), and RNeasy Lipid Tissue mini kit (Qiagen extraction method) (see references in Shukla et al., 2017; Al-Lahham et al., 2010; Cantarin et al., 2013). Qiagen, an RNAesay Mini Kit, has been used commonly to isolate total RNA from zebrafish tissues (Arslan-Ergul & Adams, 2014; Tang et al., 2007; De Felice et al., 2012; Blüthgen et al., 2012). Zebrafish brain tissue contains a high quantity of lipids (Zhang et al., 2020), so the Qiagen extraction method (RNeasy® Lipid Tissue Mini Kit) that contains QIAzol Lysis Reagent was chosen, as it is suitable for fatty tissue extractions. Gene expression and molecular biology studies require a high quality and quantity of extracted RNA (Shukla et al., 2017; Norollahi et al., 2018). RNA purity and yield were determined by total cellular breakdown and disruption, so each extracted brain was homogenised in 1 ml of QIAzol Lysis Reagent to lyse the fatty brain tissue and then disrupted using a TissueLyser II before

carrying out RNA extraction (Shukla et al., 2017). The further procedures were conducted following the manufacturer's protocol for the RNeasy® Lipid Tissue Mini Kit for RNA extraction.

3.3.2.4 Checking the quality and quantity of the extracted RNA

RNA was quantified using the Qubit 2.0 Fluorometer and Qubit RNA Assay kit (Appendix, Table 3.1). A NanoDrop 8000 was used to assess the purity and concentration of the samples (Appendix, Table 3.1). An Agilent 2100 Bioanalyzer instrument was used to determine the RIN (RNA integrity number) and the quality and purity of the extracted RNA, and the manufacturer's instructions for the Agilent RNA 6000 Nano Kit protocol were followed for this process (Appendix, Table 3.1). These quality control measures were performed before submitting the RNA samples to Novogene Leading Edge Genomic Services and Solutions for sequencing. The quality of the RNA samples was again checked by Novogene using Agarose Gel Electrophoresis, Nanodrop for preliminary quality control, Agilent 2100 for sample integrity, and Nanodrop for sample purity before sequencing (Appendix, Fig. 3.1, 3.2).

3.3.2.5 Library preparation and RNA sequencing

Novogene prepared and sequenced a library for all 24 submitted samples (type: 250–300 bp insert cDNA library, Eukaryotic Transcriptome Library). Raw data were returned in fastq format.

3.3.2.6 Data cleaning and bioinformatic method

After the data quality control was carried out by the Novogene Company, the fastq data files were transferred to the iceberg HPC at the University of Sheffield, where the initial data processing steps were performed. The RNA sequence reads were trimmed using Trimmomatic

software to remove illumine adapter reads with no quality from the data (Bolger et al., 2014). SLIDINGWINDOW:4:30 (creating a window of 4bp and trimming when the average Illuminia quality is under Q30), MINLEN:100 (depending on a specific read length, it removes reads once it is less than this length), and ILLUMINACCLIP (Illumina sequences and adapter cutter of reads) were used (Bolger et al. 2014). The trimmed RNA-sequence reads were mapped to the zebrafish reference genome (downloaded from ftp://ftp.ensembl.org/pub/release 96/fasta/danio_rerio/dna/Danio_rerio.GRCz11.dna.primary_assembly.fa.gz) using the software star (a speedy mapper of RNA sequences that result in alignment of sequence with higher accuracy) with quantMode TranscriptomeSAM GeneCounts to generate gene count data (Dobin & Gingeras, 2016).

3.3.2.7 Gene expression statistical analysis

All data statistical analyses were performed using R software (version 1.1.383, R Core Team, 2017). The *DESeq* function from DESeq2 package in R was used to analyse the gene expression differences through Wald test (reviewed in Viana et al., 2020; Subunciyan, 2019). The table of sample information was read using the *coldata* function in R (Love al., 2014). The threshold for determining differential expression was corrected for false discovery rate, based on an estimate of detected genes that are false positive in a given set of transcripts (Aubert et al., 2004), using the *res* function with adjusted p value < 0.5. The adjusted p values were done using a test correction, Benjamini-Hochberg technique used for controlling false discovery rate that was implemented in the DESeq2 package in R by default (Love et al., 2014). The BH adjusted p values are presented using the results object in R (Love et al., 2014). The data of RNA sequences was normalised before carrying out the analysis of gene expression and the mean of normalised counts of all tested genes was presented in MA plot, R software (Fig. 3.1A, B) (Kadota et al., 2012).

3.4 Results

3.4.1 Scores of the top and the bottom ranked anxious fish

The t test showed that the mean scores of top anxious fish were significantly different from the mean scores of the bottom anxious fish for both males ($t = -12.4$, $df = 56.62$, $P < 2.2e\text{-}16$) and females ($t = 12.16$, $df = 51.10$, $P < 2.2e\text{-}16$).

3.4.2 Genes differentially expressed in male and female zebrafish with different levels of anxiety

We found that the sequence reads were mapped to 29,307 transcripts, with a total read count of nonzero from the brain tissues of male and female zebrafish with different levels of anxiety. Nineteen genes (0.1%) were upregulated while 58 genes (0.2%) were downregulated. Among these, we found four genes (*BX649639.1, mctp2b, zgc:174680,* and *coagulation factor XIII*) that were expressed differentially when least anxious males were compared to least anxious females ($\log_2$ fold change > 2 and $P < 0.5$, Fig. 3.2, Table 3.1). We found three genes (*si:dkey-88j15.3, fatty acid binding protein 1b* and *wu:fk65c09*) that were expressed differentially when most anxious males were compared to most anxious females (Fig. 3.3, Table 3.1). Furthermore, we identified just one gene (*si:dkey-88j15.3*) that was significantly differentially expressed when we compared least anxious to most anxious females (Fig. 3.4, Table 3.1). Also, we found just one gene (*BX649639.1*) that was expressed differently when least anxious males were compared to most anxious males (Fig. 3.5, Table 3.1). Finally, when we compared all the samples, including least anxious and most anxious males and females, we identified just one gene (*impg1b*) that was different in its expression between those groups (Fig. 3.6, Table 3.1).

Table 3.1. Identified genes that varied in their expression between males and females with different levels of anxiety in zebrafish.

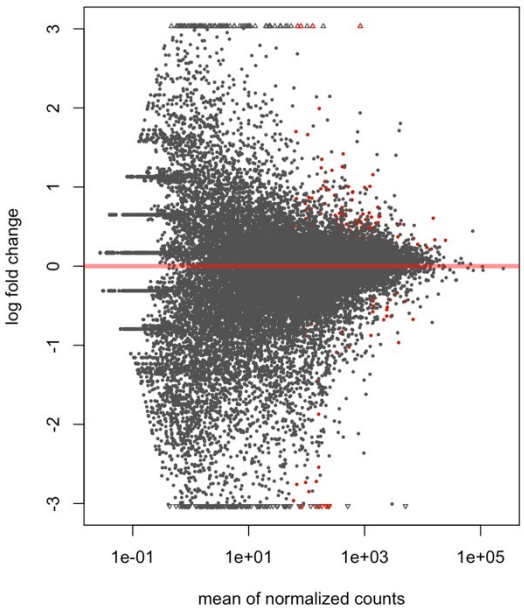
Information taken from ZFIN web page (http://zfin.org) and Ensembl (http://www.ensembl.org/Danio_rerio/).

Fish level of anxiety and sex	Gene	Annotation	Samples that have read counts for a gene		log2 Fold Change	Adjusted P value
			Females	Males		
Genes expressed between least anxious males and females	ENSDARG00000092082 †	*BX649639.1*	Two least anxious females	-	-22.119	P = 0.006
	ENSDARG00000073970	*Multiple C2 domains, transmembrane 2b (mctp2b)*	Six least anxious females	-	-5.493	P = 0.007
	ENSDARG00000100702	*zgc:174680*	Five least anxious females	-	-6.652	P = 0.009
	ENSDARG00000045453	*coagulation factor XIII, A1 polypeptide a, tandem duplicate 1 (f13a1a.1)*	Four least anxious females	-	-5.350	P = 0.021
Genes expressed between most anxious males and females	ENSDARG00000076573 ‡	*si:dkey-88j15.3*	-	Three most anxious males	23.579	P = 0.000
	ENSDARG00000103398	*fatty acid binding protein 1b, liver, tandem duplicate 2*	Three most anxious females	-	-5.792	P = 0.013
	ENSDARG00000069046	*wu:fk65c09 (wu.fk65c09, krtt1c1 Previous)*	Three most anxious females	-	-4.612	P = 0.042
Genes expressed between least anxious and most anxious females	ENSDARG00000076573 ‡	*si:dkey-88j15.3*	One least anxious females	-	21.187	P = 0.022

Genes expressed between least anxious and most anxious males	ENSDARG00000092082 †	*BX649639.1*	-	Two most anxious males	-22.271	P = 0.005
Genes expressed between least anxious and most anxious males and females	ENSDARG00000074839	*interphotoreceptor matrix proteoglycan 1b (impg1b)*	Two most anxious females Five least anxious females	Five most anxious males Five least anxious males	-8.427	P = 0.047

†, ‡, symbols indicate where the same gene was identified as significant in more than one comparison

A)



B)

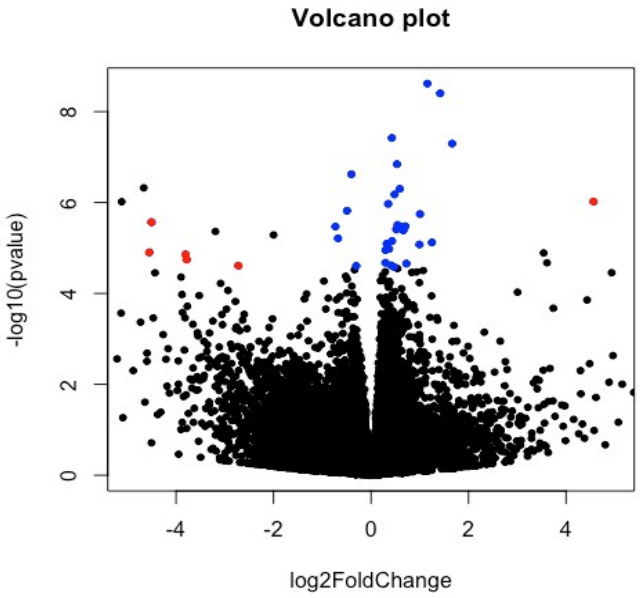


Figure 3.1. (A) The mean of normalised count reads of all tested genes in least anxious and most anxious males and females against log_2-fold changes. The red dots refer to the genes that are significantly differentially expressed. (B) the blue dots represent genes that are significant if adjusted p value < 0.01, while the red dots represent genes that are significant when log_2-fold change >1 and adjusted p <0.05 for female results for the significant differentially expressed genes in least anxious versus most anxious females.

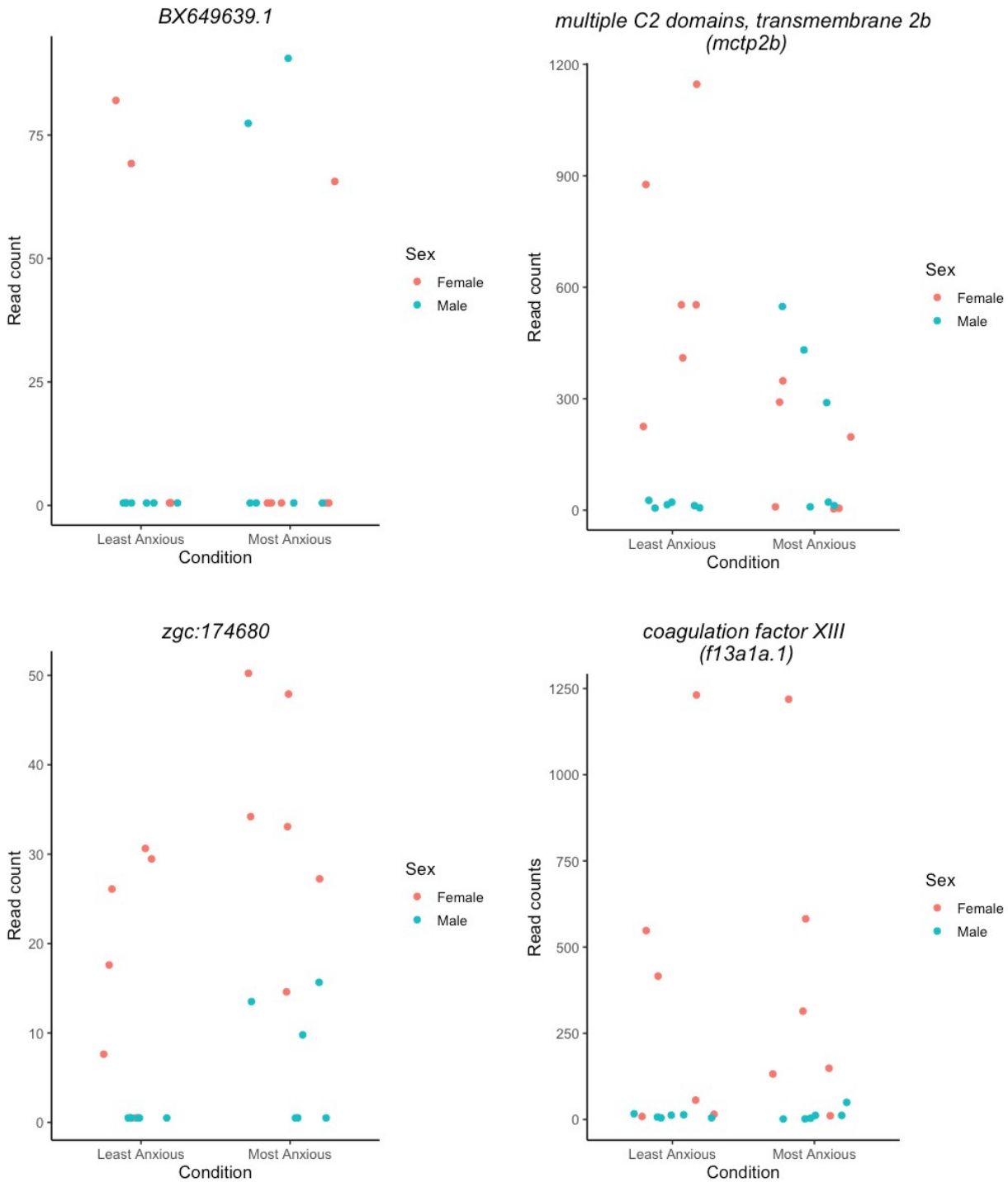


Figure 3.2. Read counts for the four significantly differentially expressed genes in least anxious males and females.

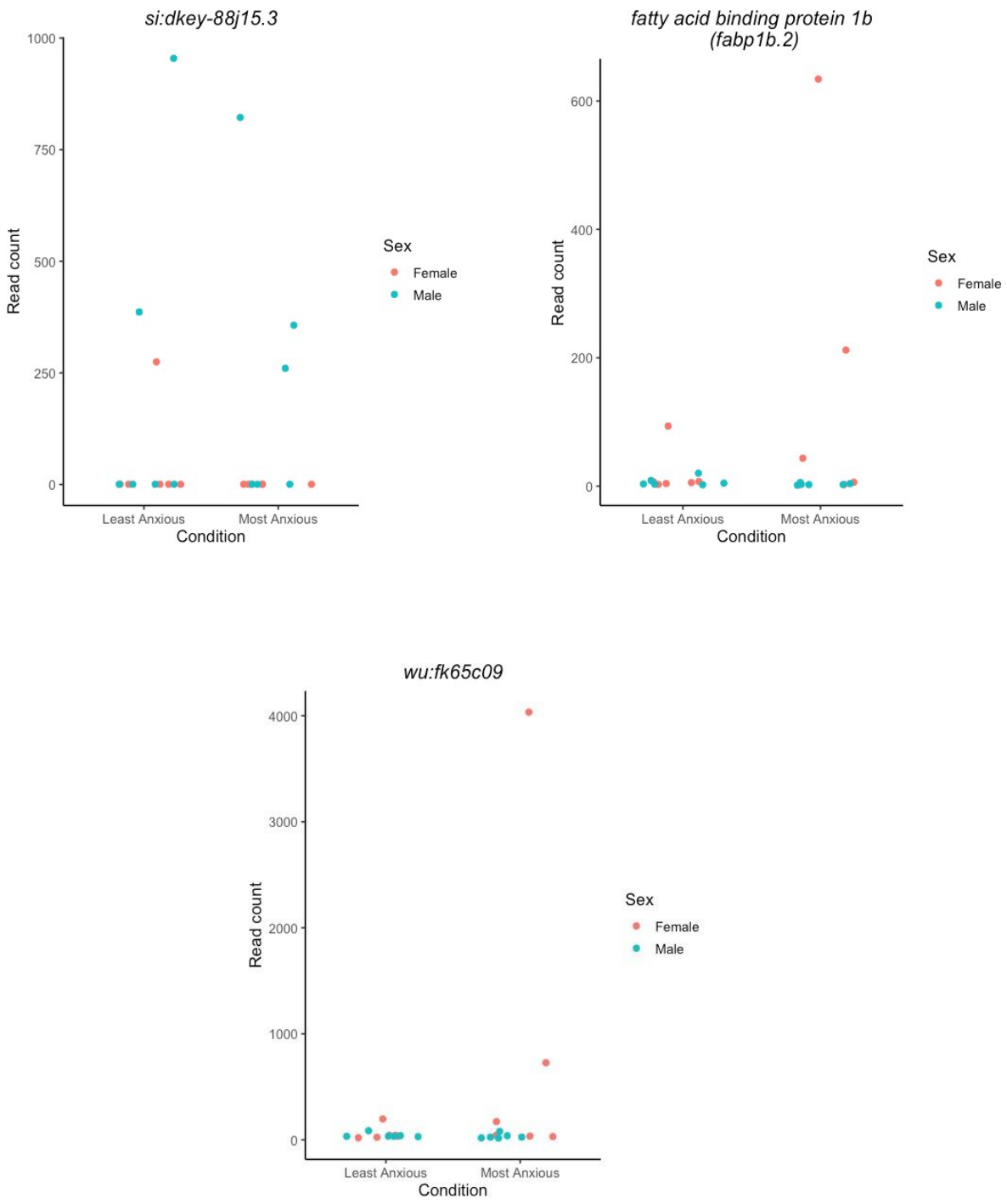


Figure 3.3. Read counts for the three significantly differentially expressed genes in most anxious males and females.

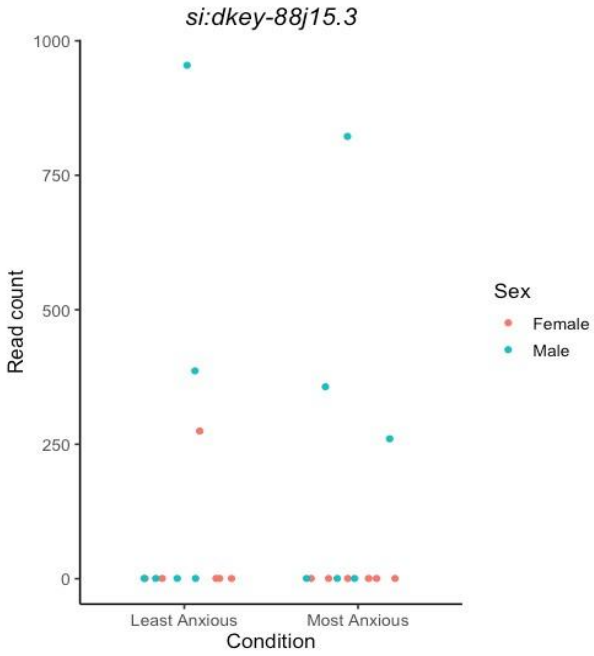


Figure 3.4. Read counts for the significantly differentially expressed gene in least anxious and most anxious females.

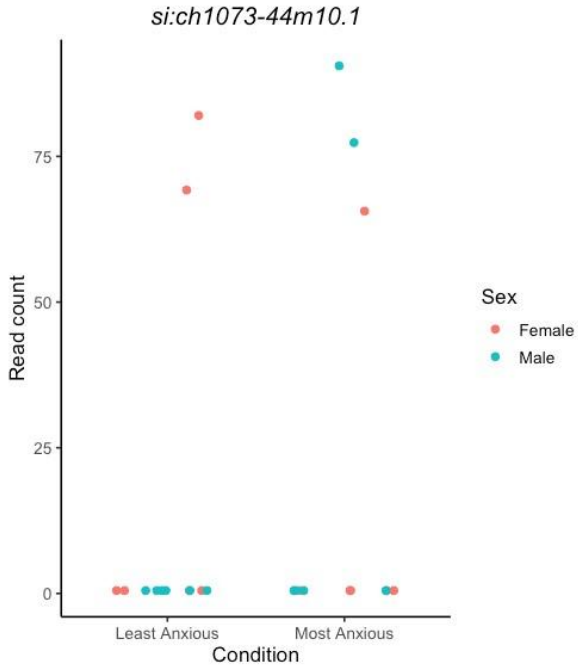


Figure 3.5. Read counts for the most significantly differentially expressed gene in least anxious and most anxious males.

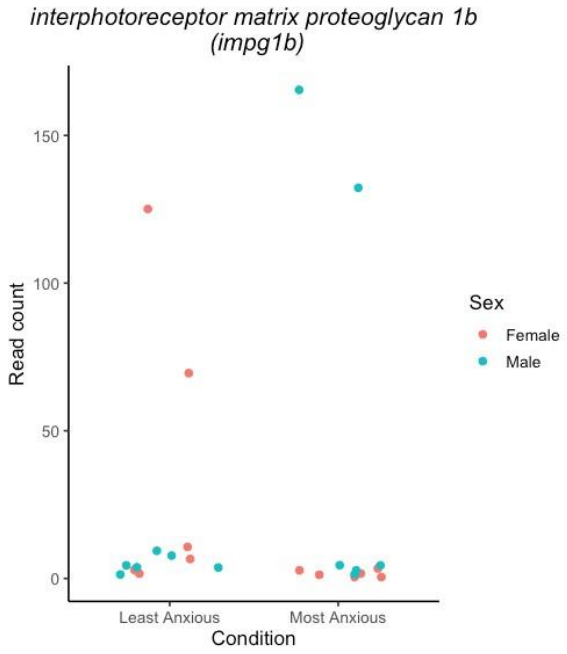


Figure 3.6. Read counts for the most significantly differentially expressed gene in least anxious and most anxious males and females.

3.5 Discussion

This experiment aimed to test for differences in gene expression between zebrafish with different levels of anxiety. The results show that there were eight genes that were expressed differentially depending on anxiety levels (least versus most anxious) and sex (males versus females), suggesting that behavioural differences in anxiety might be controlled by differences in gene expression. Moreover, two of these genes were identified again as significant differentially expressed in other comparisons (least anxious versus most anxious females and least anxious versus most anxious males). The differential expression of genes in males and females in this study is consistent with the findings of Yuan et al. (2019), who showed that about 108 genes (101 male-based and seven female-based) were differentially expressed in the brain tissue of male and female zebrafish. Variations in anxiety and depression-like behaviour have been linked to variations in gene expression in amygdala genes in mice and rats (reviewed in McCoy et al., 2017), with most of the genes being upregulated in the high-response rats compared to the low-response groups (McCoy et al., 2017).

We identified 29,307 transcripts from the zebrafish's brain tissue, and we demonstrated that four genes were significantly differentially expressed between the least anxious males and females and three genes between the most anxious males and females. These results suggest that gene expression was similar between all 24 individuals, except for the seven genes mentioned above that were sex-specific and depended on the level of anxiety. Many studies have demonstrated sex-dependent gene expression in humans and other animals, including fish (e.g., humans, reviewed in Goldstein et al., 2013, Rimol et al., 2010; *Drosophila*, Ranz et al., 2003; mice and rats, Clodfelter et al., 2006; Verma & Ahapiro, 2006; reviewed in Waxman & O'Connor, 2006; Atlantic salmon, *Salmo salar*, Barson et al., 2015). Yuan et al. (2019) suggested that sex is not the main factor controlling the variation in gene expression in the

brain tissues of zebrafish but rather that it is due to hormonal, social, and ecological factors. Some of the genes that were found to be expressed in our research have been structurally and functionally studied in humans (Qiu et al., 2015) and zebrafish (Espino-Saldaña et al., 2020). One of the genes expressed differently in both least anxious males and females is *Multiple C2 domains, transmembrane 2b (mctp2b),* and is expressed in the nervous system and muscles (Espino-Saldaña et al., 2020). Its proteins are expressed in HEK-293 cells and the spinal cord neurons of fish, and it is important in fish embryonic development (Saldana et al., 2020). *MCTP1* and *MCTP2* are *MCTP* genes in zebrafish. While the abnormality of the expression and function of *MCTP1* might have an influence on the development of the central nervous system, and thus lead to associated diseases, *MCTP2* has been associated with depression (see references in Qiu et al., 2015) and depression with anxiety (see references in Morris-Rosendahl, 2002). The anxiety disorder *MCTP* gene has been linked with some human diseases such as schizophrenia and bipolar disorder, and its abnormality may cause neuropsychiatric diseases (Qiu et al., 2015). Another gene that was expressed differently between least anxious males and females was the *coagulation factor XIII* gene, *f13ala.1* (Gerardino et al., 2006). *Factor Xlll* plays a major role in the stability of blood clots (Bronic et al., 2021; Hakimi et al., 2018; Hethershaw et al., 2014) and has been linked to some human diseases such as Alzheimer's (Gerardino et al., 2006). Furthermore, Dull et al. (2021) mentioned that this gene is not just associated with the coagulation of blood but is involved in obesity, wound recovery, and various other diseases. Other genes that were expressed differently between the least anxious males and females were *zgc:174680 and BX649639.3*, which was also expressed in the most anxious males when the expression of identified genes was compared between the most and least-anxious males. Moreover, for most anxious males and females, *si:dkey-88j15.3* was expressed as well as for the least-anxious females when the expression of genes between the most and least-anxious females was compared. Other genes that were expressed differently

between most anxious males and females were the *fatty acid binding protein 1b (fabp1b.2)* and *wu.fk65c09, krtt1c1*.

Some genes were expressed differently between the least anxious versus most anxious groups, including males and females. The findings corroborate previous studies on genes associated with personality traits, such as *catechol-O-methyltransferase* (*COMT*) in humans (Chen et al., 2011). The *COMT* gene is a prime candidate for anxiety vulnerability (reviewed in Enoch, 2003), and has been reported to be associated with anxiety in women. Furthermore, high levels of anxiety have been exhibited by homozygous *COMT*-deficient female mice (reviewed in Enoch et al., 2003). In addition, variation in the *COMT* gene has also been found to have a pharmacogenetic role in generalised anxiety disorder (Narasimhan et al., 2012).

In the current study, comparing the expression of identified genes from the 24 zebrafish brain tissue showed that there was just one gene that was expressed differently between them and this gene was *interphotoreceptor matrix proteoglycan-1b* (*impg1b*). This gene has been reported in humans as a candidate for 6q-linked to disorders of the retina (Felbor et al., 1998). Furthermore, Meunier et al. (2014), has reported that mutations of the *IMPG1* and *IMPG2* genes can lead to macular dystrophy in humans.

In the human genome, around 1.4 million single nucleotide polymorphisms (SNPs) have been recognized (Morris-Rosendahl, 2002). This recognition has enabled disequilibrium mapping in the genome of genes that affect human anxiety (Morris-Rosendahl, 2002). Zebrafish share more than 70% of human genes (Viana et al., 2020), have a brain that largely parallels the psychological and genetic structures of the mammalian brain (Stewart et al., 2014; Viana et al., 2020), and are genetically and anatomically similar to rodents and humans

regarding their anxiety traits (Stewart et al., 2014). Anxiety-like traits are considered to be a dimension of humans' normal personality traits (Morris-Rosendahl, 2002). Therefore, our findings on gene expression could have significant implications for understanding how anxiety may be controlled due to the similarity of the human and zebrafish genomes (e.g. *MCTP* gene, Qiu et al., 2015; the *coagulation factor XIII gene*, *f13ala.1*, Gerardino et al., 2006; *interphotoreceptor matrix proteoglycan-1b* (*impg1b*), Felbor et al., 1998; Meunier et al., 2014) and the existence of human homologs of the identified zebrafish genes. Morris-Rosendahl (2002) stated that anxiety-related personality traits involving fear, stress, emotion consistency, heritability, and even genetic influences are difficult to identify, but the results of our study offer evidence for differential expression of some genes, like *5-HTT* (*SLC6A4*) in human chromosome region 17q12, which might contribute to the phenotypes of anxiety (Morris-Rosendahl, 2002).

In conclusion, the aim of this research was to explore whether there were differences in gene expression between zebrafish of different sexes and levels of anxiety. From the zebrafish's brain tissue, 29,307 transcripts were detected. Among these 19 genes were upregulated and 58 genes were downregulated. Eight genes differed in their expression between least anxious and most anxious males and females. Two of these genes identified again as significant differentially expressed in different comparisons. Some of those genes have been linked to human diseases (Dull et al. 2021; Gerardino et al., 2006) and other conditions such as depression (see references in Qiu et al., 2015), schizophrenia, bipolar disorder and neuropsychiatric diseases (Qiu et al., 2015). This means that we have demonstrated that there is an association between the expressed genes and anxiety as a personality trait.

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Chapter IV

Fitness consequences of variation in anxiety in the zebrafish *(Danio rerio)*

4.1 Abstract

Individuals can vary in their personality traits and this can affect their fitness. One factor that is used to determine fitness is reproductive success. There has been a growing interest in the association of personality traits with fitness and how these traits influence reproductive success. However, determining the reproductive fitness consequences of individuals that vary in anxiety remains unclear. In this chapter, I investigate the effect of male and female anxiety on reproductive success. Four combinations of least anxious and most anxious male and female zebrafish (most anxious/most anxious, most anxious/least anxious, least anxious/most anxious, least anxious/least anxious) were crossed and the number of eggs and the proportion that were fertilised were counted. No difference was found in the total number of eggs laid by least or anxious females paired with least or anxious males. Moreover, least and anxious males did not differ in the number of eggs fertilised when paired with least or most anxious females. The least anxious females do not differ from the most anxious females and the least anxious males do not differ from the most anxious males regarding the proportion of fertilised eggs they produced. Furthermore, the results do not show any interaction between males and females with different levels of anxiety in the proportion of fertilisation of eggs they produced. These findings suggest that anxiety level has no direct effect on egg production and fertilisation, as considered here as a measure for reproductive success, in zebrafish so there are no differences between groups for this measure of fitness.

4.2 Introduction

Animal personality plays a critical role in fitness because behavioural differences can be maintained in populations and transmitted to later generations (Thomson et al., 2020). Personality traits describe individual behavioural differences that are consistent within one or multiple contexts and over time (Biro & Stamps, 2008; Frost et al., 2013; Planas-Sitjà, 2020; Réale, et al., 2009; Smith & Blumstein, 2008). An individual's fitness is shaped by natural selection (Gutiérrez et al., 2013). Depending on environmental fluctuations, a given trait may come under strong selection and exhibit different fitness consequences in line with those changes, such as the conditions of predation, food availability, and sociability (reviewed in Smith & Blumstein, 2008). Furthermore, the context in which personality traits are displayed can lead to variation in the fitness of those traits. One such measure of fitness is reproductive success, which refers to an individual's fecundity each season or over their lifetime, and the survival of their offspring to maturity (Yvan, 2009). For example, bolder individuals have been found to have an increased reproductive success compared to shyers (Smith & Blumstein. 2008).

In nonhuman animals, personality differences have a pivotal role in factors affecting fitness including mating, fecundity, and survival (Betini & Norris, 2012; Cote et al., 2008; Chira, 2014; Gutiérrez et al., 2013). There is a growing body of literature that reports that reproductive success and survival are correlated with personality traits. For example, reproductive success is related to exploratory phenotypes in great tits, *Parus major* (reviewed in Sinn et al., 2006), boldness and shyness affect fecundity and fertilisation success of dumpling squids, *Euprymna tasmanica* (Sinn et al., 2006), boldness and shyness have an impact on the reproductive success of guppies, *Poecilia reticulata* (Ariyomo & Watt, 2013) and zebrafish, *Danio rerio* (Ariyomo & Watt, 2012), as well as juvenile and adult largemouth

bass, *Micropterus salmoides* (Ballew et al., 2017), and sociability variation affects reproduction and survival in the lizards, *Lacerta vivipara* (Cote et al., 2008). Furthermore, fitness and fecundity have been attributed to the variation in personality phenotypes (proactive versus reactive) with different stripe patterns in zebrafish, with increased reproductive success exhibited by the proactive individuals (Vargas et al., 2018).

Anxiety and fear play a vital role in survival because they stimulate defence responses (Eilam et al., 2011), which span the animal kingdom and are commonly demonstrated by freezing, fleeing, and fighting. Depending on individual personalities, animals may differ in their defence responses, which in turn can lead to variations in these (Eilam et al., 2011). In challenging or novel environments, individuals vary in their tendency to take risks depending on their personalities (Dingemanse, et al., 2004). Furthermore, in risky situations, fitness can be affected by anxiety because it can be triggered by general threats that induce vigilance and help to determine the risk nature and response (Marks & Nesse, 1994).

It has been demonstrated that reproductive fitness varies in zebrafish with different levels of aggression and boldness (Ariyomo & Watt, 2012). Anxiety resulting from stress exposure has been shown to reduce egg production in zebrafish (Dewari et al., 2016), but it is not known if variation in anxiety level stimulated by novelty has an impact on reproductive success. In this study, I investigated whether zebrafish with different levels of anxiety (high and low) varied in their reproductive fitness. Male and female zebrafish with different anxiety levels were mated. Four different crosses were conducted such that females with the highest level of anxiety were mated with males with the highest or lowest level of anxiety and females with the lowest level of anxiety were mated with males with the highest or lowest level of anxiety. The number of eggs laid and fertilised was then determined. As most anxious zebrafish

show increased latency to explore a novel tank, freezing, erratic movements, and display fewer entries to the top half of the tank compared to those that are least anxious (see Chapter II; Egan et al., 2009), by crossing the four combinations, I expected that the total number of eggs laid by females with different level of anxiety and fertilised by males with different level of anxiety would be different between those combinations and that the least anxious pairs would interact more in the tank, thus producing and fertilising more eggs than the most anxious pairs. For mixed combinations, I predicted that the least anxious females paired with most anxious males would produce more eggs compared to most anxious females paired with the least anxious males and the least anxious males would fertilise more eggs when paired with most anxious females compared to the most anxious males paired with the least anxious females.

4.3 Methods

4.3.1 Animals

Male and female adult zebrafish (not used in any previous experiments) were raised in aquaria kept in the Department of Animal and Plant Sciences at the University of Sheffield. Sixty males and sixty females (age 9-11 months) were maintained separately in 10 l holding tanks (30 x 15 cm, 24.5 cm high) in a recirculatory aquarium system held at 26-27 °C and on a 12:12 h light: dark cycle. The tanks allowed males and females to be visible to each other, and chemical cues circulated throughout the system. Brine shrimp (*Artemia sp*) and commercial dry food were used to feed the fish twice a day. Ethical approval was not required in this study because it did not include any regulated procedures.

4.3.2 Pre-crossing procedure

4.3.2.1 Anxious behavioural test for male and female zebrafish

Each fish was transferred from its aquarium to the test laboratory in a small tank (18.5 x 11.5 cm, 12 cm high) containing 700 ml of heated dechlorinated water, then kept in this for 300 s for acclimation. The fish was then transferred to the novel tank diving test for assessing anxiety following the same procedure as described in the Chapter II (see: 2.3 Methodology, 2.3.2.1) except a 40 W LED panel (600 x 600 mm) (Element Lighting, Colchester, UK) was used instead to light the tank from underneath.

4.3.2.2 Behavioural processing and fish ordering

All data recorded were processed manually and using the Viewpoint program. The repeatability of the tests was analysed following the same steps as illustrated before in Chapter II (see: 2.3 Methodology, 2.3.3.2) using *rptr* function from the rptR package in R software (version 3.5.1; R development Core Team, 2018). The number of entries (original-scale R = 0.46, P = 1.1e-09, Table 4.1) and time spent in the upper half of the tank (original-scale R = 0.41, P = 3.59e-11, Table 4.1) by male and female zebrafish had higher repeatabilities than latency to enter the upper half of the tank (original-scale R = 0.04, P= 0.004, Table 4.1), and so the fish were ranked first from the largest to smallest values for the number of entries and time spent in the upper half followed by the smallest to the largest values for latency using excel software. Before the ranking step, the mean score of fish behaviours in the first and second novel tank diving test was calculated using excel software.

Table 4.1. Estimated repeatability with their corresponding 95% confidence intervals of male and female zebrafish behaviours measured in the novel tank diving test using the glmm method and log link.

Behaviour recorded in the novel tank diving test for both male and female zebrafish	Original-scale approximation of R (CI) $\pm$ SE df P value
Latency to enter the upper half of the tank	0.04 (0.01, 0.10) $\pm$ 0.02 1 0.004
Number of entries to the upper half of the tank	0.46 (0.32, 0.60) $\pm$ 0.1 1 1.1e-09
Time spent in the upper half of the tank	0.41 (0.30, 0.53) $\pm$ 0.1 1 3.59e-11

4.3.2.3. Mating pairs

The top 24 (most anxious) and the bottom 24 (least anxious) of each sex were used for the crossing procedure. Twelve males and 12 females of the middle ranks were excluded. Each fish was held separately in a 10-l tank (30 x 15 cm, 24.5 cm high) on the recirculatory system. Four different combinations of mating pairs were established by taking males and females randomly from the highest and lowest groups, so the crosses were as follows: least anxious males (12 fish) x least anxious females (12 fish); least anxious males (12 fish) x most anxious females (12 fish); most anxious males (12 fish) x most anxious females (12 fish); most anxious males (11 fish) x least anxious females (11 fish).

4.3.3 Crossing procedure

Tank holding a single male was provided with a 12 cm diameter (5 cm depth) plastic dish filled with four layers of glass marbles that acted as a breeding site (Spence et al., 2008; Fig. 4.1). A female was transferred to a male's tank for crossing and left there for 22 hours (12:30 pm-10:30 am) as zebrafish spawning is stimulated during the end and the beginning of the light period (Eaton & Farley, 1974). The following morning, the dish was gently removed from the tank and the female was returned to her tank. Marbles were removed from the dish and all the eggs were counted and pipetted into a petri dish (100 x 15 mm) containing aquarium water. Pairs that did not produce eggs in the first pairing were crossed with another randomly selected fish with the same anxiety level as the previous mate, and if no eggs were produced after three attempts, they were recorded as zero. There were seven pairs that produced no eggs after three attempts at mating: three least anxious females paired with least anxious males, two least anxious females paired with most anxious males, and two most anxious females paired with most anxious males. These pairs were included in the analysis. The eggs were checked after 24 hours and those that were dead and unfertilised (opaque) were counted and removed.

Larvae at 10 DPF were transferred to separate holding tanks (12 cm W x 22 cm L x 14 cm H) under the same laboratory conditions in Chapter II (see, 2.3 Methodology, 2.3.1).

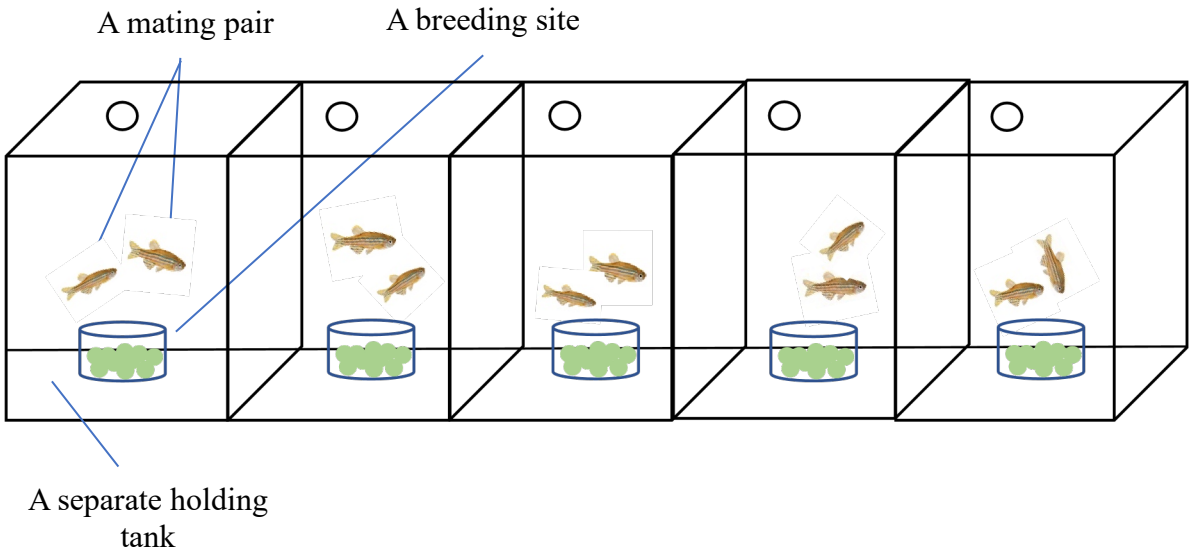

Figure 4.1. Zebrafish holding tanks and the spawning experiment set up.

4.3.4 Statistical analysis

R statistical software (version 1.1.383, R Core Team, 2018) was used to analyse the data. A generalised linear model was fitted to the data to compare the four mating pairs using *glm* function from the lme4 package in R software (vergion 1.1.383; r Core Team, 2017). The model set the group of least anxious females paired with least anxious males (intercept) as a baseline for the other groups. The other groups of mating pairs which included least anxious females paired with most anxious males (LAF X MAM), most anxious females paired with least anxious males (MAF X LAM) and most anxious females paired with most anxious males (MAF X MAM) were compared with the baseline. The model was done with the level of anxiety of males and females and the interaction between them as fixed effects. As the number of eggs laid and fertilised were counts, a Poisson distribution with log link was specified to

assess whether eggs produced by females with a different level of anxiety depended on the male level of anxiety that they were paired with. For the fertilisation proportion data, a binomial distribution was specified and calculated the proportion of the total number of eggs laid to eggs fertilised to create a one response variable limited by 1 and zero. To assess the significant differences between the four group mating crosses, TukeyHSD function in R was used with 95% confidence intervals (see Fig. 4.1, Table 4.1 Appendix).

4.4 Results

The results showed that the anxiety level of males and females has no main effect on the total number of laid, fertilised eggs and the proportion of eggs laying to fertilisation. There was no significant difference between the four groups of mating pairs in the number of eggs laid by anxious or non-anxious females paired with anxious or non-anxious males (Table 4.2). There was no significant difference between the four groups of mating pairs in the number of eggs fertilised by anxious or non-anxious males paired with anxious or non-anxious females (Table 4.2). There is no difference between the four groups in the proportion of eggs laying to fertilisation (Table, 4.2). There is also no interaction between the pairs with different level of anxiety in the number of eggs laid (estimate = -0.57, SE = 0.46, T = -1.23, P = 0.22), fertilised (estimate = -1.21, SE = 0.67, T = -1.82, P = 0.1) and the proportion of eggs laying to fertilisation (estimate = -0.4, SE = 0.51, T = -0.7, P = 0.5).

Table 4.2. The estimate, standard errors, T and P-value results of the difference between the four groups of mating pairs (least anxious females paired with least anxious males LAF X LAM, least anxious females paired with most anxious males LAF x MAM, most anxious females paired with least anxious males MAF X LAM and most anxious females paired with most anxious males MAF X MAM) from the glm model for number of eggs laid, number of eggs fertilised and the proportion of fertilised eggs.

Groups	**Estimate**	**Standard error**	**T-value**	**P-value**
Laid eggs				
intercept (group LAF X LAM)	4.61	0.22	21.14	P < 0.001
group LAF x MAM	0.15	0.31	0.49	0.63
group MAF x LAM	0.11	0.31	0.36	0.72
group MAF x MAM	-0.31	0.34	-0.9	0.4
Fertilised eggs				
intercept (group LAF X LAM)	3.34	0.4	9.32	P < 0.001
group LAF x MAM	0.64	0.5	1.40	0.2
group MAF x LAM	0.51	0.5	1.1	0.3
group MAF x MAM	-0.1	0.5	-0.13	0. 9
Proportion of fertilised eggs				
intercept (group LAF X LAM)	-1.20	0.3	-4.64	P < 0.001
group LAF x MAM	0.13	0.4	0.4	0.73
group MAF x LAM	0.21	0.4	0.61	0.54
group MAF x MAM	-0.01	0.4	-0.03	0.98

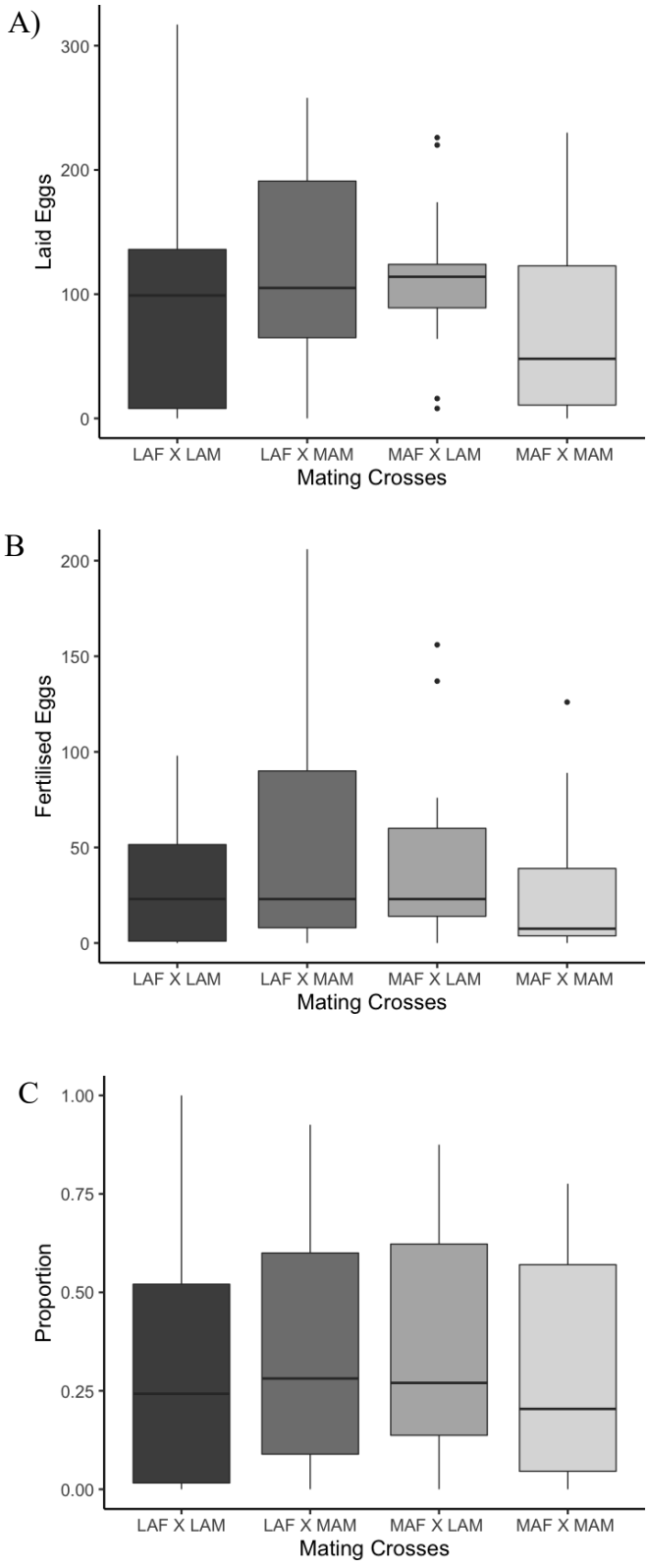

Figure 4.2. Medians (a parallel line within boxes), interquartile ranges (second quartile refers to box under median, and third quartile refers to box above median), outer quartiles (represented as vertical lines out of the boxes) and outliers (circles) for (A) laid eggs, (B) fertilised eggs and (C) proportion of fertilised eggs for least anxious and most anxious females and males in four different combination crosses.

4.5. Discussion

Contrary to expectations, there was no difference in the total number of eggs laid or fertilised by most or least anxious females paired with most or least anxious males. Moreover, no interaction was found between male and female levels of anxiety and the number of eggs laid, fertilised and the proportion of fertilised eggs they produced. The results of this chapter are contrary to those that found a link between personality traits and fitness. For instance, consistent differences in boldness and aggressiveness have been associated with variation in reproductive success in zebrafish (Ariyomo & Watt, 2012) and guppies, *Poecilia reticulata* (Ariyomo & Watt 2013). In guppies, male and female pairs with different levels of boldness led to variation in reproductive success, suggesting that such results were driven by frequency-dependent selection (Ariyomo & Watt, 2013). Moreover, differences in reproductive success have been linked with variation in exploration and boldness in blue tits, *Cyanistes caeruleus* (Mutzel et al., 2013). In blue tits, fledglings have been shown to be fed at higher rates by less aggressive male and fast-exploring female tits than more aggressive males and slow-exploring female tits, which fed fledglings at lower rates (Mutzel et al., 2013). Vargas et al. (2018) demonstrated that a proactive personality group of zebrafish (described as having a consistent level of boldness) increased the chance of females being in the reproductive site for longer than expected and had increased fitness variation compared to the reactive personality group that had a consistent level of shyness. These findings suggest that personality may play a critical role in the success of zebrafish reproduction (Vargas et al., 2018). Real et al. (2009) indicated that boldness and docility as personality traits were positively linked to reproductive success in old sheep rams (*Ovis canadensis*).

Given that our results indicate that the total number of eggs laid, fertilised and the proportion of fertilisation did not differ between the least anxious and most anxious male and

female groups, it suggests that there is no link between variations in anxiety as a personality trait and reproductive success in zebrafish. Zebrafish maximise the number of mating events to produce as many eggs as possible to increase their fitness (Ariyomo & Watt, 2012; Spence et al., 2008; Vargas et al., 2018). Thus, the lack of any significant effect of anxiety on reproductive success may be due to the similarity in the number of eggs produced resulting from the mating attempts regardless of personality differences. Another possible explanation for this result is that the crossing procedure involved just one male with one female. Previous work has found that mating just two individuals can lead to similarities in the embryo numbers of zebrafish (Vargus et al., 2018). Zebrafish reproduction is affected by many factors. For example, high density can lead to reduced numbers of eggs laid by females, increased female competition for breeding sites, increased male aggression, and decreased courtship (Hoo et al., 2016). Furthermore, territorial male zebrafish have been shown to exhibit increased reproductive success at low densities (Spence et al., 2006). Female zebrafish fecundity can be affected by competition from other females because released pheromones may reduce the egg viability of the others (Gerlach, 2006). The recommended sex ratio needed to ensure successful reproduction in zebrafish has been demonstrated to be one male to two females (Hoo et al., 2016; Vargas et al., 2018). Moreover, territoriality has been indicated to affect reproductive success; for example, it interrupted courtship and extended the time of spawning success in European bitterling, *Rhodeus sericeus* (Reichard et al., 2004). Ariyomo & Watt (2012) had pairings based on just one female to two male zebrafish and found similarity in the number of eggs produced suggesting that because of females ovulation that is affected by male gonadal pheromones. The similarity in the rate of spawning may be due to the lack of variation in female competition for spawning sites and male competition (Vargus et al., 2018).

Personality traits and their potential to be influenced after mating has been investigated (Monestier & Bell, 2020). For example, personality traits including risk-taking, activity, and social behaviour have been shown to be changed in female three-spined sticklebacks (*Gasterosteus aculeatus*) experienced physical mating and social courtship when risk-taking willingness and social behaviour are reduced compared to control group (Monestier & Bell, 2020). Furthermore, in giant pandas (*Ailuropoda melanoleuca*) reproductive success manifested in mating, and the production of offspring is enhanced and sometimes impaired by the personality traits of a mating pair (Martin-Wintle et al., 2017). Limited studies investigating the influence of similarity in pairs personality traits on mate choice and they focus on male traits when they study mate choice being influenced by personality traits (Ariyomo & Watt, 2013). Pairs with males that were more aggressive than females had more offspring and a higher chance of mating than aggressive males who mated with more aggressive females. Males with high excitation paired with females with low excitation displayed successful reproduction, while males with low fearfulness paired with females regardless of fearfulness level had better reproduction (Martin-Wintle et al., 2017). Ariyomo and Watt (2013) demonstrated that reproductive success was achieved in guppies *Poecilia reticulata*, when bold females paired with males had a similar level of boldness compared to a pair with a dissimilar level of boldness. Our results indicate that the behaviours of the most anxious and least anxious individuals were not influenced by those in the pairs with the same or different levels of anxiety. Moreover, there was no significant difference in the proportion of fertilised eggs between all four combinations.

Reproductive success can be determined by many things such as brood and offspring size (Both et al., 2005; Vincent & Giles, 2003), and vocal behaviour leads to pairs communication (Vasconcelos, et al., 2012), the survival of offspring to maturity, fecundity,

and spawning stock biomass (Yvan, 2009), high quality of males such as regarding body size (Ulrike et al., 2018) and sperm quality (Casselman et al., 2006; Schulte-Hostedde & Burness, 2005), and pair compatibility in monogamous animals (Schweitzer et al., 2017). Anxiety has been demonstrated to affect reproductive success (Zhang et al., 2016). For instance, the number of eggs laid, hatchability, and mortality were negatively influenced by anxiety in zebrafish (Xiao et al., 2018). Moreover, increased anxiety-like behaviour, negatively affecting courtship and reproductive success in zebrafish (Dewari et al., 2016; Mi et al., 2019). On the other hand, as aforementioned, in a risky situation, anxiety can positively affect fitness and survival by inducing vigilance and defence responses (Eilam et al., 2011; Marks & Nesse, 1994). In this chapter, we focus on the number of eggs and fertilisation as a measure of reproductive success due to time limitation. Contrary to previous findings, anxious responses induced by novelty using the novel tank diving test (Chapter IV) did not demonstrate a significant effect on the number of eggs laid and fertilised taken as a measure of reproductive success.

In conclusion, this study is the first investigation of the effect of anxiety as a personality trait on reproductive success in zebrafish. Overall, there was no difference in the total number of eggs laid and fertilised and the proportion of fertilised eggs among the male and female pairs that differed in anxiety. Ariyomo & Watt (2013) had pairings of two males to one female zebrafish, and they found effects on reproductive success with more eggs being fertilised by males with higher levels of aggressiveness and boldness. Further work is recommended to investigate the effects on other aspects of fitness when least anxious and most anxious zebrafish are mated with more than one male and female.

4.6 References

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Chapter V

Heritability of anxiety as a personality trait

5.1 Abstract

In the field of animal behaviour, personality traits are usually consistently expressed over time and between contexts. It has been shown in a previous chapter that there is consistency within individual zebrafish in their level of anxiety. However, the degree to which this trait is genetically or environmentally controlled is still unclear. In this experiment, the heritability of anxiety in the zebrafish, *Danio rerio*, was determined by estimating the additive genetic (V_A) and non-additive genetic factors, including maternal (V_M) effects. Crosses were conducted between one male with known level of anxiety (most anxious or least-anxious) and two randomly selected females, so for each male it was mated twice to two different females. This resulted in 40 broods from the 40 spawnings. Anxiety level of offspring aged two months were measured using the novel tank diving test with increased latency to enter the upper half of the tank, reduced number of entries to the upper half of the tank, and reduced time spent in the upper half during the novel tank diving test indicative of anxiety. We found that the estimate of additive genetic components contribution to anxiety variance was significant and the estimates (with 95 % confidence interval) were h^2 = 0.03 (4.007114e-08, 0.12), 0.11 (5.613514e-07, 0.32) and 0.18 (2.395571e-08, 0.49) for latency to enter the upper half of the tank, number of entries to the upper half of tank, and time spent in the upper half, respectively. We also found significant maternal (V_M = 0.58, 0.29, 0.46) effects contributing to anxiety variance. These results illustrated that anxiety is genetically and environmentally controlled.

5.2 Introduction

Personality traits are behaviours that are consistent within an individual in a given population over time and between contexts (Ariyomo et al., 2013, Biro & Stamps, 2008). In

Chapter II, it was found that anxiety was a repeatable trait in zebrafish individuals. For any trait, repeatability commonly sets the upper limit of heritability (Chervet et al., 2011), therefore, given that anxiety was repeatable between individuals has been shown in Chapter II, it suggests that anxiety may be under genetic control, and so could be heritable. Many researchers have linked the repeatability of personality traits with heritability (Kortet et al., 2014) to help estimate the additive genetic variation contribution to these traits (Dochtermann et al., 2015). For example, both the repeatability and heritability of exploration, aggression, boldness, and freezing in response to stress in brown trout juveniles, *Salmo trutta* (Kortet et al., 2014), and exploratory behaviour in great tits, *Parus major* (Dingemanse et al., 2002), have been investigated. The proportion of the total phenotypic variance attributed to additive genetic variance is assessed by heritability (Dochtermann et al., 2015). Therefore, the heritability of a personality trait can be defined as the proportion of personality differences resulting from additive genetic variance (Dochtermann et al., 2015). In a study conducted on research published from 2000-2012, it was found that additive genetic variation accounted for about 52% of consistent individual behavioural differences even when the heritability estimate of a personality trait was low (Dochtermann et al., 2015). The heritability of some personality traits and the additive genetic variance causing phenotypic variation in these traits have been investigated in the zebrafish (Ariyomo & Watt, 2013), for example, boldness and aggressiveness, and they have been found to be heritable (Ariyomo et al., 2013). Variation in personality or phenotypic variance could result from the influence of additive genetic variation or from lasting environmental effects, such as parental effects, epigenetic effects (Dochtermann et al., 2015), or maternal effects (Ariyomo et al., 2013). For instance, 9% of the phenotypic variance in boldness and aggressiveness in zebrafish was attributed to maternal contributions (Ariyomo et al., 2013).

Personality traits that are heritable will be acted upon by natural selection (reviewed in

Verhulst et al., 2016). From an evolutionary perspective, individual differences should have fitness consequences (Carere et al., 2010), as personality traits will affect reproduction and survival (reviewed in Staes et al., 2015; Santicchia et al., 2018). Heritable traits that are valuable to individuals are strongly favoured by natural selection. Possessing a genetic basis for any trait allows it to be responsive to selection (Sinn et al., 2006, Ariyomo et al., 2013). Because of previous natural selection, traits that are tightly linked to fitness are expected to have little additive genetic variance, and thus low heritability (Mousseau & Roff 1987, Ariyomo et al., 2013 & Liu et al., 2020). Anxiety is associated with fitness because it can lead to increased vigilance and sheltering behaviour, that in turn affects the likelihood of predation (for example, in the amphipod, *Gammarus fossarum,* Perrot-Minnot et al., 2017), and thus increased survival. However, in zebrafish, Dewari et al. (2016) found a negative impact of anxiety on reproductive success where fecundity (number of eggs laid) significantly decreased. In Chapter IV of this thesis, we found no fitness consequences of anxiety on zebrafish.

The study of heritability has been extended to include anxious responses, such as fearful-anxious endophenotypes in rhesus macaques, *Macaca mulatta* (Williamson et al., 2003, Rogers et al., 2008). However, investigations of the genetic components underlying the consistency and repeatability of such responses are lacking. This study determined whether individual differences in anxiety were heritable in the zebrafish, and so estimating the proportion of additive and non-additive genetic components was the main target of this investigation. Anxiety was assessed using the novel tank diving test since this was found to be one of the most reliable indicators of this trait (see Chapter II, 2.3.2.1 Novel tank diving test (NTDT) and Chapter IV, 4.3.2.1 Anxious behavioural test for male and female zebrafish).

5.3 Methodology

5.3.1 Study animals and crosses

Twenty males aged between 14-16 with different levels of anxiety (10 least-anxious and 10 most-anxious, based on measurements recorded in the novel tank diving test - see Chapter IV, 4.3.2.2 Behavioural processing and fish ordering) were used in this experiment. Those fish were housed separately under the same conditions designated before in Chapter II (see: 2.3 Methodology, 2.3.1) and Chapter IV (see: 4.3 Methods, 4.3.1). Each male was selected randomly and paired up with a randomly selected female (See section 4.3.3 Crossing procedure in Chapter IV**)**. After mating and one week of recovery, each male was crossed again with another randomly selected female, so there were 40 broods resulting from the 40 spawnings. Twenty-four hours after each spawning and egg collection, unfertilised and dead eggs were removed and those that were fertilised were collected and placed in petri dishes (135 x 17 mm). For each spawning, embryos were kept together, so their parental anxiety levels were known, until hatching. For the first 9 days post fertilisation (DPF), water in the petri dish was changed every day. At 10 DPF, each group of fry were moved to a holding tank (12 cm W x 22 cm L x 14 cm H). Water was changed regularly and the tank was kept in the aquarium at 26-27 °C and on a 12:12 h light: dark cycle. At 14 DPF, all the holding tanks were connected separately to the recirculatory system in the aquarium. Water flow was slow initially to prevent damage to the larvae and at 31 DPF the water flow was increased.

The larvae usually depend on the yolk for the first 5 days after fertilisation, and at 5 DPF, they start independent feeding (Hölttä-Vuori et al., 2010). In this experiment ZEBRAFEED dry diet was used. Initially, the particles of food were small, and these were gradually increased depending on the embryos' age as follows: ZEBRAFEED 100 at 6-10 DPF twice a day; ZEBRAFEED 100 and brine shrimp, *Artemia,* at 11-21 DPF twice a day;

ZEBRAFEED 100-200 and brine shrimp at 22-31 DPF, and adult food including brine shrimp and dry flakes at 31 DPF, twice a day.

5.3.2 Behavioural testing

The number of offspring within a group ranged from approximately 4-60. At the age of two months, five unsexed offspring were selected randomly from each brood and tested for anxiety using the novel tank diving test, following the same procedure described in Chapter II (see: 2.3 Methodology, 2.3.2.1) and Chapter IV (see: 4.3 Methodology, 4.3.2.1). All broods, except one containing four offspring were tested. The total number of offspring tested from the 40 broods was 199. To assess the anxiety level of the fish, each brood was transferred in its holding tank (12cm W x 22cm L x 14cm H) to the test laboratory for behavioural testing. Each fish selected from a brood was transferred from its holding tank to a tank (18.5 x 11.5 cm, 12 cm high) containing 700 ml of heated dechlorinated water, then held in this for 300 s for acclimation and then to the novel tank diving test tank (25 x 15 cm, 15 cm high). This tank was marked into two equal parts and contained 4 l of dechlorinated water heated to 27°C. A 40 W LED panel (600 x 600 mm) (Element Lighting, Colchester, UK) was used to light the tank from below and a digital camera (Panasonic HC-V160) positioned to the side of the tank using a stand and clamp was used for behavioural recording. After 60 s of acclimation, latency to enter the upper half of the tank, number of entries to the upper half of the tank, and time spent in the upper half of the tank were recorded in 300 s.

5.3.3 Statistical analysis

All data analysis was conducted using R software version 3.5.1 (R development Core Team, 2018). We used an animal model with a complete pedigree to assess the heritability of anxiety trait using a Markov Chin Monte Carlo generalised linear mixed model

(MCMCglmm) (Ariyomo *et al*. 2013; Ringo, 2022). The (*MCMCglmm*) function from the MCMglmm package in R software was used to achieve this method. The additive genetic variance (V_A) and the proportion of phenotypic variance (V_P) were calculated to estimate the narrow sense heritability (h^2) of anxiety as in the following equation: $h^2 = V_A/V_P$ using the univariate animal model run in the MCMCglmm package in R (Ariyomo *et al*., 2013; White & Wilson, 2019). The model was run with its default value (13000) of iterations = 36001:155881, thinning interval = 120, thi = 10 and burn in period = 3000 (Ariyomo *et al*. 2013). The statistical significance of the genetic components of the model was detected using deviance information criteria (DIC). The highest posterior density *HPDinterval* function from the MCMCglmm package was used to evaluate the statistical significance of the heritability estimate from the posterior distribution by computing the lower and upper limits of 95% credible intervals (CI) (Ariyomo *et al*. 2013; Ringo, 2022).

To estimate maternal effects, the maternal identity (V_M) was added to the previous model specified for anxiety as a random effect. The equation: $m^2 = V_M/V_P$ was followed to determine the maternal effects as a proportion of total variance (White & Wilson, 2019). The model was rerun with the iterations described above for the posterior distribution of the estimates of the additive genetic (V_A), maternal (V_M), and residual (V_R) variance (Ariyomo *et al*. 2013).

In this study, the offspring were not sexed. There was no variation in the number of broods selected from each pair (see Methodology, section 5.3.2. behavioural testing). Therefore, for the heritability estimates conducted here, we did not include the effect of sex and brood size to the model.

Latency to enter the upper half of the tank, number of entries, and time spent in the upper half of the tank were used as response variables in separate models with individual and maternal identity as random effects, linked to the pedigree and use of Poisson distribution.

5.4 Results

There were significant genetic components describing phenotypic variances in anxiety, and the variance estimates were different from zero. The DIC = 1517.615, 1560.06, 1995.89 for additive and non-additive components with maternal identity as random effects, for latency to enter the upper half of the tank, number of entries to the upper half of tank and time spent in the upper half, respectively.

There were significant additive genetic components and the estimates of heritability explaining 3, 11 and 18 % of the total phenotypic variance in latency to enter the upper half of the tank, number of entries to the upper half of the tank and time spent in the upper half respectively (Table 4.1).

We also found significant non-additive genetic factors explained by residual variance and maternal effects contributing to the total phenotypic variance in anxiety. Residual variance for latency to enter the upper half of the tank was $V_R = 0.39$, for number of entries to the upper half of the tank $V_R = 0.60$, and for time spent in the upper half of the tank $V_R = 0.36$. The maternal effects (V_M) explained 58, 29 and 46 % for latency to enter the upper half of the tank, number of entries to the upper half of the tank and time spent in the upper half, respectively (Table 4.1).

Table 5.1. Estimates of additive (V_A) and non-additive (V_R) components of anxiety, the proportion of phenotypic variances including maternal identity (V_M) and individuals' age (Age) as random effects with 95 % confidence intervals.

Trait	Component	Variance	Variance 95 % CI (HPD intervals)	Proportion variance (h^2)	Proportion variance 95 % CI (HPD intervals)
latency to upper	V_A	0.32	4.946e-07, 1.3	0.03	4.007114e-08, 0.12
	V_M	6.58	2.9, 11.6	0.58	0.42, 0.75
	V_R	4.13	3.02, 5.42	0.39	0.22, 0.56
number of entries	V_A	0.13	7.089e-07, 0.40	0.11	5.613514e-07, 0.32
	V_M	0.34	0.07, 0.66	0.29	0.09, 0.47
	V_R	0.67	0.42, 0.92	0.60	0.34, 0.84
time in upper	V_A	0.56	5.501e-08, 1.86	0.18	2.395571e-08, 0.49
	V_M	1.39	0.54, 2.30	0.46	0.28, 0.63
	V_R	0.99	0.00, 1.46	0.36	4.495595e-05, 0.57

5.5 Discussion

In this study, we estimated additive and non-additive genetic components contributing to anxiety phenotypic variance. The heritability estimate for anxiety was found to be attributed to genetic components that were accounting for 3, 11 and 18 % of the total variances and other non-genetic factors including residual variance and maternal effects. This finding is in line with previous studies that have reported that some personality traits are heritable, such as shyness-boldness, activity, and reactivity displayed in an antipredator context in dumpling squid, *Euprymna tasmanica* (h^2 = 0.21-0.89, Sinn *et al*., 2006), exploratory behaviour in the great tit, *Parus major* (h^2 = 0.477 $\pm$ 0.101-0.331 $\pm$ 0.114), Drent et al., 2003), aggression (h^2 = 0.12), activity (h^2 = 0.08), and docility (h^2 = 0.09) studied in North American red squirrels, *Tamiasciurus hudsonicus* (Taylor et al., 2012), boldness in wandering albatrosses, *Diomeda exulands* (h^2 = 0.24, Patrick et al., 2013), and locomotor capability in yellow bellied marmots, *Marmota flaviventris* (h^2 = 0.21), including vigilance (h^2 = 0.08, Blumstein et al., 2010).

Our study indicates that although there were additive genetic components, there were non-additive factors, or residual variance, contributing to the phenotypic variance in anxiety. An explanation for this finding is that anxiety might be related to fitness, since additive genetic variation is known to be reduced when traits are strongly associated to fitness (Stirling et al., 2002; Sinn et al., 2006), and favoured by natural selection (see references in Ariyomo *et al*., 2013). Blumstein *et al*. (2010) also found that the heritability estimate of vigilance was low in yellow bellied marmots, *Marmots flaviventris*, and suggested that this was because there was a strong link between fitness and this trait, so the trait was fixed by selection, which minimised individual genetic variation. Under threatening conditions, fitness could be increased by anxiety, because threats involve increased vigilance, physiological responses, and defence (Marks & Nesse,1994), so there may be strong selection on anxiety that then results in a low

heritability estimate. In the previous chapter (Chapter IV), we did not find a direct effect of anxiety on reproductive fitness in zebrafish. Thus, this finding involving the low estimate of heritability of anxiety raises the question whether anxiety is a trait that is fixed by selection or there are other factors underlying it. Also, some traits might be more influenced by environmental than genetic components, which could be the case with anxiety.

As mentioned, the heritability of anxiety was low, and a possible explanation might be because of the residual variance that is accounting for 39, 60, and 36 % of the proportion variances of anxiety. Differences in heritability level may result from big differences in the residual variation (Van Oers et al., 2005). Sinn *et al*. (2006), found that the heritability estimate of boldness-shyness, activity, and reactivity exhibited in a feeding test in dumpling squid, *Euprymna tasmanica* was low (h^2 = 0.05-0.08) because of the high residual factors (V_R = 0.7-7.9) of phenotypic differences that involved environmental and non-additive genetic factors. Furthermore, our results are consistent with that reported by Ariyomo *et al*. (2013) who also found that the heritability of aggressiveness in zebrafish was moderate (h^2 = 0.36) because of the high residual variance (V_R = 0.55) and non-additive genetic components explained by maternal effects. Also, Tylor et al. (2012), found low heritability estimates of aggression, docility, and activity (h^2 = 0.08-0.12) in North American red squirrels because of the contribution of other factors to the additive genetic effects, such as maternal effects (V_M = 0.07-0.15), permanent environmental effects (V_E = 0.08-0.16), and cohort effects (0.07-0.09). Another possible explanation for the low estimate of the heritability found in this study is that across biological levels, traits with a high rate of integration can result in a decreased heritability estimate of such traits (Sinn et al., 2006). Anxiety-like behaviour recorded in this chapter was elicited following novelty exposure in the form of the novel tank diving test, and

anxiety elicits neuroendocrine responses through the stress response (Wei et al., 2020; Ellis et al., 2012).

Our findings indicate that there were maternal (58, 29 and 46 %) effects contributing to the total variances of anxiety. This finding explains that the anxiety development of offspring was affected by their mother. There is no paternal care in zebrafish because they are egg scatterers, but maternal effects may occur before spawning such as exposure to mothers' hormones in very early stage of development (Spence et al., 2008; Spence, 201; Ariyomo, et al., 2013; Baker et al., 2013; White et al., 2019). Maternal effects have been indicated to contribute to non-additive genetic variance in many studies. For example, in zebrafish, Ariyomo et al. (2013) found significant maternal effects (9 and 18 %) contributed to the proportion of variance in aggressiveness and boldness respectively. Furthermore, White et al. (2019) showed that there was a significant influence of maternal effects on risk-taking behaviours in Trinidadian guppies, *Poecilia reticulata*, offspring.

Anxiety has been shown to be associated with age (Torras-Garcia et al., 2005). For instance, in mammals, Wister rats, *Rattus norvegicus*, of three months old displayed a higher level of anxiety compared to those of 24 months old in the elevated plus maze test (Torras-Garcia et al., 2005). Moreover, in zebrafish, thigmotaxis ("wall hugging" a sign of anxiety) was found to increase in a younger TU strains of 6 months old compared to 12 month old individuals, while bottom dwelling increased in older TL strains individuals (Hudock & Kenney, 2023). The age of offspring at testing in this study were two months while the parents were 9-11 months so, their behavioural responses could be different between them and this may contribute to the outcome of the heritability estimate of anxiety in this study.

However, further work is needed to investigate age effect on anxiety responses and its contribution to heritability estimate.

Environmental conditions may affect the outcome of heritability estimates. For example, brood size, season and year of an animal's birth, season of gestation period in mammals and female body size are environmental variables that can affect heritability (Gebhardt-Henrich & Noordwijk, 1991; Javed et al., 2001; Thevamanoharan et al., 2002; White & Wilson, 2019).

In conclusion, this study provided a new insight into the heritability and genetic components underlying anxiety as a personality trait. This trait has been shown to be affected by additive genetic components and residual variance, and this trait may show responses to selection. Moreover, this study showed that other factors such as maternal factors contributed to non-additive genetic variance in anxiety suggesting this trait is environmentally controlled.

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Chapter VI

Responses of zebrafish with different levels of anxiety to conspecific alarm cue

6.1 Abstract

An extensive body of literature has developed around the theme of antipredator responses to stressors in the aquatic system. One stressor that is broadly reported is the chemical alarm cue. Although many studies have reported the innate ability of fish to display antipredator behaviours in response to alarm cues, understanding the responses of fish with known personality traits has received little consideration. Here, I tested the responses of male and female zebrafish with different levels of anxiety to alarm cue. Two groups, least anxious and most anxious, of both sexes, which had had their behaviour assessed previously in the novel tank diving test (Chapter IV), were individually exposed to alarm cues and each fish was recorded again. Behaviours before and after exposure to alarm cue were compared. Individuals showed a significant decrease in latency to enter the upper half of the tank and an increase in the number of entries and in the time spent in the upper half of the tank after exposure to alarm cue. Moreover, no significant difference between pre-and post-exposure was observed in the behaviour of fish, except for the time spent in the upper half of the tank, which significantly increased post exposure, suggesting that these fish became less anxious and responded less to the alarm cue. Females showed a significant increase in latency to enter the upper half of the tank and decrease in the number of entries compared to the males. However, unexpectedly, females exhibited significantly more time in the upper half of the tank compared to males. These findings indicate that exposure to alarm cue affects how zebrafish respond to stress.

6.2 Introduction

Homeostasis is a physiological process that maintains the inner stability of an individual's body, allowing it to cope with environmental changes (Schneiderman et al., 2005), and it is at the heart of our understanding of physiological regulatory mechanisms (Modell et al., 2015). Stress has been referred to as the effect caused by external physical and environmental or internal physiological and psychological stressors (Balcombe et al., 2004). Stress is a phenomenon that individuals experience, and it elicits from them responses that affect their neural, endocrine, and immune system (Tort, 2011). The stress response is the result of a real or potential threat encounter that affects homeostasis (Clark et al., 2012; Schneiderman et al., 2005) and is regulated by the hypothalamus-pituitary-adrenal (HPA) axis in mammals (Oyola & Handa, 2017; Smith, 2006) or the hypothalamus-pituitary-interrenal (HPI) axis in fish (Ellis et al., 2011; Pijanowski et al., 2015; Fig. 6.1). Stressor exposure leads to an increase in plasma glucocorticoid concentration, such as the stress hormone cortisol (Bell et al., 2007; Ellis et al., 2011; Pijanowski et al., 2015).

Some stressors are acute and others are chronic; and they included physical, social and environmental, as well as those caused by infections (Clark et al., 2012), food limitation (Abreu et al., 2016), and chemicals, such as "alarm substance" (Sanches et al., 2015) released from alarm cells (Halbgewachs et al., 2009). In laboratories, the most widely used acute stressors in animals, including fish, are physical, such as handling, net chasing, capture (Clark et al., 2012), blood collection, and orogastric gavage (Balcombe et al., 2004).

Chemical cues are widely used by fish to monitor their predation risk. In general, there are several kinds of chemical cues that play a critical role in antipredator behaviour, such as predator odours, known as kairomones, that are naturally released and are used by prey as an

indicator of predator presence, and prey odour released from an animal being stressed or startled (but not injured), for example, urinary ammonia, which is recognized by conspecifics, and damage-released alarm cues (Vogel et al., 2017; Wisenden & Chivers, 2006), which were originally described by Von Frisch (Maximino *et al*., 2010). Von Frisch accidently observed that a chemical alarm substance released from injured minnows, *Phoxinus phoxinus*, successfully warned conspecifics by reducing their movement and causing shoaling behaviour. A chemical alarm substance is an inherited blend of components that varies depending on species (Barkhymer et al., 2018). Alarm cues are released from the epidermal club cells of damaged skin (Halbgewachs et al., 2009; Marvin & Hutchison, 1995), and their reactions are manifested in the number and duration of irregular movements and freezing incidences (Maximino et al., 2010). The ability to detect, respond to, and escape chemical substances discharged by dead or injured conspecifics is a dominant feature of aquatic organisms (Vogel et al., 2017) including fish such as the rainbow darter, *Etheostoma caeruleum* (reviewed by Abudayah & Mathis, 2016), coho salmon, *Oncorhynchus kisutch,* and fathead minnows, *Pimephales promelas* (reviewed by Maximino et al., 2010), as well as larval or adult amphibians like the red-spotted newt, *Notophthalmus-viridscens*, and fire-bellied newt, *Cynops pyrrhogaster* (Bryer et al., 2001; Crossland et al., 2019; Marvin & Hutchison, 1995). Fish that detect chemical alarm cues decrease their movement, reduce their foraging behaviour, avoid novelty (Barreto et al., 2010; Mirza & Chivers, 2003; Zenki et al., 2020), and reduce their aggressive behaviour (Barreto et al., 2010), and the detection of this threat is an essential factor for survival (Sanches et al., 2015; Vogel et al., 2017). In fish, such as the Ambon damselfish, *Pomacentrus* amboinensis (Lönnstedt & McCormick, 2011), juvenile silver catfish, *Rhamdia quelen* (reviewed in Vogel et al., 2017), and Indo-pacific gobiid fish (the starry goby), *Asterropteryx semipunctatus* (Smith, 1989), that encounter or are injured by predators, their chemical alarm substances help to warn conspecifics as a line of defence in antipredator

behaviour (Abreu et al., 2016; Mirza & Chivers, 2003; Sanches et al., 2015).

Stress responses have been studied in teleost fishes for a long time, and the zebrafish (*Danio rerio*) is a major model animal to study the physiological, behavioural, genetic and genomic aspects of stress reactions (Abreu et al., 2016; Clark et al., 2012; Eachus et al., 2017; Ord et al., 2019; Speedie & Gerlai, 2008). Zebrafish are also one of the most commonly used models for studying behaviours related to the nervous system and anxiety-like behaviours (Cachat et al., 2011). Zebrafish are sensitive to environmental manipulations, such as stress evoked by novelty, predator and alarm cues, and antigenic and anxiolytic drug exposure (Cachat et al., 2010; Cianca et al., 2013; Collier et al., 2017; Wang et al., 2020). Zebrafish are known to show antipredator responses to secreted alarm substances from injured conspecifics (Ord et al., 2019; Speedie & Gerlai, 2008). Depending on the stressor type, exposed zebrafish in a novel environment vary in their anxiety levels, which in turn affects their exploration, speed, and erratic behaviours. Anxiety levels of zebrafish can be low when they are treated with anxiolytic drugs, such as nicotine and ethanol, but higher when they are subjected to stressful stimuli, such as anxiogenic drugs, predator contact or conspecific alarm cues; this increases latency to enter the upper part of a novel tank, erratic movement, and freezing time (see references in Cachat et al., 2010). Responses to alarm cues can differ between and within species (Ide et al., 2003; Quadros et al., 2019). For instance, chronic exposure to alarm cues has been shown to induce anxious responses in zebrafish, but these were strain-specific, such that wild-type (*WT*) elicited less anxious responses compared to leopard (*leo*) (Quadros et al., 2019).

While different stressful conditions can cause various stress responses, if individuals are exposed to the same stressor, they may respond differently depending on their personality,

which is fundamentally determined by genetic inheritance and the life history of an individual (Castrol et al., 2012; Schneiderman et al., 2005). For example, in sticklebacks (*Gasterosteus aculeatus*), it has been demonstrated that the response to stressors, such as predators or conspecific cues, varies within individuals (Bell et al., 2007). Moreover, Schjolden et al. (2005), found that consistent behavioural variation in aggression in juvenile rainbow trout, *Oncorhynchus mykiss*, reflected differences in responses to a stressor. Although anxious and stress responses are well studied in zebrafish (Abreu et al., 2016; Cachat et al., 2010; Cianca et al., 2013; Rambo et al., 2017), including their responses in the novel tank diving test after alarm cue exposure (Zenki et al., 2020), focusing on personality traits (such as anxiety) underlining these responses has not been investigated.

The main aim of this experiment was to investigate whether there was a difference between least anxious and most anxious zebrafish in their stress responses induced by exposure to alarm cue. Alarm cue induces stress responses in zebrafish such as increasing of erratic movement (Speedie & Gerlai, 2008) and has been used extensively as a stressor (e.g. Eachus et al 2017; Ord et al 2020). Zebrafish with different levels of anxiety were exposed to conspecific alarm cues, then their stress responses in the novel tank diving test, including latency to move to the upper half of the tank, number of entries, and time spent in the upper half of the tank, were recorded and compared to before exposure (control conditions).

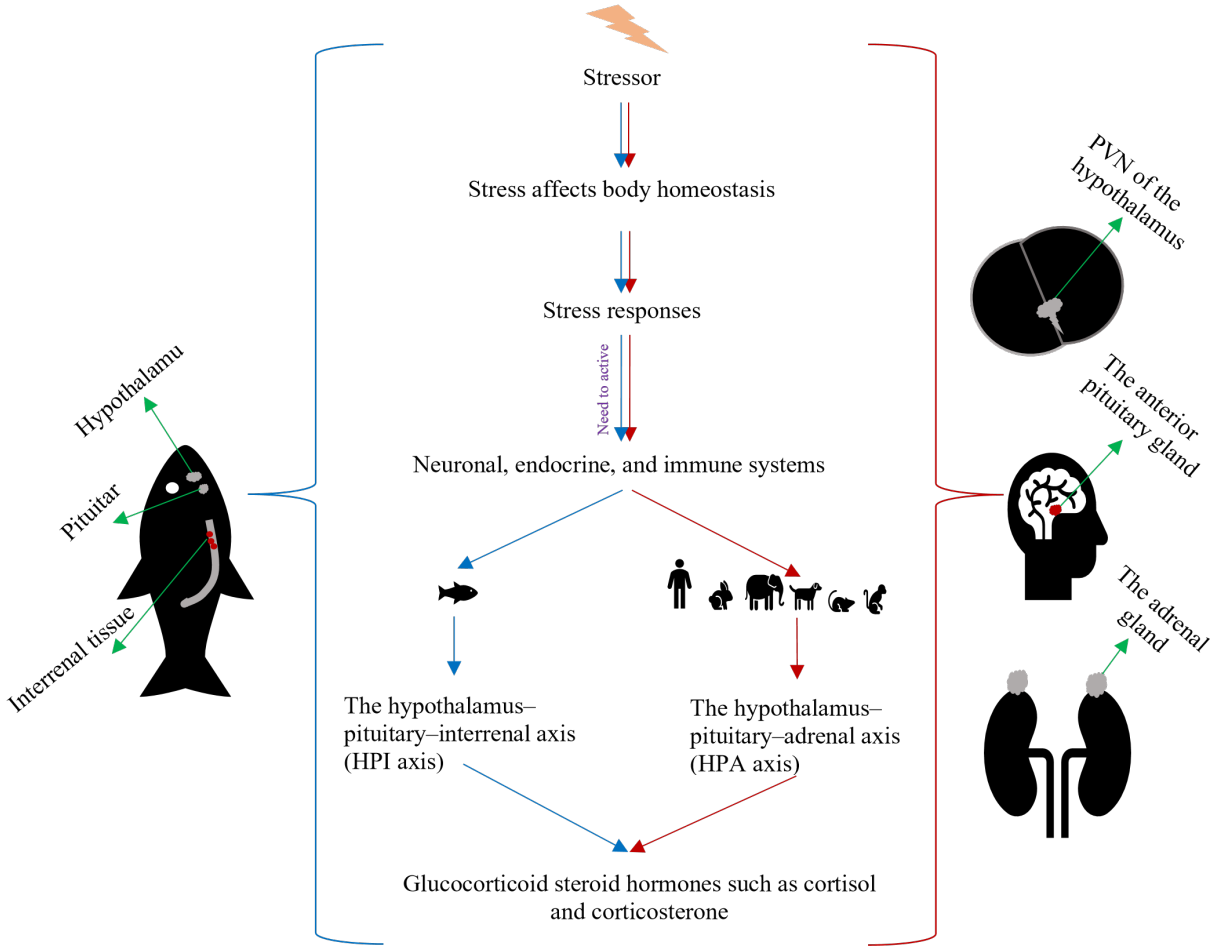

Figure 6.1. The hypothalamus-pituitary-interrnal (HPI) axis in fish and hypothalamus-pituitary-adrenal axis (HPA) in mammals when exposed to stressors (information taken from Pijanowski et al., 2015; Smith & Vale, 2006; Oyola & Handa, 2017; Ellis et al., 2011).

6.3 Methodology

6.3.1 Study animals

Adult zebrafish (18-20 months old) with known levels of anxiety used in Chapter IV were used in this experiment. Fish were held in the aquarium in the Department of Animal and Plant Sciences, University of Sheffield. Fish were kept individually in tanks (30 x 15 cm, 24.5 cm high) containing 10 l of water heated to 27 °C on a 12:12 h light: dark cycle and connected to a water recirculatory system. The length of each fish (from its snout to its caudal

peduncle) was measured using a ruler, by holding it gently in the tank using a net. Fish were fed twice a day using commercial dry food and brine shrimp (*Artemia* sp). This work was conducted under a Home Office licence. For pre-exposure test, fish used in Chapter IV were introduced to the novel tank diving test under control water conditions had already been collected following the same steps described in the Chapter II (see: 2.3 Methodology, 2.3.2.1) and Chapter IV (see: 4.3 Methodology, 4.3.2). Then the whole procedure was repeated to test the repeatability. Fish were then ordered depending on their level of anxiety (see Chapter IV, 4.3.2.2) and the 20 most anxious and the 20 least anxious males and females were used in this experiment.

6.3.2 Stressor preparation

In this experiment, alarm cues of conspecifics were used as stressors. To prepare the alarm cues, five adult zebrafish were euthanised using MS222 for at least 900 s. After death was confirmed, the fish was removed from the MS222 and rinsed using distilled water to remove the traces of the anaesthetic, and patted dry using paper towels to remove the excess water. Ten vertical cuts were gently made in each side of the fish using a razor blade. The fish was placed in 3 ml of distilled water in a glass vial and gently shaken for about 120 s. The prepared solution was moved to glass vials and heated at 95°C for 16 h. The solution was removed from the oven, run under cold water and pipetted into 1.5 ml centrifuge tubes and centrifuged at 13,000 x g for 10 min. The extracted solution was kept in the refrigerator until it was needed.

6.3.3 Exposure phase

Each test fish was transferred from its holding tank (22 l x 11 w x 14 h cm) to a small tank (17 l x 11 w x 11 h cm) containing 500 ml of heated dechlorinated water at 27 °C and was

left for 60 s to acclimatise. Each fish was moved from the small tank to a 1000 ml glass beaker, covered at the sides to prevent disturbance, containing 600 ml of heated dechlorinated water and 200 ul of alarm substance, and left for 1260 s. The fish was removed from the beaker and the water containing alarm substance was changed after each exposure (Fig. 6.2).

6.3.4 Testing phase

After exposure, the fish was immediately transferred from the glass beaker to a novel diving test tank (25 l x 15 w x 15 h) containing 4 l of dechlorinated water heated to 27 °C and left for 60 s for acclimation (Fig. 6.2). The test tank was horizontally divided into upper and lower halves and a 40 W LED panel (600 x 600 mm) (Element Lighting, Colchester, UK) was used to illuminate it from underneath. A digital camera (Panasonic HC-V160) was positioned in front of the tank and, after the acclimation period, recorded the behavioural responses of the fish for 300 s. Behaviours measured from the digital recordings were number of entries to the upper half, latency to enter the upper half, and time spent in the upper half of the tank. After the test, the fish was returned to its holding tank in the aquarium. Water in the small tank, the beaker, and the novel test tank was changed for each fish.

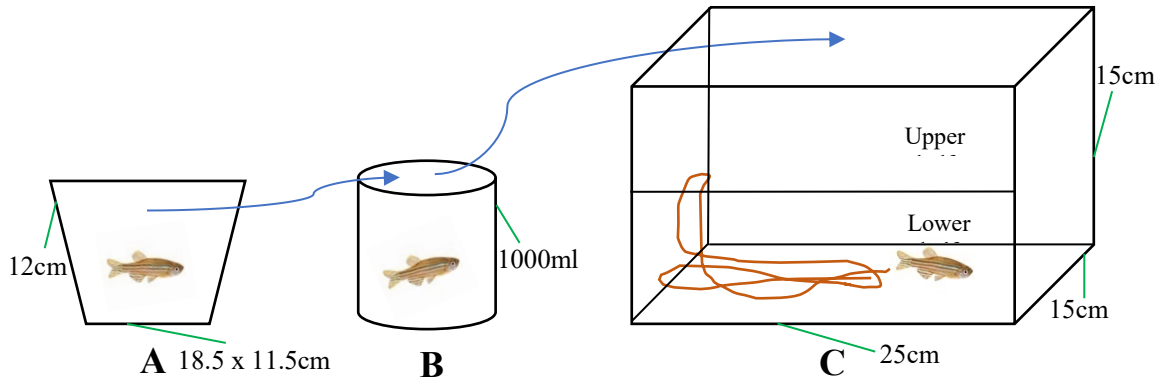

Figure 6.2. The pre-test acclimation tank (A), the 1000 ml beaker (B), and the novel diving test tank (C) used for alarm cue exposure and behavioural testing in zebrafish

6.3.5 Statistical analysis

All data analyses were performed using R software (version 1.1.383; R Core Team, 2018). A generalised linear model was fitted to the data using *glm* function from the lme4 package in R software (version 1.1.383; r Core Team, 2018). This model was done with sex (male and female), anxiety level (least anxious and most anxious), and exposure (pre-exposure; the mean of the first and second pre-exposure trials for each behavioural measure (see Chapter IV) and post-exposure, trials recorded using the novel tank diving test after exposure to the conspecific alarm cues) as fixed effects to determine their effect on behavioural responses including latency to enter the upper half of the tank, number of entries, and time spent in the upper half of the tank , and the interaction between them. Log transformation for the Poisson distribution was used because the data were not normally distributed.

6.4 Results

6.4.1 Latency to enter the upper half of the tank

Sex had a significant effect on latency to enter the upper half of the tank, with females having a higher latency to enter the upper half than males (estimate = 0. 34203, SE = 0.07788, $z = 4.392$, $p < 0.001$; Fig. 6.3A). Anxiety level also had a significant effect on latency, and most anxious individuals had a higher latency than least anxious individuals (estimate = 1.07590, SE = 0.06896, $z = 15.602$, $p < 0.001$; Fig. 6.3A). There was no significant effect of exposure to alarm cue on latency (estimate = -0.02151, SE = 0.08467, $z = -0.254$, $p = 0.799$; Fig. 6.3A). There was a significant interaction between sex and level of anxiety, with most anxious females having a lower latency to enter the upper half after exposure (estimate = -1.14363, SE = 0.09984, $z = -11.454$, $p < 0.001$; Fig. 6.4A). However, there was no significant interaction between sex and exposure (estimate = 0.15346, SE = 0.10907, $z = 1.407$, $p = 0.159$; Fig. 6.4A). There was a significant interaction between anxiety level and exposure, with most

anxious individuals having a higher latency before exposure (estimate = 61224, SE = 0.9512, z = 6.436, p < 0.001; Fig. 6.4A), There was a significant interaction between sex, anxiety level, and exposure, with most anxious females having a higher latency before exposure (estimate = 1.39030, SE = 0.12957, z = 10.730, p < 0.001, Fig. 6.4A).

6.4.2 Number of entries to the upper half of the tank

Sex had a significant effect on the number of entries to the upper half of the tank, with females having significantly less entries than males (estimate = -0. 55274, SE = 0.05391, z = -10.252, p < 0.001; Fig. 6.3B). Anxiety level had a significant effect on the number of entries and most anxious individuals had less entries than least anxious individuals (estimate = -0.38031, SE = 0.05113, z = -7.438, p < 0.001; Fig. 6.3B). There was no significant difference between individuals pre-exposure and post-exposure (estimate = 0.01684, SE = 0.04588, z = 0.367, p = 0.71357; Fig. 6.3B). There was a significant interaction between sex and level of anxiety with most anxious females having more entries after exposure (estimate = 0.26298, SE = 0.08083, z = 3.253, p < 0.01; Fig. 6.4B). There was no significant interaction between sex and exposure (estimate = 0.11600, SE = 0.07461, z = 1.555, p = 0.11999; Fig. 6.4B). However, there was a significant interaction between anxiety level and exposure with most anxious individuals having less entries before exposure than after exposure (estimate = -0.68848, SE = 0.08184, z = -8.413, p < 0.001; Fig. 6.4B). There was a significant interaction between sex, anxiety level, and exposure with most anxious females havig less entries before exposure than after exposure (estimate = -0.99733, SE = 0.14845, z = -6.718, p < 0.001, Fig. 6.4B).

6.4.3 Time spent in the upper half of the tank

Sex had a significant effect on the time spent in the upper half of the tank and females spent significantly more time there than males (estimate = 0.11336, SE = 0.02890, z = 3.922,

$p < 0.001$; Fig. 6.3C). Anxiety level had a significant effect on the time spent in the upper half, with most anxious individuals spending significantly less time than least anxious individuals (estimate = -0.32699, SE = 0.03245, z = -10.075, $p < 0.001$; Fig. 6.3C). There was a significant difference between individuals pre-exposure and post exposure in the time spent in the upper half of the tank, with individuals spending significantly less time before exposure compared to after exposure (estimate = -0.48154, SE = 0.03399, z = -14.166, $p < 0.001$; Fig. 6.3C). There was a significant interaction between sex and level of anxiety with most anxious females spending significantly less time before exposure in the upper half of the tank (estimate = -0.11092, SE = 0.04536, z = -2.445, $p < 0.05$; Fig. 6.4C) but there was no significant interaction between sex and exposure (estimate = 0.02764, SE = 0.04658, z = 0.593, p = 0.5529; Fig. 6.4C). There was a significant interaction between anxiety level and exposure where most anxious individuals displayed less time in the upper half of the tank before exposure (estimate = -0.32969, SE = 0.05608, z = -5.879, $p < 0.001$; Fig. 6.4C). There was a significant interaction between sex, anxiety level, and exposure with most anxious females spending less time in the upper half of the tank before exposure (estimate = -1.26578, SE = 0.09752, z = -12.980, $p < 0.001$; Fig. 6.4C).

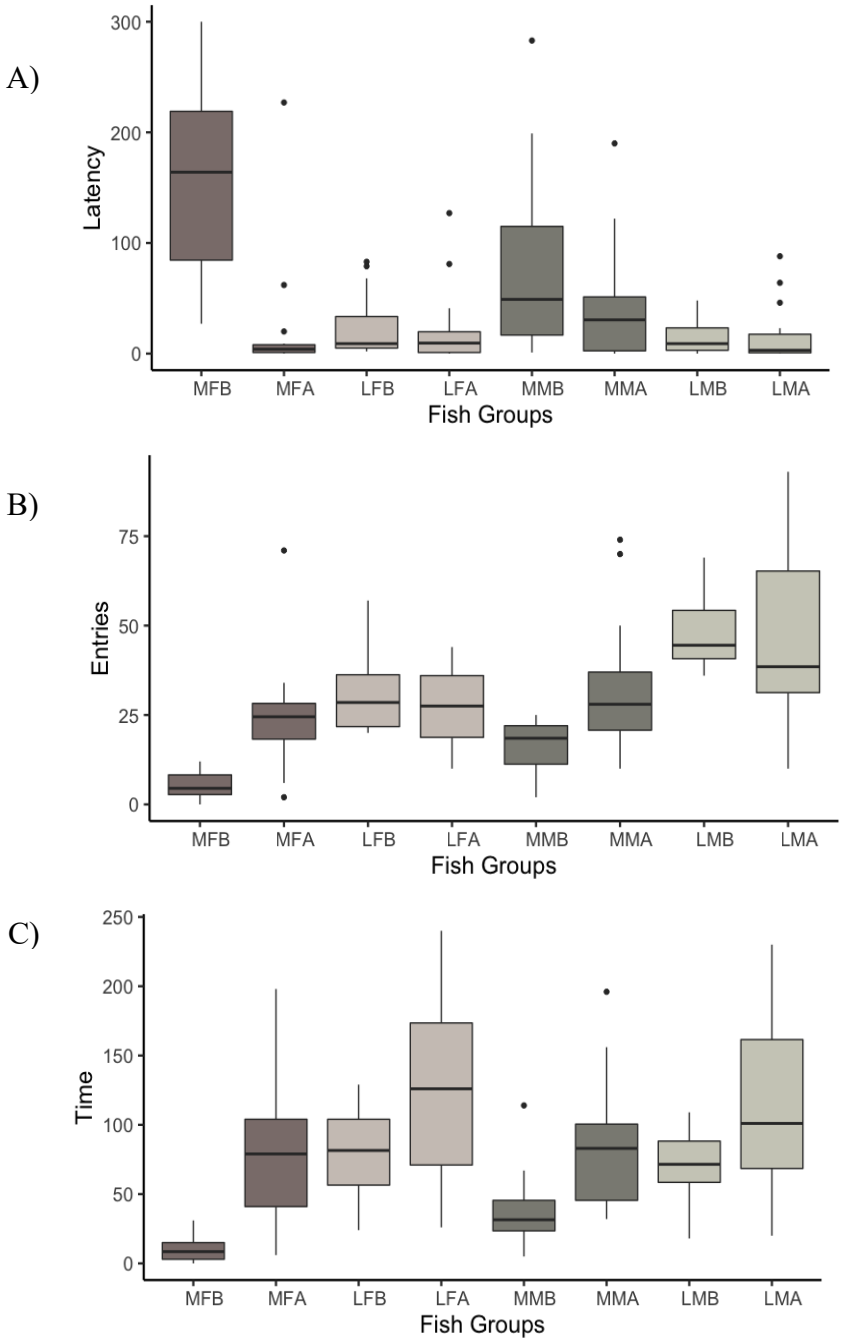

Fig. 6.3. Medians (horizontal line inside the box) and interquartile ranges (the lower area of the box to the median displays the second quartile while the upper area represents the third quartile), outer quartile (the vertical lines from the box represent the first and fourth quartiles), and outliers (dots) for (A) latency to enter the upper half of the tank, (B) number of entries into the upper half of the tank, and (C) time spent in the upper half of the tank by least anxious and most anxious female and male zebrafish in the novel tank diving test before and after exposure to conspecific alarm cue. MFB (most anxious females before exposure), MFA (most anxious females after exposure, LFB (least anxious females before exposure), LFA (least anxious females after exposure), MMB (most anxious males before exposure), MMA (most anxious males after exposure), LMB (least anxious males before exposure), and LMA (least anxious males after exposure.

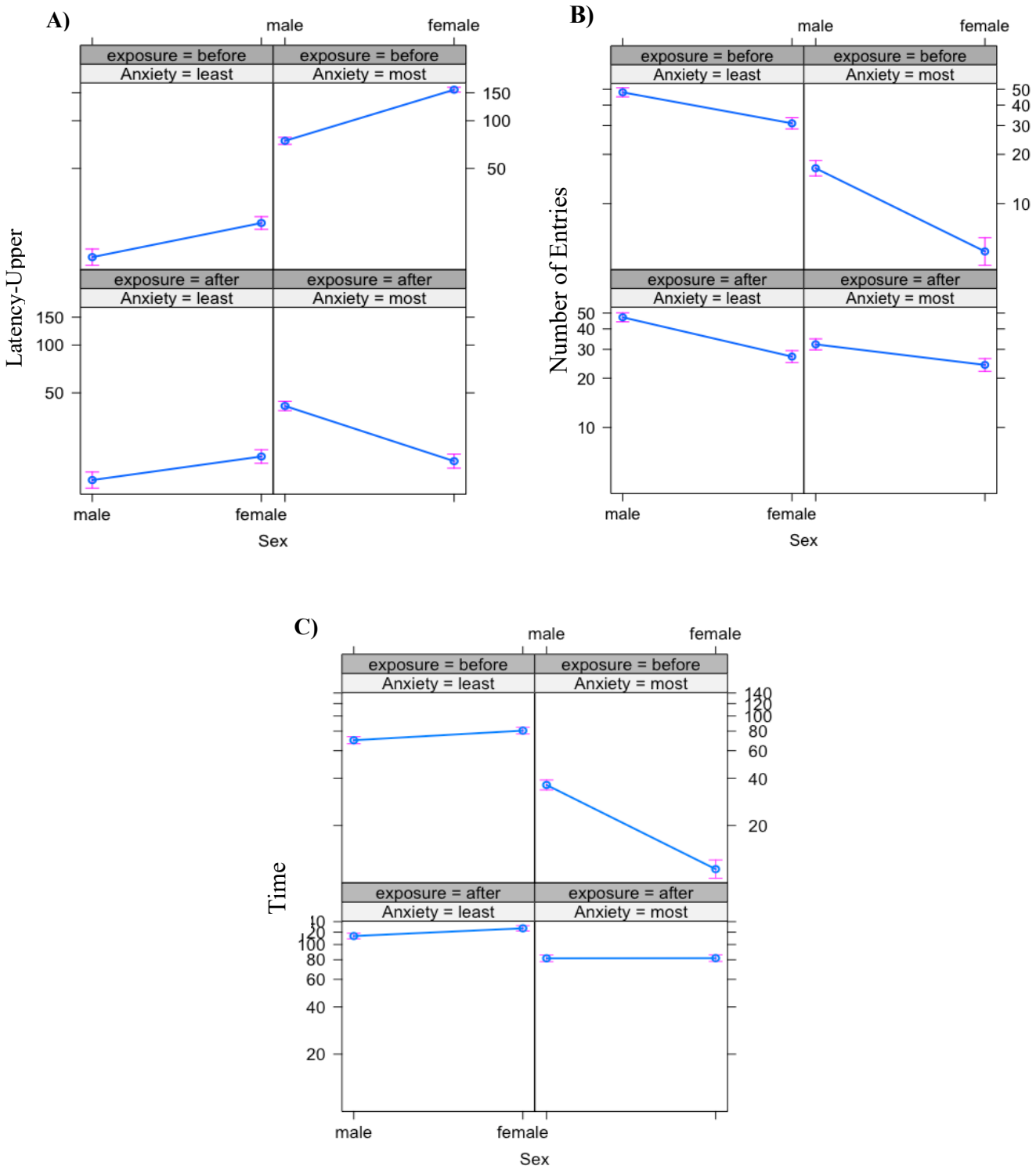

Fig. 6.4. Effect plot for interaction of sex, level of anxiety, and exposure to alarm cues for (A) latency to enter the upper half of the tank, (B) number of entries into the upper half of the tank, and (C) time spent in the upper half of the tank displayed by male and female zebrafish in the novel tank diving test pre and post exposure to alarm cue.

6.5 Discussion

The current experiment was designed to determine the effect of exposure to alarm cue as a stressor on the behaviour of male and female zebrafish with different levels of anxiety. Overall, the results revealed that exposure to alarm cue did not influence behavioural measures except time spent in the upper half of the tank. Individuals spent more time in the upper half of the tank after exposure to alarm cue than before. However, the interaction between exposure and level of anxiety, showed that most anxious individuals had reduced latency, more entries and spent more time in the upper half after exposure compared to before exposure. This suggests that fish either did not respond to the alarm cue or they became even less inhibited to enter and move into the top area. The findings in this chapter differ to those of other studies demonstrated a significant stress responses following exposure to alarm cues (e.g., Barkhymer et al., 2018; Chivers et al., 2013; Clegg & Barlow, 1982; Gardner et al., 2020; Ide et al., 2003; Jesuthasan et al., 2020; Kadye et al., 2020; Mathis & Smith, 1992; Mirza & Chivers, 2003; Speedie & Gerlai, 2008; Wisenden et al., 1995). In great tits, *Parus major*, individuals with different personality traits (slow versus fast exploration) reacted differently to social stress stimulated by aggressive male confrontation, with a greater response exhibited by slow explorers compared to fast explorers (Carere et al., 2003). Also, *P. major,* individuals with different personalities (bold versus shy) exhibited different responses to handling stress conducted at two different times, daytime and night time, immediately after capture and 5 min later, with the bold group exhibiting a lower body temperature directly after handling and a lower breathing rate than the shy ones during the night period (Carere & Van Oers, 2004). Furthermore, Veenema et al. (2003), demonstrated that wild house mice, *Mus musculus domesticus*, with different levels of aggression (short latency to attack and long latency to attack) responded differently to a stressor (forced swimming), and increased immobility behaviour was seen in less aggressive individuals compared to more aggressive individuals.

Castro *et al*. (2012), found that in adult male Sprague-Dawley rats, individuals that differed in anxiety, exploration, and activity, showed vulnerability and resilience in response to stress, with high-anxiety and low-exploration animals exhibiting higher responses to stress.

One explanation for this finding may be hippocampus plasticity caused by habituation resulting from stress exposure (see references in Ord et al., 2020). Another explanation is that fish may perceive the stressor as not threatening due to acclimation to the exposure. Bell et al. (2010), suggested that following exposure to even a mild stressor in trout caused an inability to respond due to acclimation. Furthermore, lack of response and reduction in the anxiety level of the fish that reduced latency to move to the upper half, increased number of entries and spent longer in the upper half of the tank post- compared to pre exposure might have been because of mating behaviour displayed before exposure (see Chapter IV). Personality traits, such as social behaviour and risk taking have been shown to be affected following mating in three spine sticklebacks (Monestier & Bell, 2020). The optimal expression of anxiety can be affected by reproductive behaviour, such as searching for a mate and egg-laying sites that cause a change in an animal's risks and vulnerability (Bath et al., 2020). Fish used in this experiment have been shown to display mating behaviour previously (see Chapter IV, section 4.3.2.3 and Chapter V, section 5.3.1), so regardless of exposure effect, it was possible that this reduced their anxiety levels in these experiments.

The results of this study are contrary to previous studies on fish that have suggested that responses such as freezing, increased movement, and looking for shelter are increased when individuals are exposed to chemical alarm substance (Ide et al., 2003; Kalueff et al., 2013; see references in Lönnstedt & McCormick, 2011; Wisenden et al., 1995). For instance, after exposure to conspecific alarm cues, behaviour associated with increased anxiety has been

detected, such as reduced exploration and increased erratic movements in zebrafish in the novel tank diving test (Egan et al., 2009), increased stereotypical movements in zebrafish (Speedie & Gerlai, 2008), increased latency to move and reduced activity in rainbow darters, *Etheostoma caeruleum* (Anderson et al., 2016), lack of activity by larval spotted salamanders, *Ambystoma maculatum* (Gardner et al., 2020), and reduced movements by larval southern newts, *Triturus pygmaeus* (Gonzalo et al., 2012) and reticulate sculpins, *Cottus perplexus* (Chivers et al., 2000). However, contrary to those findings, Speedie and Gerlai (2008), found that groups of zebrafish exposed to different alarm cue concentrations did not differ in their behaviour, which they ascribed to the experiment being conducted in a clear glass bottom tank and thus preventing the fish from camouflaging themselves, which they would have done in their natural habitat.

Sex had a significant effect on latency, which was higher in females than males, and number of entries, which was lower in females than males. This suggests that females were more anxious than males. This finding is consistent with that found in the second chapter of this thesis (Chapter II, Section 2.4.1.1) which showed that males had a lower latency to enter the upper half of the tank compared to females. Sex differences in stress responses have been reported before in zebrafish. For example, it has been shown that in female zebrafish cortisol level in the hypothalamic pituitary interrenal axis (HPI) was higher compared to males following exposure to the stressor triphenylphosphate (Liu et al., 2016). This could be attributed to differences between males and females in endocrine responses and steroid hormones such as cortisol produced during the stress exposure (Aoyama et al., 2003). Moreover, in mammals, the hypothalamic pituitary adrenal axis (HPA) reacts more quickly in females than in males (Goel et al., 2014). For instance, cortisol level, which is related to the stress response, was found to be higher in female Shiba goats, *Capra aegagrus hircus*,

compared to males after transportation (Aoyama et al., 2003). This may be due to females secreting oestrogen in ovaries that leads to increased (HPA) stress responses (Aoyama et al., 2003). However, it has been found that following unpredictable chronic stress, female zebrafish produced lower amounts of body cortisol than males (reviewed by Santos et al., 2021). Moreover, Wong et al. (2019) found that the whole-body cortisol level of zebrafish as a result of stress responses was higher in males than females.

Response to alarm cues is dependent on many factors, such as chemical cue concentration, with a higher concentration leading to greater antipredator and avoidance behaviours, such as is seen in the Ambon damselfish*, Pomacentrus amboinesis* (Lönnstedt & McCormick, 2011; Marcus & Brown, 2003). The concentration of the cue itself is affected by the distance between the individual and the place where the alarm cue is added, so that concentration decreases with distance, and thus the response decreases as well (Lönnstedt & McCormick, 2011; Speedie & Gerlai, 2008). Fish used in the research described in this chapter were first exposed to alarm cues in a pre-test beaker, then they were moved to a test tank without alarm substance. Therefore, the non-significant effect of exposure on most of the fish behavioural responses may have been due to low concentration.

Furthermore, alarm cue responses have also been shown to be affected by ontogenetic stage of the conspecific cue-originator, such that the greater ontogenetic age disparity between an individual and the cue donor, the weaker the response elicited (Lönnstedt & McCormick, 2011). For example, Mitchell and McCormick (2013) showed that juvenile spiny chromise, *Acanthochromis polyacanthus*, were able to detect alarm cues of juveniles paired with predator cues but were unsuccessful in detecting adult cues paired with predator cues. Body size of the donor of the cue has also been reported as a factor that may affect avoidance responses in fish,

such as in brook char, *Salvelinus fontinalis*, in which the greater the similarity in size between the donor and recipient of the cue, the stronger the avoidance responses exhibited (Mirza & Chivers, 2002). Another possibility that could explain the differences in the alarm cues with age was donor diet that changes depending on ontogenetic stage and can lead to a difference in the epidermis, and thus to epidermal alarm substance cells that produce the alarm cues (see references on Lönnstedt & McCormick, 2011; Wisenden & Smith, 1997). However, Brown et al. (2004), found that juvenile convict cichlids, *Archocentrus nigrofasciatus*, could not differentiate juvenile alarm cues from adult alarm cues. The donor fish in this experiment were selected randomly with unspecific age or size, so regardless of the variation in anxiety between individuals, the unexpected responses to the alarm cues could have been due to differences in the cue.

It is possible that hunger level can have some effect on alarm cues responses. For instance, reticulate sculpins, *Cottus perplexus*, starved for two days failed to show antipredator responses to conspecific alarm cues compared to those that were fed regularly (Chivers et al., 2000). In this study, least anxious and most anxious groups were reared in the laboratory, and they were all fed twice daily under the same conditions; therefore, the failure to respond to alarm cues of conspecifics being a result of hunger levels is improbable.

The aim of the current experiment was to examine the responses of least anxious versus most anxious male and female zebrafish to conspecific alarm cue using the novel tank diving test. One of the most significant results to arise from this experiment was that the individuals unexpectedly reduced their level of anxiety following exposure to the alarm cues, suggesting that zebrafish became less anxious or less inhibited. These results resemble recent findings demonstrating that the offspring of mothers that experienced stress significantly reduced their

anxiety-like behaviour when exposed to alarm cues (Ord et al., 2020). Moreover, the lack of stress response by these over-stressed individuals may be due to impairment of the HPI axis (Eachus et al., 2017). Our study was the first to investigate the impact of conspecific alarm cue on zebrafish with a known level of anxiety and, surprisingly, the groups responded to the alarm cue in an unexpected way. This raises important questions about how these fish respond to stressors.

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CHAPTER VII

General discussion

7.1 Anxiety as personality trait in zebrafish, *Danio rerio*

Although, researchers have investigated a number of personality traits, which are behaviours that show consistent individual variation over time and between contexts, on animals since the 1930s (reviewed in Kaiser & Müller, 2021; Santicchia et al., 2021; Stamps & Groothuis, 2010; Biro & Stamps, 2008; Neave et al., 2020), anxiety has not been studied previously in this way. The main aim of this experiment was to explore whether individual zebrafish varied consistently in anxiety over time and across contexts. Chapter II of this thesis was designed to assess the individual behavioural variation in anxiety in male and female zebrafish using three different tests: the novel tank diving test, the open field test, and the light/dark test as these tests have been broadly used to assess anxiety-like behaviours in zebrafish (Cachat et al., 2010; Gogwin et al., 2012; Stewart et al., 2011; Mathur & Guo, 2011; Magno et al., 2015; Duarte et al., 2019). The results of this chapter revealed that individuals significantly changed their behaviours measured in the three tests. In the novel tank diving test, individuals significantly exhibited lower latency to upper half of the novel tank diving test, a greater number of entries and more time in the upper half of the novel tank diving test in the second test compared to the first test suggesting they become less anxious. In the open field test, individuals spent leass time in the centre in the second test compared to the test one. In light/dark test, individuals significantly spent less time in the light half of the second light/dark test compared to the first test suggesting that they become more anxious. Although the behaviours of male and female zebrafish were inconsistent, individuals responded in the same way, resulting to a significant highly repeatability estimate. Other studies have demonstrated that the consistency and presence of behavioural differences are greater at later rather than earlier stages of life (MacDonald, 1983), such as the study reported by Budaev et al. (1999)

who found that variation in behaviours in a novel and aggressive situation exhibited by the lion-headed cichlid, *Steatocranus casuarius*, were more consistent when they were around one year of age compared than when they were tested at the age of 4-5.5 months. Polverino et al. (2016) also demonstrated that over an individual's lifespan, personality variation consistency increased, and suggested that this was because of the decrease in the plasticity of individual behaviour (age-dependent plasticity), which has also been demonstrated in the literature on human behaviour. All the fish tested in Chapter II were adults so this may have increased the repeatability of behavioural differences and reduced plasticity. In other studies, inconsistency in behaviours has been found to be due to factors including hunger, gene expression, or zebrafish strain, for example, wild-type short fin zebrafish are less anxious than leopard and albino zebrafish, and age (reviewed in Genario et al., 2020), such that female zebrafish of 10 months old exhibit lower anxious responses and movement compared to 22 months old (reviewed in Genario et al., 2020; Philpott et al., 2012). In the tests conducted in Chapter II, hunger levels were controlled to reduce behavioural inconsistency and so the lack of consistent behaviours were probably due to other factors. In Chapter III, differential gene expression was investigated in order to determine whether specific genes were controlling aspects of behaviour in the different groups and sexes of fish.

In Chapter II, principal component analysis (PCA) was used to investigate the relationship between the three repeated tests including the novel tank diving test, open field test, and light/dark test by determining the basic dimensions that caused the measured variable association (reviewed in Kaiser & Müller, 2021). The findings demonstrated that the majority of individuals' variation in anxiety was explained by the novel tank diving test. The reminder was explained by the open field test and the light dark test depending on sex. However, The PCA results demonstrated that the novel tank diving test was loaded in the first component of

the PCA, while the open field test and light dark test were loaded into the second component of the PCA. Therefore, the PCA results suggested that both the novel tank diving test and the open field test were not correlated as they were orthogonal in the analysis while the open field test and light/dark test were negatively correlated.

7.2 Genetic control underlining personality traits

Personality phenotypes usually describe the types of personality that are subtypes of personality traits, such as bolder, boldest, shyer, and shyest, which are personality types that are subtypes of the boldness trait (Kaiser & Müller, 2021). In this thesis, the anxiety trait was subdivided into anxiety types that included least anxious and most anxious individuals according to the individual level of anxiety, as identified in Chapter II. Finding different personality types of anxiety that were consistent, suggested that there may be genes that were differently expressed in the least anxious and most anxious individuals in both male and female zebrafish causing the consistent phenotypic variation in anxiety. Differential gene expression was considered in Chapter III of this thesis. In this chapter, the total RNA was extracted from the brains of least anxious and most anxious male and female zebrafish and we identified genes that were significant differentially expressed between the different groups and sexes, which suggested that variation in anxiety was linked to variation in gene expression.

7.3 Fitness consequence and heritability of personality traits

Fitness consequences, inheritance mechanism, and phenotypic differences must be shown to demonstrate that a given trait has responded to selection (see references in Sinn et al., 2006; reviewed in Ariyomo et al., 2013). The number of reproductive offspring produced determines the evolutionary fitness of an individual (see references in Sinn et al., 2006). In Chapter IV, four different groups with phenotypic variation in anxiety were established and

the number of eggs laid and fertilised after crossing was counted. Surprisingly, there was no significant difference in the number of eggs laid, fertilised and the proportion of fertilisation between anxious and non-anxious females and males.

The elimination hypothesis suggests that alleles related to higher fitness are favoured by selection, so traits being intensely selected should reflect little or no additive genetic differences, therefore, low heritability results when such alleles have become fixed in a population (reviewed in Ariyomo et al., 2013). Thus, the findings suggested that anxiety was not favoured by selection due do the lack of fitness consequences, as measured by the number of eggs laid and fertilised. Vargus et al. (2018), also found that the number of eggs laid by female zebrafish with variation in personality traits, proactive versus reactive, was similar (Vargus et al., 2018). Reproductive success may be higher when males and females in a pair have the same personality compared with those with different personalities (Ariyomo et al., 2013; Collins et al., 2019). For example, reproductive success has been shown to be higher in the guppy, *Poecilia reticulata*, when mating pairs had a similar level of boldness or shyness compared to the mating pairs with dissimilar levels of these traits (Ariyomo & Watt, 2013). In great tits, *Parus major*, fledglings have been shown to be in a higher condition when their parents have the same level of exploration (Both et al., 2005), and in black-legged kittiwakes, *Rissa tridactyla*, a higher reproductive success was shown in individuals that assortativly mated depending on boldness (Collins et al., 2019). However, zebrafish pairs with similar levels of anxiety did not show a significant increase in reproductive success compared to those with different levels of anxiety (Chapter IV). In Chapter IV, the experiment was limited by the absence of free mate choice that has been mostly used before (Aryiomo & Watt, 2013; Fox & Millam, 2014). Drickamer et al. (1999), demonstrated that house mice females, *Mus musculus*, had higher reproductive success following free mate choice of males that they favoured compared to those that they did not. Furthermore, Chen et

al. (2012), indicated that offspring fitness was higher in a pair with a male and a preferred female compared to a male paired with a non-preferred female in a bark beetle, *Dendroctonus valens*. In Chapter IV, there was no mate choice and one male was forced to mate with one female. Reproductive success of pairs depending on free choice has been shown to be higher even with disassortativly mated pairs, such as in cockatiels, *Nymphicus hollandicus*, reproductive success was shown to be higher in offspring of pairs that mated disassortatively depending on agreeableness with free mate choice (Fox & Millam, 2014). Other limitation in Chapter IV was the lack of investigation of other aspect of reproductive success not considered here that may affect fitness. Specifying eggs laid and fertilised made the results of this study less generalisable to the fitness consequences of anxiety in zebrafish.

Dochtermann et al. (2015) suggested that about 52 % of animal personality differences are attributed to additive genetic variation, and this has been demonstrated in many studies before (Oers et al., 2004; Ariyomo et al., 2013). In this thesis, as the repeatabilities of anxiety were found (Chapter II), it was hypothesised that phenotypic variation in anxiety in zebrafish would be attributed to additive genetic differences. The main objective of Chapter V of this thesis was to assess the heritability of anxiety. Our results indicated that consistent phenotypic variation in anxiety level between individuals was attributed to additive genetic variation, suggesting that anxiety is heritable (h^2 = 0.3-0.18 & V_R = 0.36-0.60). These findings supported the suggestion mentioned above that due to the lack of anxiety fitness consequences, this trait might not be selected, thus had additive genetic variance that resulted in the heritability estimates. Other factors including non-additive genetic factors has been shown to have an effect on individual behavioural variation. For example, it has been found that the heritability estimate of aggressiveness was moderate in zebrafish (h^2 = 0.36) and this was attributed to maternal effects and the high residual

variance (V_R = 0.55) (Ariyomo et al., 2013). The results of Chapter V showed that maternal effects had contributed to variation in anxiety (V_M = 0.29-58). The scope of this experiment was limited to focus on investigating the additive genetic variation and maternal effects rather than other factors that may contribute to variation in anxiety such as brood size, sex of offspring and age. Thus, further work is needed to take these factors into consideration.

7.4 Stress responses of individuals with different levels of anxiety

Different animal personalities may vary in their responses to stressors, with some showing little or no response and others showing a strong response. Stress responses of individuals with different levels of personality traits have been reported before in a variety of animals (e.g., avian birds, Cockrem, 2007; wild-caught Trinidadian guppies, Brown et al., 2014; gray squirrels, *Sciurus carolinensis*, Santicchia et al., 2020). One of the stressors that has been used in studies is the chemical alarm cue released by conspecific damaged skin, for example, the individual differences in the responses to alarm cues exhibited by African catfish (Nieuwegiessen et al., 2008). In Chapter VI, male and female zebrafish with known levels of anxiety, least anxious and most anxious groups, were exposed to conspecific alarm cues and their behavioural responses pre and post-exposure were compared. We found that, no significant difference between pre and post-exposure was evident in the behavioural reactions, except for one behaviour that was time spent in the upper half of the novel tank diving test, which increased post exposure in both males and females, suggesting that alarm cues had little effect on the individuals' responses. However, we found that there was a significant interaction between exposure and level of anxiety. Unexpectedly, the anxious group significantly decreased latency to enter the upper half of the tank, increased the number of entries to the upper half of the tank, and increased time spent in the upper half of the tank, suggesting they reduced their level of anxiety after exposure to the alarm cue. This study did not address the effect of chronic exposure to alarm cues in zebrafish, just a single,

acute exposure. Previous studies have shown that zebrafish respond differently to alarm cues depending on exposure. For example, in one study conducted on two populations of different zebrafish strains, wild-type and leopard, aggression was affected following exposure to acute and chronic exposure to alarm cues, with acute exposure resulting in a higher aggression while chronic exposure resulted in a lower aggression in those populations (Quadros et al., 2018). In addition, Wright et al. (2013), demonstrated that repeated exposure of rats to cat odour induced anxious behaviours and caused increased thigmotaxis and reduced activity in an open field test. Further work is needed to investigate the effect of chronic exposure of alarm cues to zebrafish with different levels of anxiety.

7.5 Conclusion

The research in this thesis assessed the consistency, repeatability, heritability of anxiety as a personality trait in zebrafish. In addition, it investigated whether there were specific genes underlying the consistency in anxiety. Moreover, in this thesis, the fitness consequences of anxiety on zebrafish was examined. Finally, the anxious responses of males and females with different levels of anxiety to chemical alarm cues was assessed. Overall, this study confirmed that anxiety was repeatable and heritable. Furthermore, there were some genes that were expressed differently depending on level of anxiety and sex, and anxiety was found to be heritable. Anxiety was found to have no direct effect on the number of eggs laid and fertilised by zebrafish with different levels of anxiety. Finally, when exposed to stressors, zebrafish with different levels of anxiety either did not respond or became less anxious, which was against my predictions.

7.6 References

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Appendix

Chapter II

Table 2.1. Principal component scores for individual female zebrafish fish ordered from the highest to the lowest values, based on the first component. Fish with a high value of the first component were deemed as least anxious while fish with a low value were deemed as most anxious.

Number	Fish ID	Scores.RC1	Scores.RC2
1	2F	2.18879389	-0.63900171
2	31F	2.14228289	-0.22393244
3	25F	1.91231692	-0.89173329
4	29F	1.69147841	1.160072
5	32F	1.63564045	-0.61138577
6	8F	1.50042066	1.77140037
7	37F	1.36274085	0.7856716
8	19F	1.1017484	-0.84379515
9	49F	1.03363508	-0.07541108
10	9F	0.96433478	0.51916417
11	20F	0.94396562	-1.10854341
12	43F	0.9253914	-0.61710814
13	54F	0.8331373	-1.77697582
14	18F	0.76945404	-0.32236543
15	55F	0.74052201	-0.34329189
16	5F	0.7160581	0.68565335
17	10F	0.68041828	1.235274
18	1F	0.65441991	1.13690088
19	30F	0.64610893	-0.33938248
20	34F	0.59189151	0.766658
21	23F	0.44342143	1.51471646
22	28F	0.37383609	1.46588188
23	33F	0.36078963	-0.27754784
24	53F	0.30200709	0.01697265
25	60F	0.28996145	-0.20237586
26	42F	0.12230414	0.11565519
27	44F	0.11676799	0.70264798
28	16F	0.1134261	-0.20364498

29	47F	0.06942203	-0.71676329
30	35F	-0.01461338	-0.58837623
31	14F	-0.06169893	-0.64644971
32	46F	-0.10547795	-0.40959299
33	51F	-0.2369426	-1.71046467
34	56F	-0.25497641	-0.38533739
35	12F	-0.31802228	-0.18963135
36	22F	-0.42567548	0.60633368
37	6F	-0.49847526	-0.72767983
38	21F	-0.54189085	-0.91138014
39	52F	-0.54547267	1.43135515
40	4F	-0.6081844	0.70777826
41	57F	-0.72901025	-0.83325928
42	38F	-0.75369569	-0.19595791
43	39F	-0.75431984	0.5131831
44	36F	-0.77216206	-1.09936793
45	45F	-0.84910473	1.35078353
46	13F	-0.86495568	-0.07465765
47	7F	-0.87180109	-2.32965078
48	15F	-0.87222272	0.8793506
49	24F	-0.93767259	0.07847603
50	17F	-0.99633833	1.82146596
51	11F	-1.0348317	1.70494742
52	3F	-1.05072739	0.23712917
53	26F	-1.06267187	-0.36856551
54	58F	-1.11661516	-1.95869073
55	27F	-1.4482239	-0.32827958
56	59F	-1.4559653	1.25365662
57	50F	-1.47020988	1.58691686
58	48F	-1.48632412	-1.73699383
59	41F	-1.51912445	0.3610907
60	40F	-1.56928837	-0.72154149

Table 2.2. Principal component scores for individual male zebrafish fish ordered from the highest to the lowest values, based on the first component. Fish with a high value of the first component deemed as least anxious while fish with a low value of the first component deemed as most anxious.

Number	Fish ID	Scores.RC1	Scores.RC2
1	9M	2.49407993	-1.47966963
2	41M	2.2220122	0.57395047
3	44M	1.73199844	-0.78646715
4	52M	1.20950358	-0.72644397
5	3M	1.158624	0.83040496
6	36M	1.1185098	0.11979194
7	20M	1.09531049	0.04556209
8	57M	1.0565104	-0.06165853
9	12M	1.02914266	-0.08571474
10	4M	0.99867612	-0.17779305
11	50M	0.91037796	0.98774317
12	60M	0.88001524	1.99016401
13	49M	0.79014207	-2.08090107
14	22M	0.77867893	0.18498532
15	19M	0.760385	0.89862321
16	58M	0.72498523	-0.58785794
17	28M	0.70127087	1.06514225
18	53M	0.69682278	0.64234042
19	18M	0.69430332	-1.02611334
20	23M	0.57991138	0.48627894
21	51M	0.51855317	-1.15914635
22	24M	0.50949233	0.24589718
23	42M	0.48511322	0.37517011
24	5M	0.3846442	-0.05579185
25	32M	0.37834027	-0.57382131
26	21M	0.35903877	-0.27265136
27	55M	0.30574164	-1.0298598
28	14M	0.27668713	-0.43672192
29	30M	0.14088164	1.54260165
30	35M	-0.03374883	2.07481027

31	6M	0.11298536	1.68819096
32	25M	-0.17537432	-0.31491899
33	31M	-0.21279389	-0.45136781
34	47M	-0.24704048	0.60140211
35	45M	0.31466797	0.69090509
36	17M	-0.40043203	0.67643903
37	46M	-0.47371474	-0.2857094
38	10M	-0.50745013	-0.96795856
39	37M	-0.55374969	-0.98929976
40	15M	-0.5661683	0.10884014
41	27M	-0.65136636	-0.79014429
42	8M	-0.66089597	-1.00464697
43	38M	-0.66895889	-0.60794924
44	39M	-0.77175918	-1.4130302
45	33M	-0.81551567	2.39603105
46	43M	-0.84505143	-1.32215986
47	16M	-0.86788825	-0.26168585
48	56M	-0.927094	-1.21122525
49	11M	-0.97852547	0.82681525
50	13M	-0.98138915	1.17114306
51	1M	-1.13196615	0.47499181
52	34M	-1.13441539	1.49781403
53	48M	-1.31954367	0.58028957
54	29M	-1.35997025	-0.62986821
55	2M	-1.39349541	-0.09085126
56	7M	-1.63251015	-1.70634531
57	54M	-1.66708373	-1.05558627
58	26M	-1.75952061	-0.3594357
59	40M	-1.82467731	1.22646685

Chapter III

Table 3.1. Quality control checking results using Qubit 2.0 Fluorometwe, NanoDrop 8000, and Agilent 2100 Bioanalyzer of 24 extracted RNA from 12 male & 12 female zebrafish.

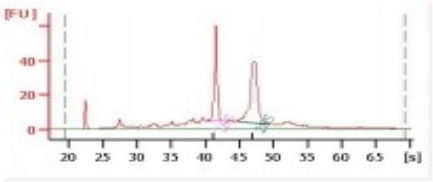
Sample ID	Amount of yield RNA (Qubit) ng/ml	Amount of yield RNA (Qubit) mg	NanoDrop results RNA purity 260/280	NanoDrop results RNA purity 260/230	RNA integrity number
3 Male	170	5.1	2.91	1.08	8.8
20 Male	91.2	2.7	1.97	1.23	8.8
29 Male	114	3.4	2.06	1.81	8.7
2 Male	69.4	2	1.90	0.25	9
7 Male	58.2	1.7	1.84	0.35	8.4
8 Female	73	2	1.98	0.32	8.9
37 Female	73.6	2	1.96	0.25	8.7
58 Female	140	4	2.16	2.21	8.7
27 Female	102	3	2.03	1.63	8.2
59 Female	138	4	2.10	2.14	8.6
50 Female	156	4	2.09	2.19	8.8
48 Female	84.4	2.5	2.17	1.99	NA
41 Female	49.6	1.5	2.24	1.09	NA
9 Male	19.5	2.9	2.91	1.08	NA
41 Male	30.4	4.5	2.21	0.79	NA
44 Male	25.4	3.8	1.90	0.47	NA
52 Male	22.9	3.4	3.18	0.60	NA
54 Male	30.9	927	2.27	0.12	NA
26 Male	14.6	2.19	2.35	0.80	NA
40 Male	14.3	2.14	3.04	1.48	NA
2 Female	164	4.92	1.94	6.51	NA
25 Female	79	2.37	1.91	4.21	NA
29 Female	104	3.12	1.92	4.95	NA
32 Female	32.6	978	2.35	0.25	NA

Table 3.2. Quality control results summary of extracted RNA Using Agarose Gel Electrophoresis, Nanodrop, Agilent 2100 conducted by Novogene.

Sample NO.	Sample Name	Novogene ID	Conc (ng/μl)	Vol. (μL)	Amt. (μg)	260/280	260/230	RIN	Conclusion	Note
1	9 Male	UKR19040524	179	23	4.117			7.9	Pass	
2	41 Male	UKR19040525	141	28	3.948			8.3	Pass	
3	44 Male	UKR19040526	150	22	3.300			8.3	Pass	
4	52 Male	UKR19040527	166	23	3.818			8.6	Pass	
5	3 Male	UKR19040528	240	18	4.320			9.5	Pass	
6	20 Male	UKR19040529	99	18	1.782			8.7	Pass	
7	29 Male	UKR19040530	122	18	2.196			9.3	Pass	
8	2 Male	UKR19040531	78	18	1.404			9.5	Pass	
9	7 Male	UKR19040532	86	21	1.806			9.3	Pass	
10	54 Male	UKR19040533	31	18	0.558			9.2	Hold	Insufficient total amount
11	26 Male	UKR19040534	148	22	3.256			9.2	Pass	
12	40 Male	UKR19040535	57	29	1.653			8.8	Pass	
13	2 Female	UKR19040536	28	27	0.756			7.6	Hold	Insufficient total amount
14	25 Female	UKR19040537	19	22	0.418			7.7	Hold	Insufficient total amount
15	29 Female	UKR19040538	35	27	0.945			9.5	Pass	
16	32 Female	UKR19040539	34	19	0.646			8.2	Hold	Insufficient total amount
17	8 Female	UKR19040540	83	20	1.660			8.9	Pass	
18	37 Female	UKR19040541	88	21	1.848			8.9	Pass	
19	58 Female	UKR19040542	155	20	3.100			9.5	Pass	
20	27 Female	UKR19040543	111	18	1.998			9.1	Pass	

21	59 Female	UKR19040544	170	19	3.230			9.4	Pass	
22	50 Female	UKR19040545	147	19	2.793			9	Pass	
23	48 Female	UKR19040546	97	28	2.716			9.2	Pass	
24	41 Female	UKR19040547	66	23	1.518			8.6	Pass	

UKR19040524



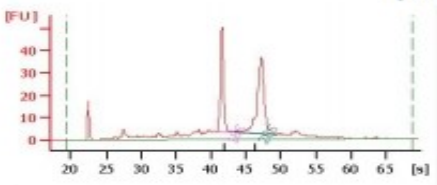
Overall Results for sample 1 : UKR19040524

RNA Area:	416.6
RNA Concentration:	179 ng/µl
rRNA Ratio [28s / 18s]:	1.2
RNA Integrity Number (RIN):	7.9 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.90

Fragment table for sample 1 : UKR19040524

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.22	42.58	72.2	17.3
28S	44.80	49.48	86.9	20.9

UKR19040525



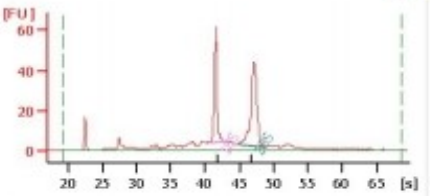
Overall Results for sample 2 : UKR19040525

RNA Area:	330.2
RNA Concentration:	141 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.3 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.30

Fragment table for sample 2 : UKR19040525

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.76	43.15	63.2	19.1
28S	43.25	49.44	83.3	25.2

UKR19040526



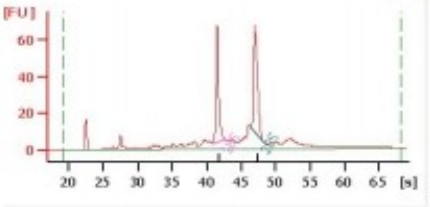
Overall Results for sample 3 : UKR19040526

RNA Area:	349.4
RNA Concentration:	150 ng/µl
rRNA Ratio [28s / 18s]:	1.4
RNA Integrity Number (RIN):	8.3 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.30

Fragment table for sample 3 : UKR19040526

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.82	42.66	69.6	19.9
28S	44.75	48.73	94.7	27.1

UKR19040527



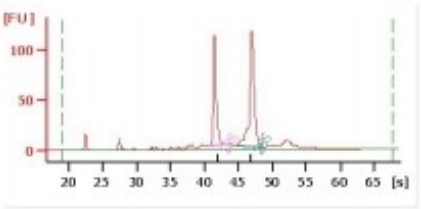
Overall Results for sample 4 : UKR19040527

RNA Area:	387.9
RNA Concentration:	166 ng/µl
rRNA Ratio [28s / 18s]:	1.2
RNA Integrity Number (RIN):	8.6 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.60

Fragment table for sample 4 : UKR19040527

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.80	42.58	75.1	19.4
28S	46.38	48.36	90.0	23.2

UKR19040528



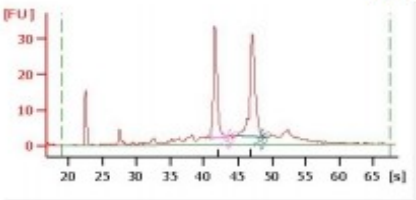
Overall Results for sample 5 : UKR19040528

RNA Area:	560.7
RNA Concentration:	240 ng/µl
rRNA Ratio [28s / 18s]:	1.7
RNA Integrity Number (RIN):	9.5 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.50

Fragment table for sample 5 : UKR19040528

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.82	43.13	127.1	22.7
28S	44.71	48.83	212.2	37.9

UKR19040529



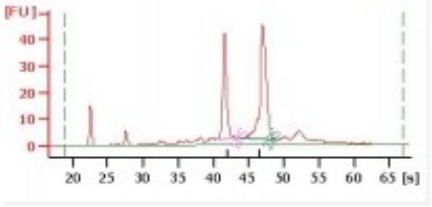
Overall Results for sample 6 : UKR19040529

RNA Area:	230.5
RNA Concentration:	99 ng/µl
rRNA Ratio [28s / 18s]:	1.5
RNA Integrity Number (RIN):	8.7 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 6 : UKR19040529

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.84	43.24	42.8	18.6
28S	44.80	48.86	62.8	27.2

UKR19040530



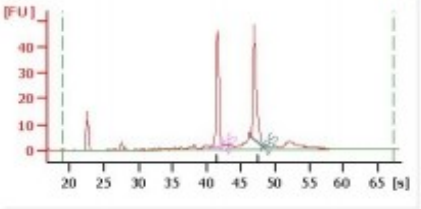
Overall Results for sample 7 : UKR19040530

RNA Area:	285.5
RNA Concentration:	122 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	9.3 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.30

Fragment table for sample 7 : UKR19040530

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.77	43.06	55.0	19.3
28S	44.03	48.98	97.5	34.2

UKR19040531



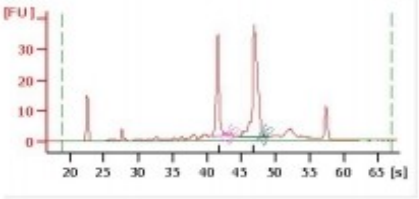
Overall Results for sample 8 : UKR19040531

RNA Area:	180.9
RNA Concentration:	78 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	9.5 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.50

Fragment table for sample 8 : UKR19040531

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.31	42.61	43.5	24.0
28S	46.42	48.42	55.3	30.5

UKR19040532



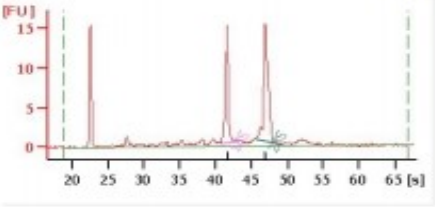
Overall Results for sample 9 : UKR19040532

RNA Area:	199.6
RNA Concentration:	86 ng/µl
rRNA Ratio [28s / 18s]:	1.9
RNA Integrity Number (RIN):	9.3 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.30

Fragment table for sample 9 : UKR19040532

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.77	42.57	36.9	18.5
28S	44.76	48.79	71.4	35.8

UKR19040533



Overall Results for sample 10 : UKR19040533

RNA Area:	72.7
RNA Concentration:	31 ng/µl
rRNA Ratio [28s / 18s]:	1.5
RNA Integrity Number (RIN):	9.2 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.20

Fragment table for sample 10 : UKR19040533

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.83	42.53	15.7	21.6
28S	45.44	48.44	24.2	33.3

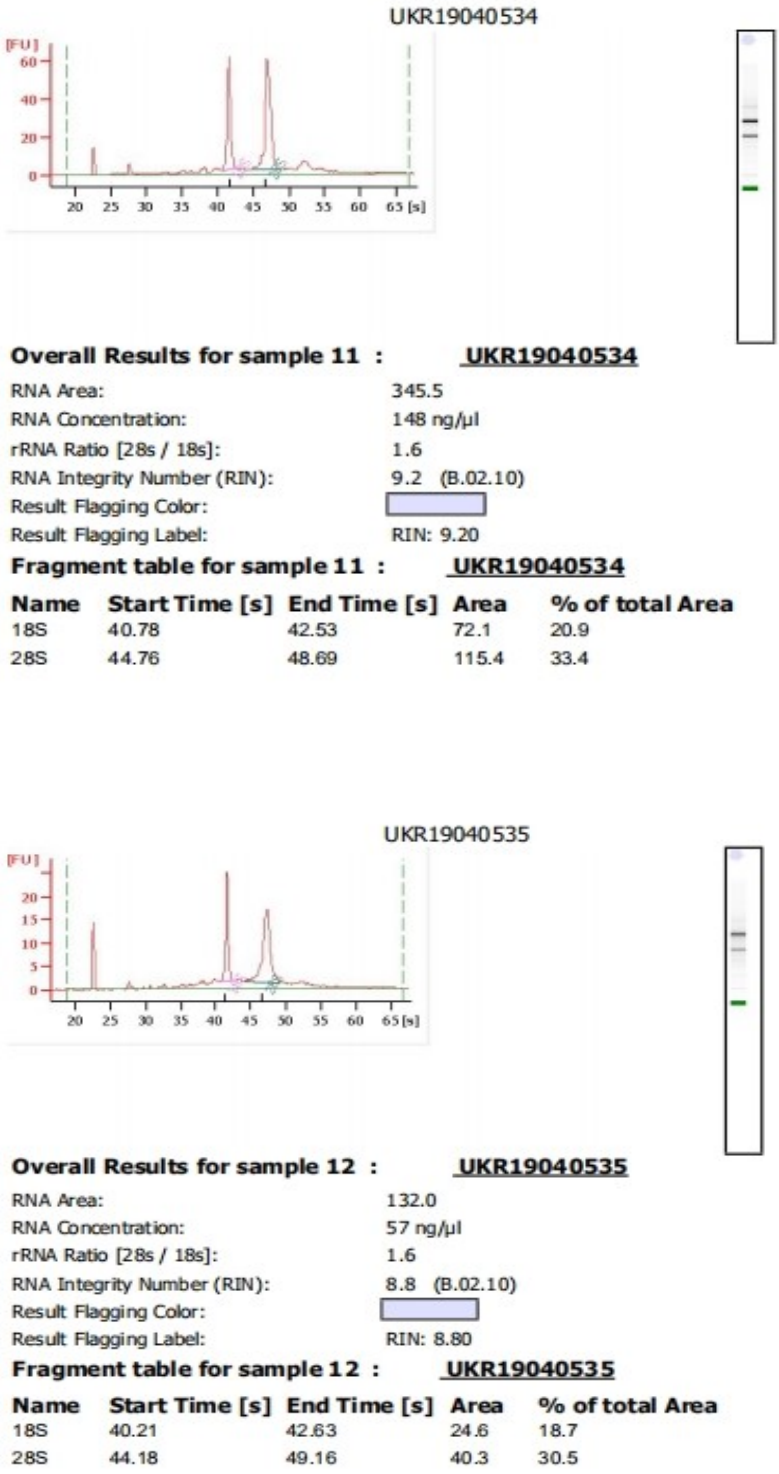
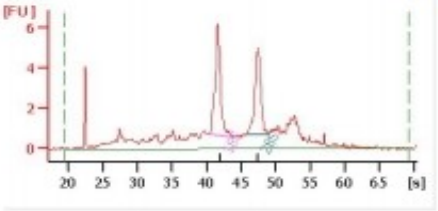


Figure 3.1. The result of extracted RNA samples from male zebrafish brains using Agilent 2100 analysis by Novogene.

UKR19040536



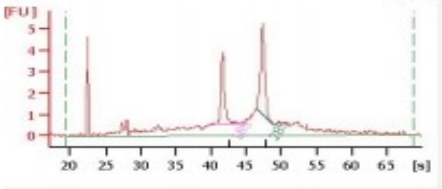
Overall Results for sample 1 : UKR19040536

RNA Area:	73.2
RNA Concentration:	28 ng/µl
rRNA Ratio [28s / 18s]:	0.7
RNA Integrity Number (RIN):	7.6 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.60

Fragment table for sample 1 : UKR19040536

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.48	43.54	12.6	17.2
28S	45.90	48.97	8.7	11.8

UKR19040537



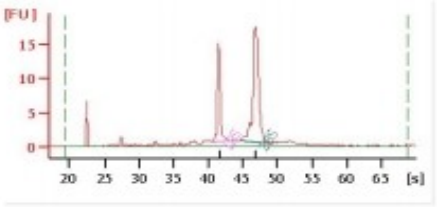
Overall Results for sample 2 : UKR19040537

RNA Area:	49.5
RNA Concentration:	19 ng/µl
rRNA Ratio [28s / 18s]:	1.2
RNA Integrity Number (RIN):	7.7 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 7.70

Fragment table for sample 2 : UKR19040537

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.71	44.45	5.8	11.6
28S	46.50	48.89	6.8	13.7

UKR19040538



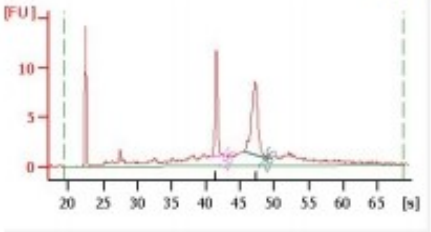
Overall Results for sample 3 : UKR19040538

RNA Area:	90.3
RNA Concentration:	35 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	9.5 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.50

Fragment table for sample 3 : UKR19040538

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.51	42.95	19.1	21.1
28S	44.65	49.09	34.0	37.7

UKR19040539



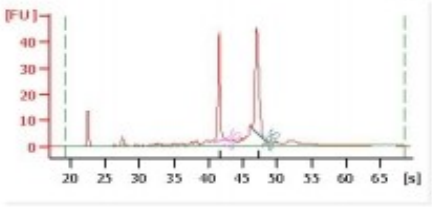
Overall Results for sample 4 : UKR19040539

RNA Area:	87.3
RNA Concentration:	34 ng/µl
rRNA Ratio [28s / 18s]:	1.4
RNA Integrity Number (RIN):	8.2 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.20

Fragment table for sample 4 : UKR19040539

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.76	42.31	11.8	13.6
28S	45.55	48.99	16.3	18.7

UKR19040540



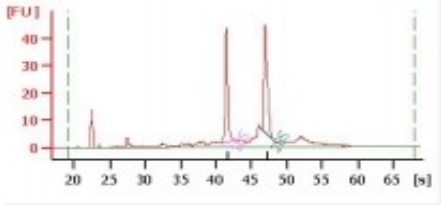
Overall Results for sample 5 : UKR19040540

RNA Area:	213.9
RNA Concentration:	83 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.9 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.90

Fragment table for sample 5 : UKR19040540

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.83	42.57	43.1	20.1
28S	46.39	48.43	56.9	26.6

UKR19040541



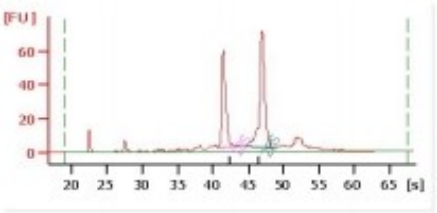
Overall Results for sample 6 : UKR19040541

RNA Area:	228.4
RNA Concentration:	88 ng/µl
rRNA Ratio [28s / 18s]:	1.2
RNA Integrity Number (RIN):	8.9 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.90

Fragment table for sample 6 : UKR19040541

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.76	42.53	44.4	19.4
28S	46.38	48.16	54.9	24.1

UKR19040542



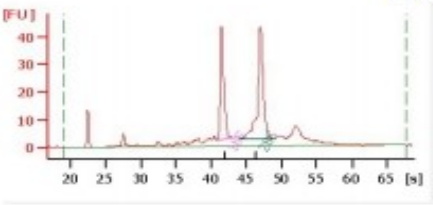
Overall Results for sample 7 : UKR19040542

RNA Area:	401.4
RNA Concentration:	155 ng/µl
rRNA Ratio [28s / 18s]:	1.7
RNA Integrity Number (RIN):	9.5 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.50

Fragment table for sample 7 : UKR19040542

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.74	44.12	81.9	20.4
28S	44.66	48.63	136.0	33.9

UKR19040543



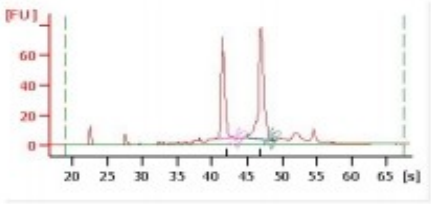
Overall Results for sample 8 : UKR19040543

RNA Area:	287.3
RNA Concentration:	111 ng/µl
rRNA Ratio [28s / 18s]:	1.6
RNA Integrity Number (RIN):	9.1 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.10

Fragment table for sample 8 : UKR19040543

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.78	43.23	54.7	19.1
28S	44.07	48.68	88.5	30.8

UKR19040544



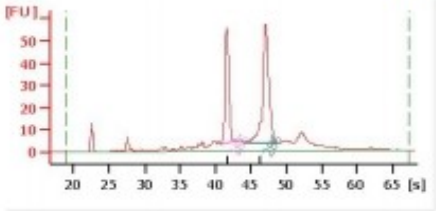
Overall Results for sample 9 : UKR19040544

RNA Area:	440.7
RNA Concentration:	170 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	9.4 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.40

Fragment table for sample 9 : UKR19040544

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.79	43.04	87.0	19.7
28S	44.71	48.96	153.4	34.8

UKR19040545

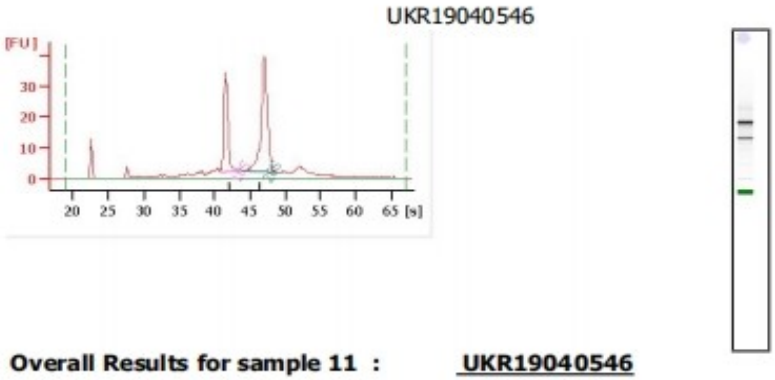


Overall Results for sample 10 : UKR19040545

RNA Area:	381.2
RNA Concentration:	147 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	9 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN:9

Fragment table for sample 10 : UKR19040545

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.80	42.56	69.1	18.1
28S	44.02	48.81	121.6	31.9

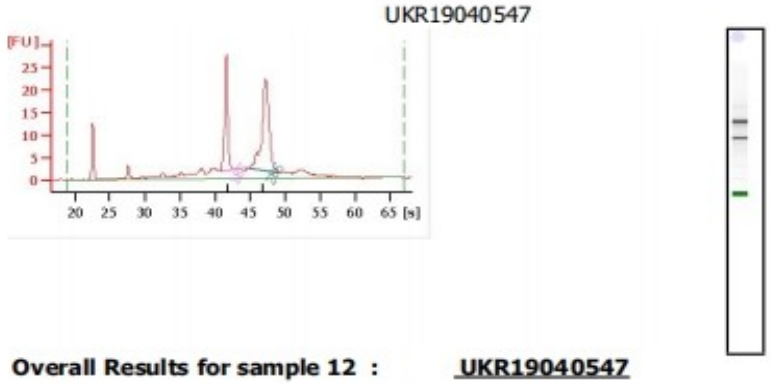


Overall Results for sample 11 : UKR19040546

RNA Area:	251.2
RNA Concentration:	97 ng/µl
rRNA Ratio [28s / 18s]:	1.9
RNA Integrity Number (RIN):	9.2 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 9.20

Fragment table for sample 11 : UKR19040546

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.81	43.15	46.7	18.6
28S	44.07	49.04	88.0	35.1



Overall Results for sample 12 : UKR19040547

RNA Area:	170.2
RNA Concentration:	66 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	8.6 (B.02.10)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.60

Fragment table for sample 12 : UKR19040547

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.82	42.57	28.7	16.9
28S	44.76	49.13	51.8	30.5

Figure 3.2. The result of extracted RNA samples from female zebrafish brains using Agilent 2100 analysis by Novogene.

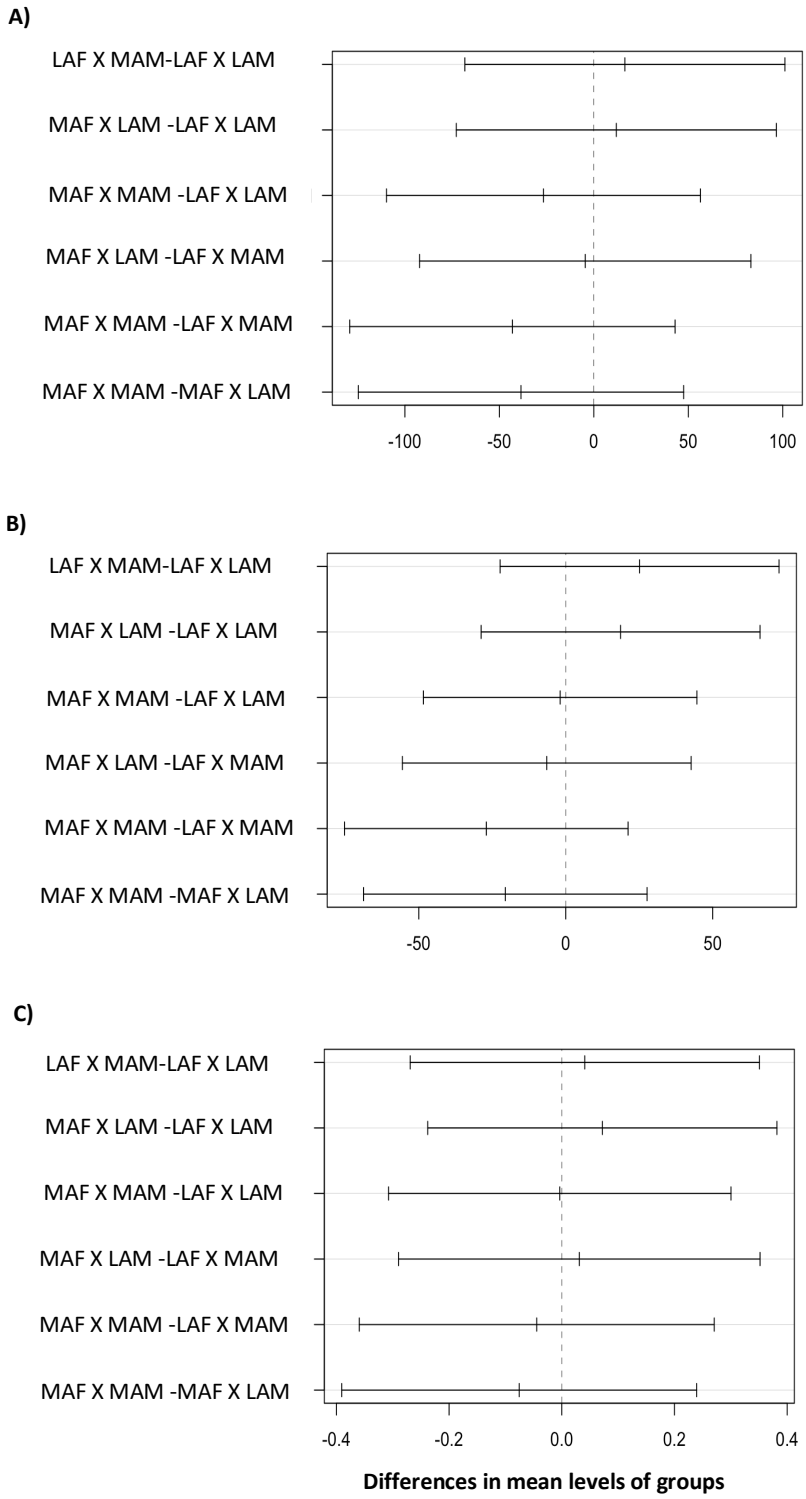


Figure 4.1. The confidence intervals comparing the mean of (A) total laid eggs, (B) fertilised eggs, and (C) the proportion of fertilised eggs across the four groups of mating crosses (least anxious x most anxious, least anxious x least anxious, most anxious x least anxious, most anxious x most anxious) zebrafish.

Table 4.1. The difference interval results on mating crosses differences in the mean values of total number of laid eggs, fertilised eggs and proportion of fertilised eggs using Tukey Honest method.

Mating crosses	**diff**	**lwr**	**upr**	**P adj**
Laid eggs				
LAF X MAM-LAF X LAM	16.441	-68.270	101.152	0.955
MAF X LAM -LAF X LAM	11.902	-72.808	96.613	0.982
MAF X MAM -LAF X LAM	-26.652	-109.726	56.422	0.829
MAF X LAM -LAF X MAM	-4.538	-92.222	83.145	0.999
MAF X MAM -LAF X MAM	-43.093	-129.197	43.010	0.548
MAF X MAM -MAF X LAM	-38.554	-124.659	47.549	0.636
Fertilised eggs				
LAF X MAM-LAF X LAM	25.117	-22.335	72.571	0.501
MAF X LAM -LAF X LAM	18.656	-28.797	66.109	0.724
MAF X MAM -LAF X LAM	-1.909	-48.446	44.627	0.999
MAF X LAM -LAF X MAM	-6.461	-55.580	42.657	0.985
MAF X MAM -LAF X MAM	-27.027	-75.261	21.206	0.451
MAF X MAM -MAF X LAM	-20.565	-68.799	27.668	0.671
Proportion of fertilised eggs				
LAF X MAM-LAF X LAM	0.040	-0.269	0.350	0.985
MAF X LAM -LAF X LAM	0.071	-0.237	0.381	0.926
MAF X MAM -LAF X LAM	-0.003	-0.307	0.300	0.999
MAF X LAM -LAF X MAM	0.031	-0.289	0.351	0.993
MAF X MAM -LAF X MAM	-0.044	-0.359	0.270	0.981
MAF X MAM -MAF X LAM	-0.075	-0.390	0.239	0.919